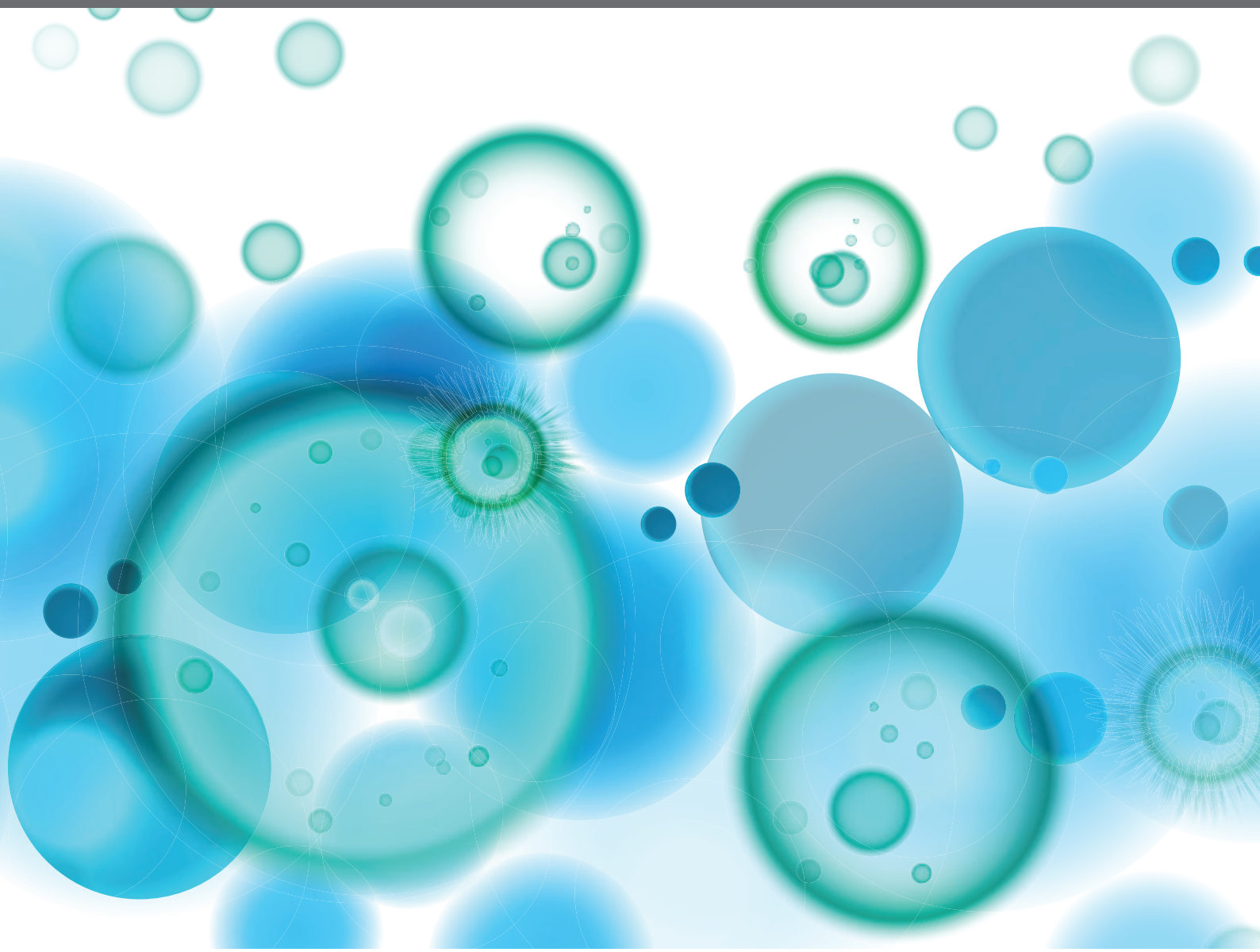
CANCER STEM CELLS AND THEIR ROLE IN TUMOR DORMANCY AND IMMUNOSURVEILLANCE

EDITED BY: Cristina Maccalli, Ira Ida Skvortsova, Sergio Rutella and Maurizio Callari

PUBLISHED IN: Frontiers in Immunology and Frontiers in Oncology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-83250-434-5
DOI 10.3389/978-2-83250-434-5

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

CANCER STEM CELLS AND THEIR ROLE IN TUMOR DORMANCY AND IMMUNOSURVEILLANCE

Topic Editors:
Cristina Maccalli, Sidra Medicine, Qatar
Ira Ida Skvortsova, Innsbruck Medical University, Austria
Sergio Rutella, Nottingham Trent University, United Kingdom
Maurizio Callari, University of Cambridge, United Kingdom

Citation: Maccalli, C., Skvortsova, I. I., Rutella, S., Callari, M., eds. (2022). Cancer Stem Cells and Their Role in Tumor Dormancy and Immunosurveillance. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-434-5

Table of Contents

frontiers in Immunology

MINI REVIEW
published: 05 February 2020
doi: 10.3389/fimmu.2020.00140

Bidirectional Crosstalk Between Cancer Stem Cells and Immune Cell Subsets

*Luise Müller[1], Antje Tunger[1,2], Ioana Plesca[1], Rebekka Wehner[1,2,3], Achim Temme[2,3,4], Dana Westphal[5], Friedegund Meier[2,3,5], Michael Bachmann[2,3,6] and Marc Schmitz[1,2,3]**

[1] Faculty of Medicine Carl Gustav Carus, Institute of Immunology, TU Dresden, Dresden, Germany, [2] National Center for Tumor Diseases, Partner Site Dresden, Dresden, Germany, [3] German Cancer Consortium (DKTK), Partner Site Dresden, and German Cancer Research Center, Heidelberg, Germany, [4] Department of Neurosurgery, Section Experimental Neurosurgery and Tumor Immunology, University Hospital Carl Gustav Carus, TU Dresden, Dresden, Germany, [5] Department of Dermatology, University Hospital Carl Gustav Carus, TU Dresden, Dresden, Germany, [6] Department of Radioimmunology, Institute of Radiopharmaceutical Cancer Research, Helmholtz Center Dresden-Rossendorf, Dresden, Germany

Cancer stem cells (CSCs), also known as tumor-initiating cells, are characterized by an increased capacity for self-renewal, multipotency, and tumor initiation. While CSCs represent only a small proportion of the tumor mass, they significantly account for metastatic dissemination and tumor recurrence, thus making them attractive targets for therapy. Due to their ability to sustain in dormancy, chemo- and radiotherapy often fail to eliminate cancer cells with stemness properties. Recent advances in the understanding of the tumor microenvironment (TME) illustrated the importance of the immune contexture, determining the response to therapy and clinical outcome of patients. In this context, CSCs exhibit special properties to escape the recognition by innate and adaptive immunity and shape the TME into an immunosuppressive, pro-tumorigenic landscape. As CSCs sculpt the immune contexture, the phenotype and functional properties of the tumor-infiltrating immune cells in turn influence the differentiation and phenotype of tumor cells. In this review, we summarize recent studies investigating main immunomodulatory properties of CSCs and their underlying molecular mechanisms as well as the impact of immune cells on cancer cells with stemness properties. A deeper understanding of this bidirectional crosstalk shaping the immunological landscape and determining therapeutic responses will facilitate the improvement of current treatment modalities and the design of innovative strategies to precisely target CSCs.

Keywords: tumor microenvironment, cancer stem cells, macrophages, T cells, myeloid-derived suppressor cells

OPEN ACCESS

Edited by:
Sergio Rutella, Nottingham Trent University, United Kingdom

Reviewed by:
Pier Paolo Claudio, University of Mississippi, United States
Benjamin Bonavida, University of California, Los Angeles, United States

***Correspondence:**
Marc Schmitz
marc.schmitz@tu-dresden.de

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *26 November 2019*
Accepted: *20 January 2020*
Published: *05 February 2020*

Citation:
Müller L, Tunger A, Plesca I, Wehner R, Temme A, Westphal D, Meier F, Bachmann M and Schmitz M (2020) Bidirectional Crosstalk Between Cancer Stem Cells and Immune Cell Subsets. Front. Immunol. 11:140. doi: 10.3389/fimmu.2020.00140

INTRODUCTION

In recent years, the role of the tumor microenvironment (TME) has gained an increasing amount of attention, allowing the discovery of new concepts and development of novel therapeutic approaches. Especially immune checkpoint inhibitors emerged as a promising tool to use the power of pre-existing tumor-specific T cells to target cancer cells (1, 2). Despite these advances, a significant fraction of patients fails to respond to those therapies or develops resistance and even patients with a complete response often present with recurrence and metastatic lesions later on. While there are several factors determining the response to therapy and long-term outcome,

cancer stem cells (CSCs) are considered to play an important role in the resistance, metastasis, and recurrence of tumors (3). These cells, also known as tumor-initiating cells, exhibit an enhanced capacity for self-renewal, multipotency, and tumorigenicity and were first described in acute myeloid leukemia (4). Subsequently, CSCs were also identified in a multitude of solid cancers and further characterized by various cell surface markers (5). The most common markers are CD44 and CD133, with CD44 being utilized to isolate CSCs from breast, prostate, gastric, as well as head and neck squamous cell cancer (HNSCC). CD133 on the other hand is widely used to identify CSCs in glioblastoma, lung cancer, and sarcomas. Recently, more markers have been established, for example CD90 for breast cancer and glioblastoma, CD117 for breast, ovarian, lung cancer, and glioblastoma, and CD29 for breast and colon cancer (6). Importantly, due to the high plasticity of CSCs and the shared marker expression on other cells, none of these molecules is sufficient to be used as a standalone marker. So far, no consensus regarding marker combinations for the characterization and isolation of CSCs has been reached, complicating the comparison of different studies and partly explaining conflicting results. Other characteristics of CSCs that can be used for their identification are an increased activity of aldehyde dehydrogenase (ALDH) and the high expression of efflux pumps, which allows discrimination based on the exclusion of vital dyes (7, 8). The upregulation of efflux pumps is, together with a decreased sensitivity to apoptosis, an altered cell cycle, and DNA damage repair, associated with an enhanced resistance to chemotherapy (9). Considering all of these characteristics, the interaction between immune cells and CSCs is highly important, as they show distinct properties to shape the TME and represent an attractive target for therapy to improve the long-term outcome of patients.

IMPACT OF CSCs ON THE FUNCTIONAL PROPERTIES OF IMMUNE CELLS

Previous studies revealed the importance of the TME for both prognosis and treatment of malignant diseases and improved the understanding of the highly complex crosstalk between the TME and tumor cells (10, 11). It has been shown that tumor cells themselves can influence the immune contexture by expressing cell membrane-associated coinhibitory receptors or secreting various soluble factors to modulate certain immune subsets, shaping the TME into an immunosuppressive landscape. Due to the outstanding importance of CSCs regarding resistance, metastasis, and recurrence, their role in modulating the TME in general and major immune subsets in particular are discussed below (**Figure 1**).

Macrophages

Tumor-associated macrophages (TAMs) can exhibit both pro- and antitumor properties depending on the microenvironment and their polarization. The current understanding of macrophage polarization includes the classically activated, pro-inflammatory M1 phenotype and the alternatively activated, anti-inflammatory M2 phenotype (12–14). In general, M1 macrophages exhibit anti-tumor immunity, while M2 macrophages display immunosuppressive properties and contribute to tumor progression. Therefore, a high M1/M2 ratio is associated with improved survival in multiple cancer entities (15–17). Tumor cells are known to influence the composition of intratumoral macrophages by directly recruiting M2 macrophages or driving the polarization of both tissue-resident macrophages and recruited macrophages toward a M2 phenotype (18, 19). Several studies pointed out a particular importance of CSCs in driving the recruitment of macrophages and subsequent M2 polarization. For example, Yi et al. showed that glioma-derived CSCs are associated with an increased density of intratumoral macrophages and exhibit a stronger capacity to recruit macrophages into the TME (20). Periostin, a secreted extracellular matrix protein, produced by glioma stem cells was shown to attract M2 TAMs, promoting tumor growth (21). Another study also proposed that M2 macrophages are recruited by tumor-initiating cells already at the single cell stage and that the elimination of these macrophages may abolish early tumorigenesis (22). Besides the recruitment of macrophages, CSCs are able to promote the macrophage polarization toward the M2 phenotype via secretion of various cytokines and growth factors (23). For example, the CSC-mediated M2 polarization of macrophages was shown to be driven by cyclooxygenase-2 and CCL2 in ovarian cancer (24). Similarly, glioma CSCs polarize macrophages and microglia by the production of colony-stimulating factors (CSFs), transforming growth factor β (TGF-β), and macrophage inhibitory cytokine 1 (MIC-1), and glioma CSC-conditioned medium promoted the immunosuppressive properties of macrophages (25–27). In particular, chemoresistant CSCs have a unique capacity to produce proinflammatory cytokines and soluble factors such as CCL2 and CSF-1, known to recruit and polarize macrophages (28). Altogether, this data suggests that the CSC-mediated accumulation of M2 macrophages may contribute to the generation of an immunosuppressive, pro-tumorigenic TME.

T Cells

CSCs are also able to alter the composition and functional properties of tumor-specific effector T cells and promote the expansion of immunosuppressive, pro-tumorigenic regulatory T cells (Tregs). While a dense infiltration of $CD3^+$ T cells generally correlates with a good clinical outcome in many cancers, Tregs in particular are mainly associated with a poor prognosis, with the exception of colorectal and gastric cancers (11). For example, glioblastoma CSCs were shown to produce TGF-β, promoting the induction of Tregs, and CCL2, a chemokine involved in the recruitment of Tregs (29). Furthermore, CSC-conditioned medium inhibited T cell proliferation and this effect could be reversed by blocking the signal transducer and activator of transcription 3 (STAT3) pathway. Similarly, $CD44^+$ CSCs from HNSCC exhibited a significantly increased secretion of interleukin (IL)-8, granulocyte-CSF, and TGF-β compared to their $CD44^-$ counterparts, suppressing the proliferation of T cells and Th1 responses while supporting the generation of Tregs (30). Another study revealed that the levels

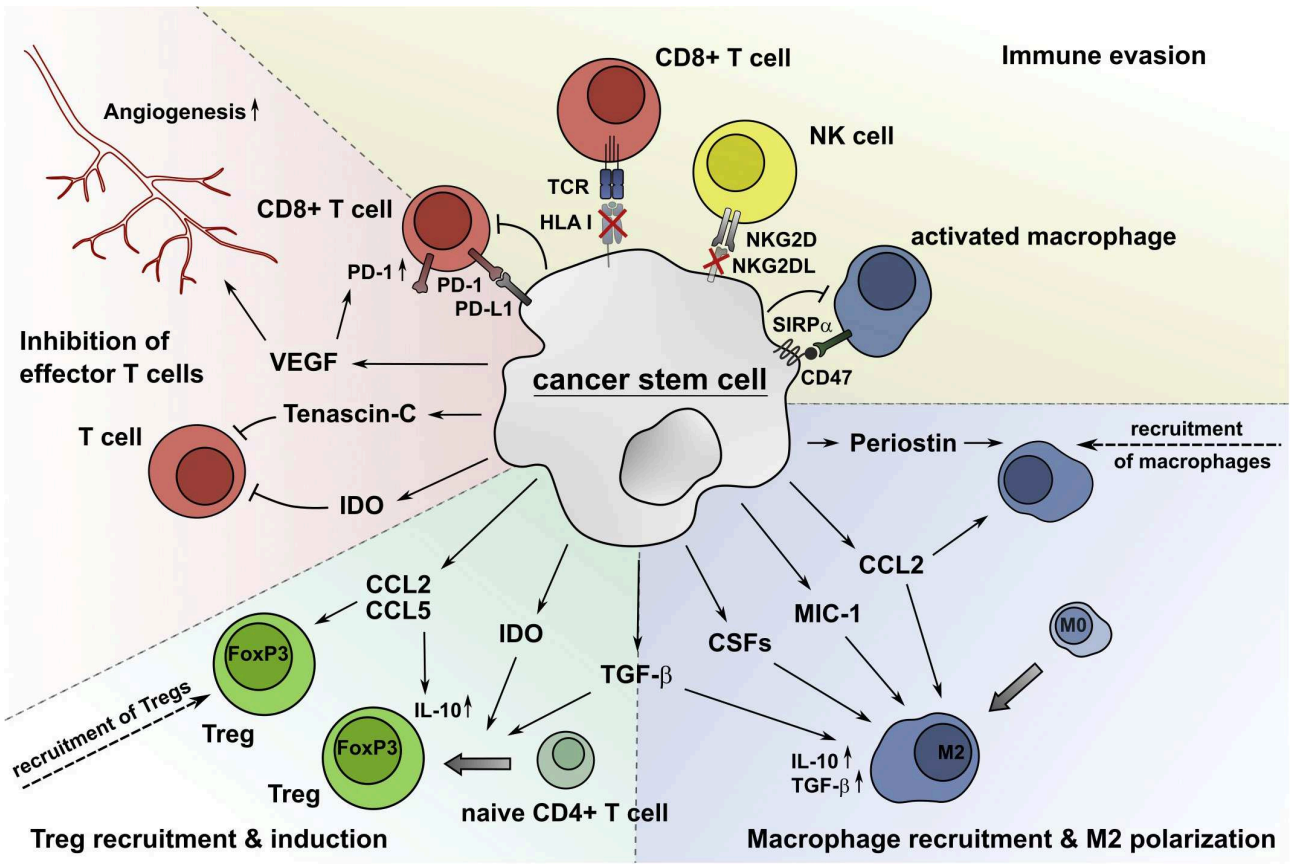


FIGURE 1 | CSCs alter the composition and functional properties of immune cell subsets, which results in an impaired CSC recognition and elimination by the immune system. CSCs produce soluble molecules such as periostin, CCL2, MIC-1, CSFs, and TGF-β to attract macrophages and drive their M2 polarization. The secretion of CCL2 and CCL5 recruits Tregs into the TME, and IDO and TGF-β facilitate the generation of Tregs from naïve $CD4^+$ T cells. The extracellular matrix protein tenascin-C inhibits T cell proliferation by interfering with the T cell receptor signaling pathway. Additionally, the immunomodulatory enzyme IDO impairs the expansion of effector T cells through accumulation of tryptophan catabolites, which also increases the generation of Tregs. VEGF produced by CSCs supports the induction of angiogenesis, which is critical for tumor growth, and promotes the expression of PD-1 by $CD8^+$ T cells. Additionally, CSCs avoid the recognition by $CD8^+$ T cells and NK cells by reducing the surface expression of HLA class I molecules and NKG2DL, respectively. The ligation of PD-L1 expressed by CSCs to PD-1 on effector T cells decreases their proliferation and IFN-γ production or leads to apoptosis. Furthermore, CSCs express the coinhibitory molecule CD47, which inhibits phagocytosis by macrophages due to repositioning of its ligand SIRPα.

of indoleamine-2,3-dioxygenase (IDO) were particularly elevated in CSCs from breast cancer, prostate cancer, and mesothelioma cell lines, as well as primary human glioblastoma cells (31). The immunomodulatory enzyme IDO substantially suppresses T cell expansion and is involved in the generation and activation of Tregs (32). Recently, exosomes containing the extracellular matrix component tenascin-C were shown to be secreted by brain tumor CSCs, inhibiting T cell activity (33). Furthermore, You et al. proposed that ovarian $CD133^+$ CSCs recruit Tregs via CCL5 and additionally enhance their immunosuppressive properties, namely the secretion of IL-10 (34). Moreover, CSCs produced elevated levels of vascular endothelial growth factor (VEGF), which is known to promote angiogenesis and increase the expression of programmed cell death 1 (PD-1) on $CD8^+$ T cells (35–37). Importantly, emerging evidence suggests that the immune checkpoint molecule programmed cell death ligand 1 (PD-L1) is expressed by CSCs from glioblastoma (38), HNSCC (39), breast, and colon cancer (40, 41).

IMMUNE EVASION BY CSCs

In addition to the modulation of the immune contexture, CSCs exhibit various properties to directly evade effector mechanisms of immune cells. The prerequisite for the elimination of tumor cells by activated $CD8^+$ T cells is the presentation of peptides on the cell surface via human leukocyte antigen (HLA) class I molecules. Various molecules are involved in this process, creating a multitude of possibilities to alter the HLA class I peptide complex expression to evade the recognition by the adaptive immune system. While tumor cells in general are well-known to downregulate components of the antigen processing and presenting machinery, for example, the transporter associated with antigen processing (TAP), CSCs appear particularly specialized in this mechanism. Several groups reported a reduced expression of HLA class I or TAP molecules of CSCs in HNSCC (42), melanoma (43), glioblastoma (38), lung cancer (44), and colorectal cancer (45) in comparison to their

non-stem-cell counterparts. However, Chikamatsu et al. showed a decreased expression of TAP2 in HNSCC CSCs, but were not able to find a significant difference between $CD44^+$ and $CD44^-$ cells with regard to other TAP molecules or the overall HLA I expression (30). Another study also failed to show a difference in the HLA class I expression of CSCs and non-CSCs in colon cancer (46). These discrepancies are likely to arise from strongly differing protocols for the isolation and culture of CSCs and also the heterogeneity of the CSC population.

In theory, missing HLA class I expression makes cancer cells more susceptible to the recognition and elimination by natural killer (NK) cells. However, conflicting results have been published, either arguing that NK cells display an enhanced recognition of CSCs, or reporting an increased evasion of NK cell-mediated killing by CSCs for example due to downregulation of activating NKG2D ligands (NKG2DL) (38, 47–50). A recent study reported that $CD34^+CD38^-$ leukemic stem cells express lower levels of NKG2DL compared to their $CD34^-$ counterparts (51). Further analysis revealed that only $NKG2DL^-$ AML cells exhibited chemoresistance toward cytarabine and patients with a higher proportion of $NKG2DL^+$ AML cells showed a better response to chemotherapy and an improved overall survival.

Moreover, CSCs were shown to express CD47 which engages with signal regulatory protein α (SIRPα) on macrophages, inhibiting phagocytosis. Several groups reported an increased expression of CD47 on leukemic stem cells compared to non-CSCs and that the blocking of CD47 induced efficient phagocytosis (52–54). Although this mechanism seems to be predominantly found in hematologic malignancies, CD47 overexpression was also reported in lung, pancreatic, and hepatocellular carcinoma (HCC) (54–56).

IMMUNE CELLS DRIVE THE FORMATION AND MAINTENANCE OF CSCs

As CSCs shape the TME, infiltrating immune cells can in turn influence the characteristics of CSCs. In this chapter, the impact of major immune subsets on stemness properties, metastatic potential, and tumorigenicity of CSCs is summarized (**Figure 2**).

Macrophages

While CSCs are able to recruit macrophages and promote their M2 polarization, TAMs seem to play a predominant role in the maintenance of CSCs within their niche (57). IL-6, which is mainly produced by M1 macrophages, but also by subtypes of M2 macrophages, is upregulated in breast, ovarian, prostate, pancreatic, and colorectal cancer and can confer resistance against apoptosis as well as promote proliferation, invasion, metastasis, and angiogenesis (58–64). Emerging evidence also suggests an important role for IL-6 in the induction and maintenance of CSCs. For example, IL-6 produced by TAMs supported the expansion and drug resistance of CSCs through STAT3 signaling in non-small-cell lung cancer (NSCLC) and HCC (65, 66). Moreover, multiple studies showed an IL-6-STAT3-dependent conversion of non-stem-cell breast cancer cells into CSCs, partially by promoting epithelial to mesenchymal transition (EMT) (67–69). Similarly, TGF-β secreted by M2-polarized TAMs promoted EMT and acquisition of stem-like properties in HCC (70). These findings are in line with data from Wu et al. indicating that chronic TGF-β stimulation in the course of liver cirrhosis induces expression of CSC-associated genes in liver progenitor cells and therefore promotes the development of cancer (71). Moreover, especially M2 macrophages are known to secrete VEGF, which is also produced by CSCs themselves and promotes angiogenesis, tumorigenicity, and their stem-like phenotype (72–74). Additionally, the secretion of pleiotrophin by $CD163^+$ TAMs in glioma fostered CSC-mediated tumor growth and the inhibition of this pathway led to decreased tumor growth and prolonged survival in mouse xenografts (75). Besides the paracrine interaction via soluble molecules, M2 macrophages in breast cancer interact with CSCs in a cell-cell-contact dependent manner (76). The ligation of CD90 to ephrin type-A receptor 4 (EphA4) on cancer cells induced downstream signaling that resulted in the production of IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF), thus facilitating the maintenance of the stem-cell-like niche. In summary, these findings indicate a predominant role of macrophages in driving the induction and maintenance of CSCs.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) play an important role in cancer-related immune suppression and are shown to significantly contribute to tumor progression, angiogenesis, and metastasis (77). Recently, several findings indicated that MDSCs are also involved in supporting the stem-cell features of CSCs. For example, MDSCs induce the expression of microRNA101 in ovarian cancer cells, promoting their stemness-properties and increasing the tumorigenic and metastatic potential (78). Additionally, MDSCs fostered the stemness of cervical cancer cells via the secretion of prostaglandin E2 (PGE2) (79). In breast cancer, MDSCs were shown to enhance the stem-like qualities of tumor cells by secretion of IL-6 and nitric oxide (NO) in a STAT3-dependent manner (80). Immunohistochemical analysis revealed that the presence of MDSCs positively correlated with the density of CSCs in breast cancer tissues and a high infiltration of MDSCs was associated with shorter overall survival, indicating their clinical relevance. Interestingly, STAT3 is also involved in the induction of monocytic-MDSCs in a mouse model of pancreatic cancer and monocytic-MDSCs subsequently increased the frequency of $ALDH1^{bright}$ CSCs (81).

T Cells

In general, tumors with a dense infiltration of $CD3^+$ T cells are associated with a good clinical outcome, yet recent advances in the understanding of the immune contexture revealed various mechanisms by which infiltrating T cells can act in an immunosuppressive manner, promoting tumor progression. The most prominent mediators of immunosuppression within the T cell compartment are Tregs, exhibiting both contact-dependent and cytokine-mediated actions to inhibit effector T cells and promote tumor progression (82, 83). While CSCs are able to support Treg accumulation in the tumor, in turn, Tregs can influence the CSC-niche. For example, Treg-conditioned

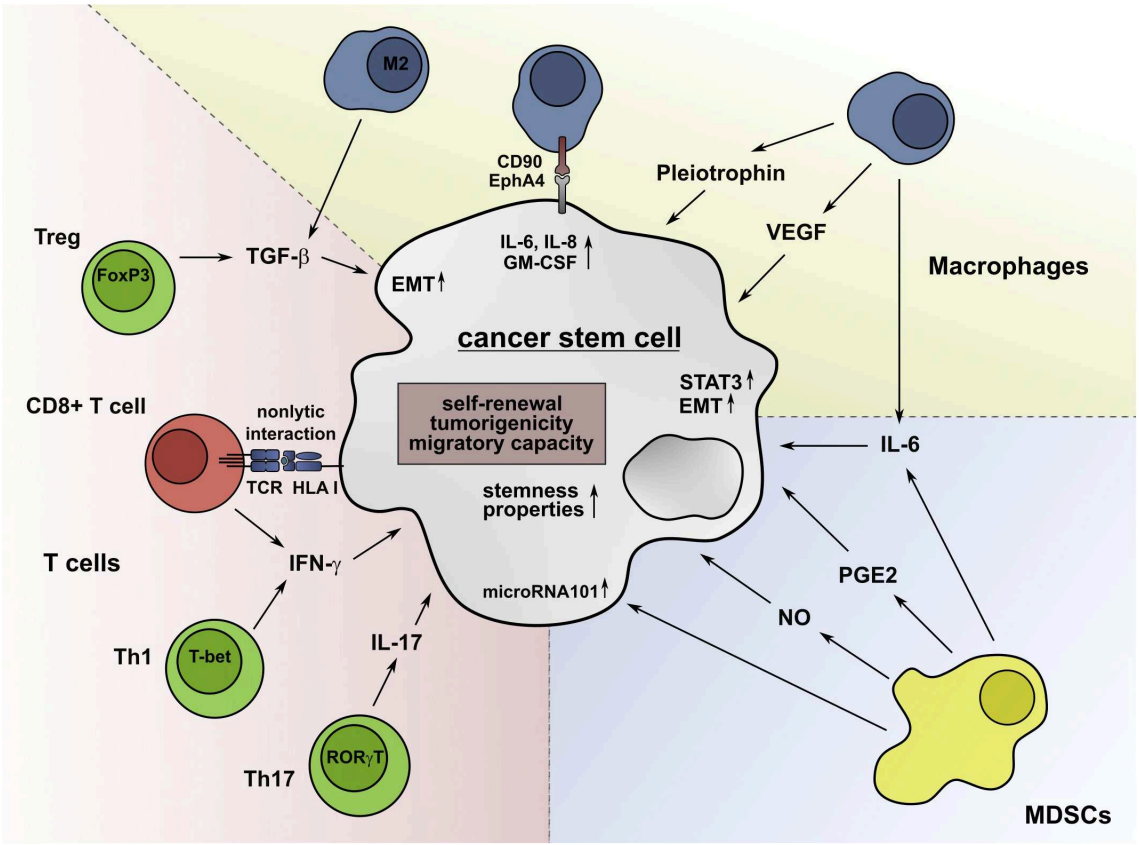


FIGURE 2 | Immune cell subsets promote stemness properties in CSCs. IL-6 secreted by TAMs can convert non-stem cancer cells into CSCs and foster their drug resistance. The secretion of TGF-β by M2 macrophages can support the EMT process and the acquisition of stem-cell properties. M2 macrophages also produce VEGF, which promotes angiogenesis and the tumorigenicity of CSCs. Furthermore, the secretion of pleiotrophin by TAMs supports the CSC-driven tumor growth through activation of the Akt pathway. Besides soluble molecules, macrophages can interact with CSCs via CD90 and EphA4 in a cell-cell-contact-dependent manner, inducing the production of IL-6, IL-8, and GM-CSF. MDSCs can facilitate the expression of microRNA101 in CSCs, which increases their tumorigenicity and metastatic potential. The secretion of NO and IL-6 by MDSCs leads to a STAT3-dependent increase of the stemness properties of CSCs. Furthermore, Tregs can enhance the expression of genes, which are associated with CSCs and secrete TGF-β, which promotes the EMT and dedifferentiation of cancer cells. Moreover, Th17 cells contribute to the formation of CSCs by secretion of IL-17. Low IFN-γ levels produced by Th1 cells and $CD8^+$ T cells enhance the stemness of tumor cells by activation of the Akt pathway. Non-lytic interactions of $CD8^+$ T cells further promote this process by inducing the expression of genes that are associated with cancer cell dedifferentiation.

medium increased the side population of mouse breast cancer cell lines, supported their sphere forming capability and enhanced the expression of Sox2, Nanog, and Oct4 genes, which are associated with stemness-properties (84). Additionally, TGF-β, a hallmark cytokine of Tregs, is well-known to mediate the EMT process and therefore support the generation of CSCs (85). Besides the mechanism of EMT, a recent study described the acquisition of stem cell properties due to a TGF-β driven dedifferentiation process in colorectal cancer cells (86).

In the past years, the T helper cell subset Th17 has also been implicated in mediating not only anti-tumor activity, but also immunosuppression (87, 88). The characteristic cytokine of this subset IL-17 promoted the self-renewal of ovarian CSCs and induced stem cell features in pancreatic cancer cells (89, 90). Similarly, IL-17 was associated with an increased capacity for invasion, migration, and tumorigenicity in both *in-vitro* and *in-vivo* studies of gastric cancer cells (91). Further experiments revealed that these effects are accompanied by an increase of phosphorylated STAT3, while the results significantly decreased upon blocking the STAT3 pathway, suggesting that IL-17 acts in a STAT3-dependent manner. Importantly, these studies were conducted irrespective of the source of IL-17. Even though Th17 cells are thought to be the main producers of IL-17, some studies suggest that innate immune cells account for the majority of IL-17^+ cells (92, 93). Additionally, hypoxia-induced expression of IL-17 by $FoxP3^+$ Tregs fostered the development of CSCs in colorectal cancer, although all of these findings emerged from *in-vitro* experiments (94). Besides immunosuppressive T cell subsets and cytokines, also low doses of interferon (IFN)-γ, which is mainly produced by activated Th1 cells or $CD8^+$ T cells, can increase the stemness of tumor cells in NSCLC (95). Furthermore, Stein and colleagues demonstrated that ineffective, non-lytic interactions of $CD8^+$ T cells with breast cancer cells induced the expression of genes associated with stemness and

dedifferentiation (96). Subsequent analysis of the generated tumors showed an increased proliferation, tumorigenicity, and capacity for metastasis.

Taken together, different T cell subsets, in addition to macrophages and MDSCs, assist CSCs to maintain their stem-cell-like state. The finding that CSCs themselves facilitate the recruitment or induction of Tregs within the tumor illustrates the strong bidirectional crosstalk between CSCs and various immune cell subsets which shapes both the TME and the CSC niche.

CONCLUSION

Emerging evidence suggests that not only genetic alterations determine the development and fate of the tumor, but also the phenotype and functional properties of infiltrating immune cells. As discussed in this review, CSCs are able to shape the TME by attracting immunosuppressive cell subsets and inhibiting effector T cells. Vice versa, infiltrating immune cells interact with CSCs in various ways to promote their self-renewal, tumorigenicity, and metastasis. These findings emphasize the unique role of CSCs and the immense potential that lies in targeting them. Consequently, therapeutic strategies leading to the elimination of CSCs in addition to non-stem cancer cells may further improve the clinical outcome for tumor patients. Many of the aforementioned CSC-immune cell interactions, including the generation of M2 macrophages and MDSCs, the CSC-dependent T cell suppression, the effect of IL-6 and IL-17 on the stemness properties of CSCs, and the expression of PD-L1 are dependent on active STAT3 signaling in CSCs or immune cells. Many of these effects could be reversed by inhibition of STAT3, rendering this molecule an attractive therapeutic target to tackle both the induction of an immunosuppressive TME and the emerging consolidation of the CSC-niche (25, 29, 39, 91). For example, the STAT3 inhibitor napabucasin was shown to reduce stemness gene expression and sphere formation in different entities (97–99). Furthermore, the SIRPα ligand CD47 is overexpressed by CSCs and represents another target structure for therapy. Several studies showed an increased phagocytosis of CSCs by macrophages upon blocking of CD47 and multiple CD47 inhibitors are tested in ongoing clinical trials (53–55, 100, 101). Additionally, CSCs were shown to express increased levels of the immune checkpoint PD-L1 and PD-L1 in turn promoted the generation of CSCs, creating a rationale for combination therapies with checkpoint inhibitors (1, 102). Furthermore, TGF-β secreted by Tregs and M2 macrophages or CSCs themselves is a crucial mediator of immunosuppression that can be targeted by neutralizing antibodies or receptor kinase inhibitors (103). The inhibition of the pro-angiogenic molecule VEGF has also been proven beneficial as combinational therapy in multiple entities and could be used to disrupt both the CSC-mediated angiogenesis and the induction of stemness-properties by macrophages (104, 105). In addition to targeting the crosstalk between CSCs and the TME, CSCs can be eliminated by using specific immunotherapeutic approaches, such as drug-conjugated monoclonal antibodies, bispecific antibodies, and chimeric antigen receptor- or T cell receptor-engineered T cells, targeting antigens that are characteristically expressed by CSCs (106–109).

The described studies exploring important immunmodulatory capabilities of CSCs and the impact of various immune cell subsets on cancer cells with stemness properties led to a deeper understanding of the bidirectional crosstalk between CSCs and the immune system. However, many studies used isolated CSCs to determine their phenotype and properties, which has several limitations and can significantly influence the results (110, 111). For example, the dissociation of solid tumors usually requires enzymatic treatment, which may result in the reduction or elimination of phenotypically and/or functionally relevant CSC-associated surface molecules. The different CSC isolation procedures may also reduce the viability of the purified cells. In addition, the characteristics of CSCs in solid tumors rely on the direct interaction of CSCs with various cellular components of the TME and the extracellular matrix, which is not appropriately considered, when utilizing isolated CSCs. To circumvent these limitations, advanced technologies to explore CSCs in intact tumors, such as lineage tracing approaches, may help to gain novel insights into the phenotype and properties of CSCs and may enable the design of improved therapies to target CSCs.

AUTHOR CONTRIBUTIONS

LM and ATu drafted the manuscript. IP, RW, ATe, DW, FM, MB, and MS reviewed and edited the manuscript.

REFERENCES

1. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science.* (2018) 359:1350–5. doi: 10.1126/science.aar4060
2. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J Clin Oncol.* (2015) 33:1974–82. doi: 10.1200/JCO.2014.59.4358
3. Kreso A, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell.* (2014) 14:275–91. doi: 10.1016/j.stem.2014.02.006
4. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature.* (1994) 367:645–8. doi: 10.1038/367645a0
5. Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, La Noce M, et al. Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. *FASEB J.* (2013) 27:13–24. doi: 10.1096/fj.12-218222
6. Karsten U, Goletz S. What makes cancer stem cell markers different? *Springerplus.* (2013) 2:301. doi: 10.1186/2193-1801-2-301
7. Moitra K. Overcoming multidrug resistance in cancer stem cells. *Biomed Res Int.* (2015) 2015:635745. doi: 10.1155/2015/635745
8. Vassalli G. Aldehyde dehydrogenases: not just markers, but functional regulators of stem cells. *Stem Cells Int.* (2019) 2019:3904645. doi: 10.1155/2019/3904645
9. Izumiya M, Kabashima A, Higuchi H, Igarashi T, Sakai G, Iizuka H, et al. Chemoresistance is associated with cancer stem cell-like properties and epithelial-to-mesenchymal transition in pancreatic cancer cells. *Anticancer Res.* (2012) 32:3847–53.

10. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med.* (2018) 24:541–50. doi: 10.1038/s41591-018-0014-x
11. Fridman WH, Zitvogel L, Sautès-Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. *Nat Rev Clin Oncol.* (2017) 14:717–34. doi: 10.1038/nrclinonc.2017.101
12. Cassetta L, Cassol E, Poli G. Macrophage polarization in health and disease. *Sci World J.* (2011) 11:2391–402. doi: 10.1100/2011/213962
13. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity.* (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
14. Murray PJ. Macrophage polarization. *Annu Rev Physiol.* (2017) 79:541–66. doi: 10.1146/annurev-physiol-022516-034339
15. Pantano F, Berti P, Guida FM, Perrone G, Vincenzi B, Amato MMC, et al. The role of macrophages polarization in predicting prognosis of radically resected gastric cancer patients. *J Cell Mol Med.* (2013) 17:1415–21. doi: 10.1111/jcmm.12109
16. Zhang M, He Y, Sun X, Li Q, Wang W, Zhao A, et al. A high M1/M2 ratio of tumor-associated macrophages is associated with extended survival in ovarian cancer patients. *J Ovarian Res.* (2014) 7:19. doi: 10.1186/1757-2215-7-19
17. Zhang S, Zhang E, Long J, Hu Z, Peng J, Liu L, et al. Immune infiltration in renal cell carcinoma. *Cancer Sci.* (2019) 110:1564–72. doi: 10.1111/cas.13996
18. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* (2002) 23:549–55. doi: 10.1016/S1471-4906(02)02302-5
19. Rhee I. Diverse macrophages polarization in tumor microenvironment. *Arch Pharm Res.* (2016) 39:1588–96. doi: 10.1007/s12272-016-0820-y
20. Yi L, Xiao H, Xu M, Ye X, Hu J, Li F, et al. Glioma-initiating cells: a predominant role in microglia/macrophages tropism to glioma. *J Neuroimmunol.* (2011) 232:75–82. doi: 10.1016/j.jneuroim.2010.10.011
21. Zhou W, Ke SQ, Huang Z, Flavahan W, Fang X, Paul J, et al. Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. *Nat Cell Biol.* (2015) 17:170–82. doi: 10.1038/ncb3090
22. Guo X, Zhao Y, Yan H, Yang Y, Shen S, Dai X, et al. Single tumor-initiating cells evade immune clearance by recruiting type II macrophages. *Genes Dev.* (2017) 31:247–59. doi: 10.1101/gad.294348.116
23. Jinushi M. Role of cancer stem cell-associated inflammation in creating pro-inflammatory tumorigenic microenvironments. *Oncoimmunology.* (2014) 3:e28862. doi: 10.4161/onci.28862
24. Zhang Q, Cai D-J, Li B. Ovarian cancer stem-like cells elicit the polarization of M2 macrophages. *Mol Med Rep.* (2015) 11:4685–93. doi: 10.3892/mmr.2015.3323
25. Wu A, Wei J, Kong L-Y, Wang Y, Priebe W, Qiao W, et al. Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro Oncol.* (2010) 12:1113–25. doi: 10.1093/neuonc/noq082
26. Kokubu Y, Tabu K, Muramatsu N, Wang W, Murota Y, Nobuhisa I, et al. Induction of protumoral CD11c(high) macrophages by glioma cancer stem cells through GM-CSF. *Genes Cells.* (2016) 21:241–51. doi: 10.1111/gtc.12333
27. Nusblat LM, Carroll MJ, Roth CM. Crosstalk between M2 macrophages and glioma stem cells. *Cell Oncol.* (2017) 40:471–82. doi: 10.1007/s13402-017-0337-5
28. Yamashina T, Baghdadi M, Yoneda A, Kinoshita I, Suzu S, Dosaka-Akita H, et al. Cancer stem-like cells derived from chemoresistant tumors have a unique capacity to prime tumorigenic myeloid cells. *Cancer Res.* (2014) 74:2698–709. doi: 10.1158/0008-5472.CAN-13-2169
29. Wei J, Barr J, Kong L-Y, Wang Y, Wu A, Sharma AK, et al. Glioblastoma cancer-initiating cells inhibit T-cell proliferation and effector responses by the signal transducers and activators of transcription 3 pathway. *Mol Cancer Ther.* (2010) 9:67–78. doi: 10.1158/1535-7163.MCT-09-0734
30. Chikamatsu K, Takahashi G, Sakakura K, Ferrone S, Masuyama K. Immunoregulatory properties of CD44+ cancer stem-like cells in squamous cell carcinoma of the head and neck. *Head Neck.* (2011) 33:208–15. doi: 10.1002/hed.21420
31. Stapelberg M, Zobalova R, Nguyen MN, Walker T, Stantic M, Goodwin J, et al. Indoleamine-2,3-dioxygenase elevated in tumor-initiating cells is suppressed by mitocans. *Free Radic Biol Med.* (2014) 67:41–50. doi: 10.1016/j.freeradbiomed.2013.10.003
32. Prendergast GC, Smith C, Thomas S, Mandik-Nayak L, Laury-Kleintop L, Metz R, et al. Indoleamine 2,3-dioxygenase pathways of pathgenic inflammation and immune escape in cancer. *Cancer Immunol Immunother.* (2014) 63:721–35. doi: 10.1007/s00262-014-1549-4
33. Mirzaei R, Sarkar S, Dzikowski L, Rawji KS, Khan L, Faissner A, et al. Brain tumor-initiating cells export tenascin-C associated with exosomes to suppress T cell activity. *Oncoimmunology.* (2018) 7:e1478647. doi: 10.1080/2162402X.2018.1478647
34. You Y, Li Y, Li M, Lei M, Wu M, Qu Y, et al. Ovarian cancer stem cells promote tumour immune privilege and invasion via CCL5 and regulatory T cells. *Clin Exp Immunol.* (2018) 191:60–73. doi: 10.1111/cei.13044
35. Bao S, Wu Q, Sathornsumetee S, Hao Y, Li Z, Hjelmeland AB, et al. Stem cell–like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res.* (2006) 66:7843–8. doi: 10.1158/0008-5472.CAN-06-1010
36. Folkins C, Shaked Y, Man S, Tang T, Lee CR, Zhu Z, et al. Glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1. *Cancer Res.* (2009) 69:7243–51. doi: 10.1158/0008-5472.CAN-09-0167
37. Voron T, Colussi O, Marcheteau E, Pernot S, Nizard M, Pointet A-L, et al. VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. *J Exp Med.* (2015) 212:139–48. doi: 10.1084/jem.20140559
38. Tomaso TD, Mazzoleni S, Wang E, Sovena G, Clavenna D, Franzin A, et al. Immunobiological characterization of cancer stem cells isolated from glioblastoma patients. *Clin Cancer Res.* (2010) 16:800–13. doi: 10.1158/1078-0432.CCR-09-2730
39. Lee Y, Shin JH, Longmire M, Wang H, Kohrt HE, Chang HY, et al. CD44+ cells in head and neck squamous cell carcinoma suppress T-cell-mediated immunity by selective constitutive and inducible expression of PD-L1. *Clin Cancer Res.* (2016) 22:3571–81. doi: 10.1158/1078-0432.CCR-15-2665
40. Wu Y, Chen M, Wu P, Chen C, Xu ZP, Gu W. Increased PD-L1 expression in breast and colon cancer stem cells. *Clin Exp Pharmacol Physiol.* (2017) 44:602–4. doi: 10.1111/1440-1681.12732
41. Zhi Y, Mou Z, Chen J, He Y, Dong H, Fu X, et al. B7H1 expression and epithelial-to-mesenchymal transition phenotypes on colorectal cancer stem-like cells. *PLoS ONE.* (2015) 10:e0135528. doi: 10.1371/journal.pone.0135528
42. Grau JJ, Mesía R, Iglesia-Vicente M de la, Williams ES, Taberna M, Caballero M, et al. Enrichment of cells with cancer stem cell-like markers in relapses of chemoresistant patients with locally advanced head and neck squamous cell carcinoma. *OCL.* (2016) 90:267–72. doi: 10.1159/000445118
43. Schatton T, Schütte U, Frank NY, Zhan Q, Hoerning A, Robles SC, et al. Modulation of T cell activation by malignant melanoma initiating cells. *Cancer Res.* (2010) 70:697–708. doi: 10.1158/0008-5472.CAN-09-1592
44. Morrison BJ, Steel JC, Morris JC. Reduction of MHC-I expression limits T-lymphocyte-mediated killing of Cancer-initiating cells. *BMC Cancer.* (2018) 18:469. doi: 10.1186/s12885-018-4389-3
45. Volonté A, Tomaso TD, Spinelli M, Todaro M, Sanvito F, Albarello L, et al. Cancer-initiating cells from colorectal cancer patients escape from T cell–mediated immunosurveillance *in vitro* through membrane-bound IL-4. *J Immunol.* (2014) 192:523–32. doi: 10.4049/jimmunol.1301342
46. Inoda S, Hirohashi Y, Torigoe T, Morita R, Takahashi A, Asanuma H, et al. Cytotoxic T lymphocytes efficiently recognize human colon cancer stem-like cells. *Am J Pathol.* (2011) 178:1805–13. doi: 10.1016/j.ajpath.2011.01.004
47. Tseng H-C, Arasteh A, Paranjpe A, Teruel A, Yang W, Behel A, et al. Increased lysis of stem cells but not their differentiated cells by natural killer cells; de-differentiation or reprogramming activates NK cells. *PLoS ONE.* (2010) 5:e11590. doi: 10.1371/journal.pone.0011590
48. Ames E, Canter RJ, Grossenbacher SK, Mac S, Chen M, Smith RC, et al. NK cells preferentially target tumor cells with a cancer stem cell phenotype. *J Immunol.* (2015) 195:4010–9. doi: 10.4049/jimmunol.1500447
49. Wang B, Wang Q, Wang Z, Jiang J, Yu S-C, Ping Y-F, et al. Metastatic consequences of immune escape from NK cell cytotoxicity by human breast cancer stem cells. *Cancer Res.* (2014) 74:5746–57. doi: 10.1158/0008-5472.CAN-13-2563

50. She M, Niu X, Chen X, Li J, Zhou M, He Y, et al. Resistance of leukemic stem-like cells in AML cell line KG1a to natural killer cell-mediated cytotoxicity. *Cancer Lett.* (2012) 318:173–9. doi: 10.1016/j.canlet.2011.12.017
51. Paczulla AM, Rothfelder K, Raffel S, Konantz M, Steinbacher J, Wang H, et al. Absence of NKG2D ligands defines leukaemia stem cells and mediates their immune evasion. *Nature.* (2019) 572:254–9. doi: 10.1038/s41586-019-1410-1
52. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD, et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell.* (2009) 138:286–99. doi: 10.1016/j.cell.2009.05.045
53. Theocharides APA, Jin L, Cheng P-Y, Prasolava TK, Malko AV, Ho JM, et al. Disruption of SIRPα signaling in macrophages eliminates human acute myeloid leukemia stem cells in xenografts. *J Exp Med.* (2012) 209:1883–99. doi: 10.1084/jem.20120502
54. Liu L, Zhang L, Yang L, Li H, Li R, Yu J, et al. Anti-CD47 antibody as a targeted therapeutic agent for human lung cancer and cancer stem cells. *Front Immunol.* (2017) 8:404. doi: 10.3389/fimmu.2017.00404
55. Cioffi M, Trabulo S, Hidalgo M, Costello E, Greenhalf W, Erkan M, et al. Inhibition of CD47 effectively targets pancreatic cancer stem cells via dual mechanisms. *Clin Cancer Res.* (2015) 21:2325–37. doi: 10.1158/1078-0432.CCR-14-1399
56. Lee TK-W, Cheung VC-H, Lu P, Lau EYT, Ma S, Tang KH, et al. Blockade of CD47-mediated cathepsin S/protease-activated receptor 2 signaling provides a therapeutic target for hepatocellular carcinoma. *Hepatology.* (2014) 60:179–91. doi: 10.1002/hep.27070
57. Sainz B, Carron E, Vallespinós M, Machado HL. Cancer stem cells and macrophages: implications in tumor biology and therapeutic strategies. *Mediators Inflamm.* (2016) 2016:9012369. doi: 10.1155/2016/9012369
58. Culig Z, Puhr M. Interleukin-6: a multifunctional targetable cytokine in human prostate cancer. *Mol Cell Endocrinol.* (2012) 360:52–8. doi: 10.1016/j.mce.2011.05.033
59. Waldner MJ, Foersch S, Neurath MF. Interleukin-6 - a key regulator of colorectal cancer development. *Int J Biol Sci.* (2012) 8:1248–53. doi: 10.7150/ijbs.4614
60. Chang CH, Hsiao CF, Yeh YM, Chang GC, Tsai YH, Chen YM, et al. Circulating interleukin-6 level is a prognostic marker for survival in advanced nonsmall cell lung cancer patients treated with chemotherapy. *Int J Cancer.* (2013) 132:1977–85. doi: 10.1002/ijc.27892
61. Dethlefsen C, Højfeldt G, Hojman P. The role of intratumoral and systemic IL-6 in breast cancer. *Breast Cancer Res Treat.* (2013) 138:657–64. doi: 10.1007/s10549-013-2488-z
62. Macciò A, Madeddu C. The role of interleukin-6 in the evolution of ovarian cancer: clinical and prognostic implications–a review. *J Mol Med.* (2013) 91:1355–68. doi: 10.1007/s00109-013-1080-7
63. Miura T, Mitsunaga S, Ikeda M, Shimizu S, Ohno I, Takahashi H, et al. Characterization of patients with advanced pancreatic cancer and high serum interleukin-6 levels. *Pancreas.* (2015) 44:756–63. doi: 10.1097/MPA.0000000000000335
64. Kumari N, Dwarakanath BS, Das A, Bhatt AN. Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumor Biol.* (2016) 37:11553–72. doi: 10.1007/s13277-016-5098-7
65. Jinushi M, Chiba S, Yoshiyama H, Masutomi K, Kinoshita I, Dosaka-Akita H, et al. Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells. *PNAS.* (2011) 108:12425–30. doi: 10.1073/pnas.1106645108
66. Wan S, Zhao E, Kryczek I, Vatan L, Sadovskaya A, Ludema G, et al. Tumor-associated macrophages produce interleukin 6 and signal via STAT3 to promote expansion of human hepatocellular carcinoma stem cells. *Gastroenterology.* (2014) 147:1393–404. doi: 10.1053/j.gastro.2014.08.039
67. Iliopoulos D, Hirsch HA, Wang G, Struhl K. Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. *Proc Natl Acad Sci USA.* (2011) 108:1397–402. doi: 10.1073/pnas.1018898108
68. Xie G, Yao Q, Liu Y, Du S, Liu A, Guo Z, et al. IL-6-induced epithelial-mesenchymal transition promotes the generation of breast cancer stem-like cells analogous to mammosphere cultures. *Int J Oncol.* (2012) 40:1171–9. doi: 10.3892/ijo.2011.1275
69. Kim S-Y, Kang JW, Song X, Kim BK, Yoo YD, Kwon YT, et al. Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. *Cell Signal.* (2013) 25:961–9. doi: 10.1016/j.cellsig.2013.01.007
70. Fan Q-M, Jing Y-Y, Yu G-F, Kou X-R, Ye F, Gao L, et al. Tumor-associated macrophages promote cancer stem cell-like properties via transforming growth factor-beta1-induced epithelial–mesenchymal transition in hepatocellular carcinoma. *Cancer Lett.* (2014) 352:160–8. doi: 10.1016/j.canlet.2014.05.008
71. Wu K, Ding J, Chen C, Sun W, Ning B-F, Wen W, et al. Hepatic transforming growth factor beta gives rise to tumor-initiating cells and promotes liver cancer development. *Hepatology.* (2012) 56:2255–67. doi: 10.1002/hep.26007
72. Oka N, Soeda A, Inagaki A, Onodera M, Maruyama H, Hara A, et al. VEGF promotes tumorigenesis and angiogenesis of human glioblastoma stem cells. *Biochem Biophys Res Commun.* (2007) 360:553–9. doi: 10.1016/j.bbrc.2007.06.094
73. Elaimy AL, Guru S, Chang C, Ou J, Amante JJ, Zhu LJ, et al. VEGF–neuropilin-2 signaling promotes stem-like traits in breast cancer cells by TAZ-mediated repression of the Rac GAP β2-chimaerin. *Sci Signal.* (2018) 11:eaao6897. doi: 10.1126/scisignal.aao6897
74. Mercurio AM. VEGF/Neuropilin signaling in cancer stem cells. *Int J Mol Sci.* (2019) 20:490. doi: 10.3390/ijms20030490
75. Shi Y, Ping Y-F, Zhou W, He Z-C, Chen C, Bian B-S-J, et al. Tumour-associated macrophages secrete pleiotrophin to promote PTPRZ1 signalling in glioblastoma stem cells for tumour growth. *Nat Commun.* (2017) 8:15080. doi: 10.1038/ncomms15080
76. Lu H, Clauser KR, Tam WL, Fröse J, Ye X, Eaton EN, et al. A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. *Nat Cell Biol.* (2014) 16:1105–17. doi: 10.1038/ncb3041
77. Gabrilovich DI. Myeloid-derived suppressor cells. *Cancer Immunol Res.* (2017) 5:3–8. doi: 10.1158/2326-6066.CIR-16-0297
78. Cui TX, Kryczek I, Zhao L, Zhao E, Kuick R, Roh MH, et al. Myeloid-derived suppressor cells enhance stemness of cancer cells by inducing microRNA101 and suppressing the corepressor CtBP2. *Immunity.* (2013) 39:611–21. doi: 10.1016/j.immuni.2013.08.025
79. Kuroda H, Mabuchi S, Yokoi E, Komura N, Kozasa K, Matsumoto Y, et al. Prostaglandin E2 produced by myeloid-derived suppressive cells induces cancer stem cells in uterine cervical cancer. *Oncotarget.* (2018) 9:36317–30. doi: 10.18632/oncotarget.26347
80. Peng D, Tanikawa T, Li W, Zhao L, Vatan L, Szeliga W, et al. Myeloid-derived suppressor cells endow stem-like qualities to breast cancer cells through IL6/STAT3 and NO/NOTCH cross-talk signaling. *Cancer Res.* (2016) 76:3156–65. doi: 10.1158/0008-5472.CAN-15-2528
81. Panni RZ, Sanford DE, Belt BA, Mitchem JB, Worley LA, Goetz BD, et al. Tumor-induced STAT3 activation in monocytic myeloid-derived suppressor cells enhances stemness and mesenchymal properties in human pancreatic cancer. *Cancer Immunol Immunother.* (2014) 63:513–28. doi: 10.1007/s00262-014-1527-x
82. Takeuchi Y, Nishikawa H. Roles of regulatory T cells in cancer immunity. *Int Immunol.* (2016) 28:401–9. doi: 10.1093/intimm/dxw025
83. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol.* (2010) 10:490–500. doi: 10.1038/nri2785
84. Xu Y, Dong X, Qi P, Ye Y, Shen W, Leng L, et al. Sox2 communicates with tregs through CCL1 to promote the stemness property of breast cancer cells. *Stem Cells.* (2017) 35:2351–65. doi: 10.1002/stem.2720
85. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. *Cell Res.* (2009) 19:156–72. doi: 10.1038/cr.2009.5
86. Nakano M, Kikushige Y, Miyawaki K, Kunisaki Y, Mizuno S, Takenaka K, et al. Dedifferentiation process driven by TGF-beta signaling enhances stem cell properties in human colorectal cancer. *Oncogene.* (2019) 38:780–93. doi: 10.1038/s41388-018-0480-0
87. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res.* (2011) 71:1263–71. doi: 10.1158/0008-5472.CAN-10-2907
88. Guéry L, Hugues S. Th17 cell plasticity and functions in cancer immunity. *Biomed Res Int.* (2015) 2015:314620. doi: 10.1155/2015/314620

89. Zhang Y, Zoltan M, Riquelme E, Xu H, Sahin I, Castro-Pando S, et al. Immune cell production of interleukin 17 induces stem cell features of pancreatic intraepithelial neoplasia cells. *Gastroenterology*. (2018) 155:210–23.e3. doi: 10.1053/j.gastro.2018.03.041
90. Xiang T, Long H, He L, Han X, Lin K, Liang Z, et al. Interleukin-17 produced by tumor microenvironment promotes self-renewal of $CD133^+$ cancer stem-like cells in ovarian cancer. *Oncogene*. (2015) 34:165–76. doi: 10.1038/onc.2013.537
91. Jiang Y-X, Yang S-W, Li P-A, Luo X, Li Z-Y, Hao Y-X, et al. The promotion of the transformation of quiescent gastric cancer stem cells by IL-17 and the underlying mechanisms. *Oncogene*. (2017) 36:1256–64. doi: 10.1038/onc.2016.291
92. Punt S, Fleuren GJ, Kritikou E, Lubberts E, Trimbos JB, Jordanova ES, et al. Angels and demons: Th17 cells represent a beneficial response, while neutrophil IL-17 is associated with poor prognosis in squamous cervical cancer. *Oncoimmunology*. (2015) 4:e984539. doi: 10.4161/2162402X.2014.984539
93. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol*. (2010) 10:479–89. doi: 10.1038/nri2800
94. Yang S, Wang B, Guan C, Wu B, Cai C, Wang M, et al. Foxp3+IL-17+ T cells promote development of cancer-initiating cells in colorectal cancer. *J Leukocyte Biol*. (2011) 89:85–91. doi: 10.1189/jlb.0910506
95. Song M, Ping Y, Zhang K, Yang L, Li F, Zhang C, et al. Low-dose IFNγ induces tumor cell stemness in tumor microenvironment of non-small cell lung cancer. *Cancer Res*. (2019) 79:3737–48. doi: 10.1158/0008-5472.CAN-19-0596
96. Stein RG, Ebert S, Schlahsa L, Scholz CJ, Braun M, Hauck P, et al. Cognate nonlytic interactions between CD8+ T cells and breast cancer cells induce cancer stem cell-like properties. *Cancer Res*. (2019) 79:1507–19. doi: 10.1002/9781119645214
97. Han D, Yu T, Dong N, Wang B, Sun F, Jiang D. Napabucasin, a novel STAT3 inhibitor suppresses proliferation, invasion and stemness of glioblastoma cells. *J Exp Clin Cancer Res*. (2019) 38:289. doi: 10.1186/s13046-019-1289-6
98. MacDonagh L, Gray SG, Breen E, Cuffe S, Finn SP, O'Byrne KJ, et al. BBI608 inhibits cancer stemness and reverses cisplatin resistance in NSCLC. *Cancer Lett*. (2018) 428:117–26. doi: 10.1016/j.canlet.2018.04.008
99. Li Y, Rogoff HA, Keates S, Gao Y, Murikipudi S, Mikule K, et al. Suppression of cancer relapse and metastasis by inhibiting cancer stemness. *Proc Natl Acad Sci USA*. (2015) 112:1839–44. doi: 10.1073/pnas.1424171112
100. Sikic BI, Lakhani N, Patnaik A, Shah SA, Chandana SR, Rasco D, et al. First-in-human, first-in-class phase i trial of the anti-CD47 antibody Hu5F9-G4 in patients with advanced cancers. *J Clin Oncol*. (2019) 37:946–53. doi: 10.1200/JCO.18.02018
101. Uger R, Johnson L. Blockade of the CD47-SIRPα axis: a promising approach for cancer immunotherapy. *Expert Opin Biol Th*. (2019) 20:5–8. doi: 10.1080/14712598.2020.1685976
102. Gupta HB, Clark CA, Yuan B, Sareddy G, Pandeswara S, Padron AS, et al. Tumor cell-intrinsic PD-L1 promotes tumor-initiating cell generation and functions in melanoma and ovarian cancer. *Sig Transduct Target Ther*. (2016) 1:1–9. doi: 10.1038/sigtrans.2016.30
103. Chen Y, Di C, Zhang X, Wang J, Wang F, Yan J-F, et al. Transforming growth factor β signaling pathway: a promising therapeutic target for cancer. *J Cell Physiol*. (2020) 235:1903–14. doi: 10.1002/jcp.29108
104. Cardones AR, Banez LL. VEGF inhibitors in cancer therapy. *Curr Pharm Des*. (2006) 12:387–94. doi: 10.2174/138161206775201910
105. Chen DS, Hurwitz H. Combinations of bevacizumab with cancer immunotherapy. *Cancer J*. (2018) 24:193–204. doi: 10.1097/PPO.0000000000000327
106. Weiner GJ. Building better monoclonal antibody-based therapeutics. *Nat Rev Cancer*. (2015) 15:361–70. doi: 10.1038/nrc3930
107. Trabolsi A, Arumov A, Schatz JH. T cell–activating bispecific antibodies in cancer therapy. *J Immunol*. (2019) 203:585–92. doi: 10.4049/jimmunol.1900496
108. Fesnak AD, June CH, Levine BL. Engineered T cells: the promise and challenges of cancer immunotherapy. *Nat Rev Cancer*. (2016) 16:566–81. doi: 10.1038/nrc.2016.97
109. Guo Y, Feng K, Wang Y, Han W. Targeting cancer stem cells by using chimeric antigen receptor-modified T cells: a potential and curable approach for cancer treatment. *Protein Cell*. (2018) 9:516–26. doi: 10.1007/s13238-017-0394-6
110. Batlle E, Clevers H. Cancer stem cells revisited. *Nat Med*. (2017) 23:1124–34. doi: 10.1038/nm.4409
111. Abbaszadegan MR, Bagheri V, Razavi MS, Momtazi AA, Sahebkar A, Gholamin M. Isolation, identification, and characterization of cancer stem cells: a review. *J Cell Physiol*. (2017) 232:2008–18. doi: 10.1002/jcp.25759

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 31 March 2020
doi: 10.3389/fimmu.2020.00526

The Interactions Between Cancer Stem Cells and the Innate Interferon Signaling Pathway

Laura Martin-Hijano [1,2,3] *and Bruno Sainz Jr.* [1,2,3]*

[1] *Cancer Stem Cell and Tumor Microenvironment Group, Department of Biochemistry, Universidad Autónoma de Madrid (UAM), Madrid, Spain,* [2] *Cancer Stem Cell and Tumor Microenvironment Group, Department of Cancer Biology, Instituto de Investigaciones Biomédicas "Alberto Sols" (IIBM), CSIC-UAM, Madrid, Spain,* [3] *Cancer Stem Cell and Tumor Microenvironment Group, Chronic Diseases and Cancer—Area 3, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain*

Interferons (IFNs) form a family of cytokines with pleiotropic effects that modulate the immune response against multiple challenges like viral infections, autoimmune diseases, and cancer. While numerous anti-tumor activities have been described for IFNs, IFNs have also been associated with tumor growth and progression. The effect of IFNs on apoptosis, angiogenesis, tumor cell immunogenicity, and modulation of immune cells have been largely studied; however, less is known about their specific effects on cancer stem cells (CSCs). CSCs constitute a subpopulation of tumor cells endowed with stem-like properties including self-renewal, chemoresistance, tumorigenic capacity, and quiescence. This rare and unique subpopulation of cells is believed to be responsible for tumor maintenance, metastatic spread, and relapse. Thus, this review aims to summarize and discuss the current knowledge of the anti- and pro-CSCs effects of IFNs and also to highlight the need for further research on the interplay between IFNs and CSCs. Importantly, understanding this interplay will surely help to exploit the anti-tumor effects of IFNs, specifically those that target CSCs.

Keywords: interferons, cancer stem cells, immune response, plasticity, immunoediting, quiescence

OPEN ACCESS

Edited by:
Cristina Maccalli,
Sidra Medicine, Qatar

Reviewed by:
Carlo Catapano,
Institute of Oncology Research (IOR), Switzerland
Kawaljit Kaur,
University of California, Los Angeles, United States

***Correspondence:**
Bruno Sainz Jr.
bsainz@iib.uam.es

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *12 December 2019*
Accepted: *09 March 2020*
Published: *31 March 2020*

Citation:
Martin-Hijano L and Sainz B Jr (2020) The Interactions Between Cancer Stem Cells and the Innate Interferon Signaling Pathway. Front. Immunol. 11:526. doi: 10.3389/fimmu.2020.00526

INTRODUCTION

Interferons

Interferons (IFNs) constitute a family of cytokines first described in the late 1950s for their ability to trigger a very potent anti-viral response in cells (1). All IFNs are class II α-helical cytokines that are classified into three main types: IFN-I (mainly IFN-α and -β) (2), IFN-II (IFN-γ) (3), and IFN-III (IFN-λ) (4) and their canonical signaling consists of activation of the JAK/STAT pathway (5).

IFNs are fundamental players in the modulation of both innate and adaptive immune responses. Although they were first identified as molecules with a strong capability of inducing viral resistance in cells, many other activities have been discovered for this family of cytokines over the years, including their involvement in pathologies such as autoimmune diseases [e.g., systemic lupus erythematosus (6–9) and rheumatoid arthritis (8, 10, 11)] and cancer (discussed below). IFNs, regardless of the specific receptor they activate, are able to exert pleiotropic effects, suggesting a rich signaling network coupled to IFN stimulation and undoubtedly adds complexity to understanding its effects on cell function and its contributions to immune response regulation.

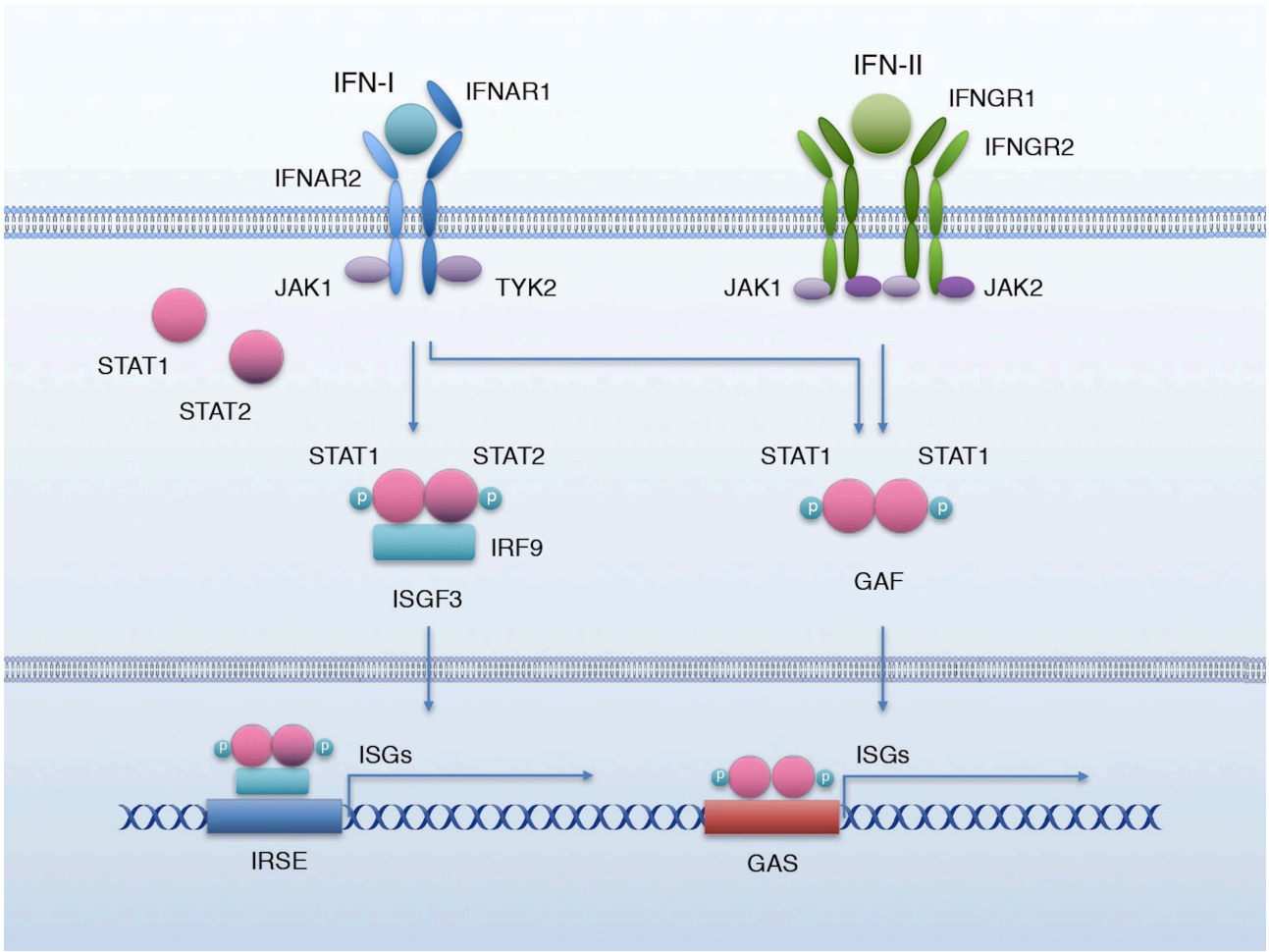


FIGURE 1 | IFN-I and IFN-II signaling. Canonical IFN-I and IFN-II signaling pathway. Binding of IFN-I to IFNAR activates JAK1 and TYK2 to phosphorylate STAT1 and STAT2, which associate with IRF9 to form the transcriptional complex ISGF3; ISGF3 translocates to the nucleus to bind the IRSE elements and activate the transcription of a battery of ISGs. IFN-II biding to its receptor IFNGR activates kinases JAK1 and JAK2, which phosphorylate STAT1; p-STAT1 forms a homodimer named GAF that translocates to the nucleus and activates the transcription of ISGs by binding GAS elements. IFN-I can also lead to the formation of GAF.

Type I Interferon (IFN-I)

IFN-I comprises multiple and diverse members; in mammals, 9 subtypes have been described: IFN-α (of which there are 13 known subtypes), IFN-β, -ε, -κ, -ω, -δ, -τ, -ν, and -ζ; all of them except -δ and -τ exist in humans (12). The level of homology between these members can range from 20% to nearly 100% (2). However, they all signal through the same receptor, the IFN-α receptor (IFNAR). IFN-I binds a heterodimeric receptor formed by IFNAR1 and IFNAR2 chains, causing their constitutively associated Janus kinases TYK2 and JAK1, respectively, to activate and phosphorylate signal transducer and activator of transcription 1 (STAT1) and 2 (STAT2). pY-STAT1 and pY-STAT2 then form a heterodimer that associates IRF9 to form a transcriptional activator complex named IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus, where it binds interferon-stimulated response elements (IRSE) to activate the transcription of a battery of interferon-stimulated genes (ISGs) (**Figure 1**). However, IFN-I also activates other non-canonical signaling pathways such as the MAPK (13, 14), PI3-Kinase (15–17), and NF-κB pathways (18, 19), as well as unphosphorylated STAT1 (U-STAT1) (5), that prolongs the expression of a subset of interferon-induced immune regulatory genes (20).

Regarding their immunomodulatory nature, IFN-I fulfills roles in both innate and adaptive immune responses, that include inducing cell-autonomous antiviral activity (21), stimulating immune cells, including natural killer (NK) (22–24) and T cells (25–29), and increasing antigen presentation by macrophages and dendritic cells (30), in order to help orchestrate an efficient immune response (5).

Type II Interferon (IFN-II)

IFN-II has only one member, IFN-γ, which is remarkably different from IFN-I in structure and has a different receptor, but was originally grouped in the IFN family due to its ability to trigger an antiviral response (31). Like other IFNs, IFN-γ also activates the JAK/STAT signaling pathway, through

IFNGR. This receptor is formed by two chains: IFNGR1 and IFNGR2. Binding of IFN-γ to its receptor activates the associated Janus kinases JAK1 and JAK2, respectively to IFNGR-1 and−2, to phosphorylate STAT1. pY-STAT1 forms homodimers, also known as interferon gamma-activated factor (GAF), that translocate to the nucleus to activate the transcription of a set of ISGs by binding the interferon-activated sites (GAS) (5) (**Figure 1**). Nonetheless, like IFN-I, IFN-γ can also activate other non-canonical signaling pathways such as MAPK (32, 33), PI3-Kinase (32, 33), and NF-κB (34, 35) and U-STAT1 (5, 20).

Functionally, IFN-γ also importantly contributes to the development of innate and adaptive immune responses, targeting mainly macrophages (36–39) and T cells (40–42). IFN-γ signaling induces the expression of many transcription factors, that can amplify the IFN response (5). Importantly, IFN-γ has a substantial role in modulating macrophage activation, as it upregulates the expression of gene products with microbicidal activity (43–46) and interacts with other cytokines and signaling molecules to enhance or antagonize their effect (47–50). Also, IFN-γ is capable of modulating helper T cell (Th) responses (51–53) and promoting class switching in B cells (54, 55). In addition, IFN-γ modulates the activity and recruitment of NK cells (56, 57). Interestingly, IFN-γ has been reported to either promote or repress NK cell-mediated lysis of tumor cells derived from diverse pediatric tumor cell lines (58). Treatment of the tumor cell lines with IFN-γ induced differential upregulation of MHC-class I and ICAM-I, which seemed to determine tumor cells' resistance or sensitivity, respectively, to NK cell-mediated lysis.

Type III Interferon (IFN-III)

Finally, interferon type III or IFN-λ is the latest class to be described, and it also shares the same antiviral functions as that of type I IFNs (2). The focus of this review will be on the effect that type I and type II IFNs have on cancer stem cells (CSCs) in different cancer entities.

Interferons in Cancer

Decades of research have demonstrated that IFNs are able to display a wide range of anti-tumor activities, including induction of apoptosis, inhibition of angiogenesis and proliferation, cell terminal differentiation and immune regulation. At the level of tumor cell survival, IFNs can induce tumor cell apoptosis through various mechanisms, such as the TRAIL pathway (59, 60), via CD95/Fas (61, 62) and the activation of pro-apoptotic members of the Bcl-2 family [reviewed by Kotredes and Gamero (63)]. Likewise, IFNs can impede tumor expansion by inducing cell cycle arrest. IFNs can up- or down-regulate CDK inhibitors and c-Myc expression, respectively, to inflict an anti-proliferative effect on tumor cells, amongst other mechanisms (64–67). However, IFNs have other indirect forms of fighting tumors, such as inducing oxygen and nutrients supply deprivation of tumor cells by suppressing angiogenesis, thus creating a hypoxic and acidic microenvironment. IFNs are also able to elicit inhibition of angiogenesis by downregulating the expression of potent angiogenic factors in endothelial and stromal cells, including IL-8, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), and in tumor cells, such as fibroblast growth factors (FGFs) (68–72). Furthermore, angiogenesis inhibition can result from IFN-mediated impairment of proliferation and migration of endothelial cells (70, 71, 73, 74).

Importantly, as already mentioned, IFNs are key regulators of the immune response against tumors. IFN-α, -β, and -γ are able to directly upregulate the expression of surface tumor-associated antigens (75, 76) via augmentation of MHC I class and MHC II class molecules (77), thus increasing the immunogenicity of tumor cells and making them more vulnerable to identification and subsequent destruction by the immune system. Indirect/unspecific immunoregulatory effects of IFNs encompass activation of dendritic cells to cross-present tumor antigens to T cells (78), promotion of full $CD8^+$ maturation necessary for them to elicit their cytotoxic effects (29, 79, 80), prevention of the proliferation of T regulatory cells, as well as enhancement of T helper cell function (81, 82) and promotion of macrophage polarization toward an M1-like pro-inflammatory state instead of the M2 pro-tumoral state (83), thus eluding their immunosuppressive effect (83, 84), amongst other mechanisms (85).

Alternatively, pro-tumoral properties have also been described for IFNs. While classically considered as pro-apoptotic agents, it has been shown that IFN-α/β activate the NF-κB pathway, inducing cell survival and protecting tumor cells against apoptotic stimuli in a variety of cancer types (86, 87). Also, IFNs can upregulate survival factors that protect cells against apoptotic stimuli, including MCL1, increased in myeloma presumably via STAT3 (88), and G1P3, which has been reported to promote tumor cell survival and contribute to poor outcome of patients in estrogen-receptor positive breast cancer (89). IFNs can also act as proliferative stimuli (90). For example, IFITM1 is an IFN-induced protein whose expression was shown to enhance lung cancer cell proliferation *in vitro* and tumor growth *in vivo* (91). In addition, IFITM1 expression was reported to promote invasion in head and neck cancer (92). Interestingly, IFN-α has been reported to induce endothelial cell proliferation, thus fomenting angiogenesis (93). One of the most recognized pro-tumoral activities of IFNs is the induction or overexpression of a subset of ISGs in distinct cancers, identified as an IFN-related DNA damage-resistant signature (IRDS), that confers tumor cell resistance to therapy (94, 95). Also, high expression of IRDS genes has been shown to promote tumor growth and metastasis (92, 96). Another major role of IFNs in cancer is immunomodulation and, in this regard, IFNs have been shown to promote immunoevasion via upregulation of the expression of MHC I class molecules, thus decreasing sensitivity to NK cells (97), downregulation of tumor-associated antigen presentation (98, 99), upregulation of the cytotoxic T cell inhibitor PDL-1 in tumor cells (100, 101), and promotion of a tumorigenic TME milieu (102).

Interferons as Anticancer Therapy

Intensive research focused on IFNs' anti-tumor activities finally led to the approval of IFN-α by the FDA as the first cancer immunotherapy in 1986 (103). In spite of being

discovered for their anti-viral activities, IFN-α2a and IFN-α2b have been used as anticancer therapeutic agents across multiple cancer types, including hairy cell leukemia, chronic myelogenous leukemia (CML) (104), AIDS-related Kaposi's sarcoma, follicular lymphoma, multiple myeloma, melanoma, condyloma acuminate, hepatocellular carcinoma (HCC), and cervical intraepithelial neoplasms (105, 106). IFN-β use as an anticancer drug is still under study, although ongoing phase III trials for melanoma (107, 108) and for glioma (109) and glioblastoma (110) are being conducted with promising results. However, IFN-β treatment studies in metastatic breast cancer have not been as successful (111). IFN-γ has also been explored as a therapy for cancer, showing some contrasting results. While IFN-γ treatment has proved to increase survival in ovarian cancer (112) and prevent recurrence in bladder cancer (113), it did not achieve the same results in other malignancies such as melanoma (114), leukemia (115), colorectal (116), and pancreatic cancers (117). Unfavorably, other preclinical studies have shown how IFN-γ upregulation leads to increased metastasis in melanoma (97) and breast cancer (118). It is worth noting that IFN treatment presents adverse side-effects ranging from a flu-like syndrome consisting of fever, chills, headache, myalgia, arthralgia, anorexia and fatigue (119), to neuropsychiatric symptoms, being depression a frequent disorder with a prevalence of 30–70% (120). These adverse effects are dose-limiting and may lead to treatment cessation in severe or longstanding treatment cases.

CSC Model

Cancer stem cells (CSCs) constitute a subpopulation of tumor cells endowed with stem-like properties such as tumorigenesis, metastatic dissemination potential, chemoresistance, and relapse (121). Nowadays, the most accepted CSC model proposes that, on the one hand, CSCs remain in a de-differentiated state, maintain their pluripotency and have unlimited self-renewal capacity. However, on the other hand, they can also differentiate into all possible cancer cell states that form a continuum, thus building the tumor hierarchy and giving rise to intratumoral heterogeneity (122). These unique abilities define CSCs as the sole drivers of tumorigenesis and tumor maintenance, and subsequently the cell entity that drives metastatic spread.

CSCs are pluripotent due to the reactivation of embryonal signaling pathways, such as Sonic Hedgehog (SHH), WNT, NOTCH, and Bone Morphogenetic Protein (BMP) (123). Other classical pluripotent genes expressed by these cells include *KLF4* (124), *NANOG* (125), *OCT3/4* (125, 126), *SOX2* (125–127), and the NODAL/ACTIVIN axis (128). CSCs are also characterized by the expression of stem-like markers, some of which are associated with a cancer type and some of which are more broadly expressed. Some of the most commonly used stem-like markers to identify CSCs are CD24, CD44, CD133, ALDH1, and CXCR4 (129–131). However, not every CSC express the same stem-like markers, the latter being due to the heterogeneity that exists within the CSC population. Genetically and/or epigenetically diverse CSC subpopulations possess different characteristics, that allow them to or preclude them from adapting to challenging situations such as nutrient deprivation, hypoxia, chemotherapy, or immune pressure. This unequal fitness of each CSC subpopulation drives either their clonal expansion or retreat (121), thus driving tumor evolution.

CSCs maintenance is supported by specific niches within the tumor. Importantly, interaction with the TME is crucial for CSC niche formation. A dynamic communication and influence occur between CSCs and the TME, thus assembling a balanced loop of reciprocal modeling. Of note, CSCs represent only a small percentage of the total number of tumor cells, but they are dispersedly located within different CSC niches and present distinct phenotypes. While some niches are spatially distinct (e.g., hypoxic and perivascular regions), others are defined by cellular interactions (e.g., immune niche). These tumor ecology dynamics have been elegantly described and reviewed by Prager et al. (132).

Implicit in this model is the idea/concept of CSC plasticity. Classically, the cellular populations with the ability to differentiate or transition into different lineages (e.g., hematopoietic stem cells) possess phenotypic plasticity. However, we now know this is not a unidirectional process, and that progenitor, transient and differentiated cells are able to regain stem-like properties that drives them to a pluripotent state (121). The latter is greatly influenced by stem cell niche factors. In this same way, CSC niches provide the needed signals for more differentiated cells to activate their plasticity and go back to a stem-like state if necessary. From a clinical perspective, not only CSC targeting but also CSC niche elimination would be necessary for complete cancer eradication.

EMT is a crucial process for activating plasticity and stemness. Between the pure epithelial (E) and pure mesenchymal (M) states, there is a spectrum of intermediate conditions, being the hybrid E/M state, with both epithelial and mesenchymal features, the state that represents the population of cells with the highest plasticity and stemness, increased therapy resistance, tumor-initiating capacity and metastatic potential (133). Importantly, these E/M hybrids with stem-like properties are able to form clusters, with increased apoptosis-resistance, that enter into the bloodstream where they collectively migrate to distant sites and colonize them more successfully than pure mesenchymal-state motile tumor cells (134, 135). As part of CSC plasticity, these cells are also able to enter a state of reversible quiescence, that is actively maintained. Quiescence protects CSCs from cell-cycle targeted therapies and grants them long-term survival through activation of environmental stress adaptive responses, including metabolic reprogramming and mechanisms that favor genomic integrity protection. In addition, these cells present high tumorigenic potential (136). Quiescent CSCs can exist within the tumor, as a subpopulation that does not contribute to tumor growth but that is greatly resistant to adverse conditions and can reactivate and re-enter the cell cycle when in the presence of certain ques or when more favorable conditions are achieved (137). They can also appear as disseminated dormant tumor cells, that are maintained in a non-proliferating state for long periods of time and can reactivate driving relapse and metastasis (136).

Chemoresistance is another hallmark of CSCs (138, 139). CSCs are invulnerable to conventional anticancer therapies, as they have an intrinsic chemo- and radio-resistant profile that enables their survival and clonal expansion over those cells

unable to resist therapeutic pressures. Expression of drug efflux pumps, such as ABCG2 and MDR1, not only allows CSCs to evade the lethal impact of chemotherapy (140), but these pumps also seem to promote stem-like capacities via facilitating the clearance of endogenous anti-tumorigenic molecules from the cell while redirecting pro-tumorigenic molecules to the cell's surface receptors (141). Other common mechanisms of chemoresistance are ALDH activity, expression of pro-survival BCL-2 protein family members and activation of several signaling pathways involved in chemo- and radio-resistance, including MYC and AKT1 (142).

Without a doubt, CSCs represent a population of highly complex tumor cells with unique properties that are responsible for tumor progression, chemoresistance, dormancy, and metastasis. At the same time, these cells are divided into unique subpopulations whose nature is driven/influenced by CSC niches, thus promoting different phenotypes (i.e., plasticity). With this in mind, the only way to successfully eradicate cancer would be to eliminate CSCs and simultaneously target the cues that promote CSC maintenance/plasticity or the source of those cues (i.e., CSCs niches).

It is well-known that IFNs can exert many different anti-tumor effects that negatively affect tumor viability, but less is known about their specific impact on CSCs. In fact, the few studies that have tested the relationship between IFNs and CSCs have yielded opposite and contradictory conclusions, showing both pro and anti-tumor activity. Thus, this review will try to set the story straight by discussing this complex relationship and provided data to support both sides.

STEMNESS AND TUMORIGENIC POTENTIAL

Interferon Type I

A recent study by Castiello et al. (143) showed how IFNAR1 silencing had a significant impact on the CSC subset in an HER2/neu transgenic mouse model (neuT) of breast cancer. Loss of functional IFNAR1 not only resulted in earlier onset and increased tumor multiplicity, but also in the presentation of a gene expression profile associated with aggressive human breast cancer. In line with these results, IFNAR$^{-/-}$ tumors showed an enrichment in the ALDH1^{+} CSC compartment, which demonstrated a greater self-renewal capacity *in vitro* and tumorigenic potential *in vivo*. These results clearly propose IFN-I as a negative regulator of stemness in breast cancer tumor cells. Accordingly, Doherty et al. (144) obtained similar conclusions when studying the role of IFN-β on triple-negative breast cancer (TNBC) CSCs, using an *in vitro* model of primary human mammary epithelial cells (HMEC) virally transduced with transforming factors. Within transformed cells, a subpopulation of mesenchymal-like cells with CSCs properties emerged (Mes/CSC), while the remaining cells maintained an epithelial phenotype and did not present such properties (Ep/non-CSC). Regarding IFN signaling, Mes/CSCs presented a basal repression of numerous ISGs, while Ep/non-CSCs had an IFN gene expression signature. Inhibition of ISG expression was attributed to upregulation of unphosphorylated ISG3F (U-ISG3F) in Mes/CSCs, which is part of the alternative IFN-I signaling pathway, although the origin of its activation remains unclear. In order to test whether IFN-β was able to reactivate the canonical IFN pathway, Mes/CSCs and Ep/non-CSCs were treated with IFN-β and CSCs properties were tested *in vitro*, showing a reversion of the CSC status of Mes/CSC cells. Moreover, IFN-β reactivated the expression of ISGs in Mes/CSCs by upregulating P-ISG3F. Therefore, activation of the canonical IFN-I pathway by IFN-β inhibited the stem-like capacities of Mes/CSCs in this model.

In support of this, Yuki et al. (145) had previously reported IFN-β to reduce proliferation, self-renewal capacity, and tumorigenesis in human glioma-initiating cells (GICs) by inducing their terminal differentiation into oligodendrocytes via STAT3 activation. Treatment of patient tumor-derived cells with IFN-β induced the phosphorylation and subsequent activation of STAT3, leading to a cell-cycle arrest in G0/G1, decreased clonogenic capacity, reduction of the expression of stem markers and, importantly, terminal differentiation of the GICs into oligodendrocytes. Significantly, STAT3 had been previously linked to gliogenesis by Bonni et al. (146) and Rajan and McKay (147), who described how Ciliary Neurotrophic Factor (CNTF)-mediated activation of STAT3 promoted the differentiation of cortical precursor cells and multipotent stem cells of the central nervous system, respectively, into astrocytes. More recently, STAT3 activation has been linked to regulation of human neural stem cell differentiation (148) and to promotion of the differentiation of NG2 cells (oligodendrocytes progenitors) into oligodendrocytes after a contusive spinal cord injury (149). Likewise, STAT3 has been shown to mediate IL-6-induced neuroendocrine differentiation in prostate cancer cells (150). This pro-differentiating role for STAT3 contradicts previous work describing its role in promoting CSCs traits among different cancer types (151–155), thus highlighting the importance of the tumor context. Illustrating this complex regulation, another study underscored the role of IFN-I as a repressor of glioblastoma stem-like cells (GSCs), as it appeared to inhibit the proliferation and self-renewal capacity of GSCs. However, the authors claim that IFN-I also inhibits the ability of GSCs to differentiate into astrocytes, since it only induces a transient activation of STAT3, while induction of astrocytic differentiation results from sustained activation of STAT3 (156).

Another interesting study tested the effects of IFN-β produced intracellularly on lung cancer murine cells (LL), avoiding external treatment of cells with the recombinant cytokine (157). For that purpose, LL cells were transduced with the mouse *ifn-b* (rBV/IFN-β) gene using a baculovirus vector (BV) and subjected to several tumor-specific assays. rBV/IFN-β cells showed a lower proliferation rate and, importantly, decreased anchorage-independent growth (i.e., CSC self-renewal), compared to control cells. Consistent with these results, a reduction in the tumorigenic and metastatic capacity of rBV/IFN-β cells was observed, strengthening the link between IFN-β and inhibition of stem-like capacities.

IFN-α has also been reported to specifically target the side population (SP) of ovarian cancer cells, a subset of cells endowed

with stem-like properties (i.e., CSCs) (158). In an attempt to exploit the anti-tumor effects of IFN-α, ovarian PDXs were subjected to gene therapy with IFN-α, and results showed a marked increase in survival rate in those PDXs bearing a high proportion of SP cells compared to those containing a low proportion, indicating that IFN-α specifically and negatively affects the CSC compartment. Accordingly, treatment of isolated SP cells with IFN-α resulted in decreased proliferation and self-renewal capacity of these cells and in a dramatic change in their transcriptional profile, compared to non-SP cells. Moreover, these findings were tested in CRC and Daoy medulloblastoma cells with similar results, indicating that this negative regulation of the CSC compartment could be extended to other cancer types.

In contrast to the CSC inhibitory role of IFN-I described above, other studies have come to different conclusions. For example, Ma et al. (159) revealed that IFN-α fostered stem-like properties in oral squamous cell carcinoma (OSCC) cells. Treatment of implanted tumor xenografts with IFN-α resulted in increased expression of stemness markers and tumor growth. Similar results at the level of stemness markers and increased self-renewal capacity were also observed *in vitro* with OSCC cells treated with IFN-α.

More recently, a robust link between death receptor CD95/Fas, IFN-I-dependent activation of STAT1 and stemness in different cancer types has been described by Qadir et al. (160). CD95 is an apoptosis-inducing death receptor, although it can also participate in a variety of tumor promoting activities. In fact, chronic stimulation of CD95 in tumor cells has been reported to increase the number of CSCs in breast cancer (161). In this work, the authors observed that long-term stimulation of CD95 in tumor cells led to type IFN-I production and secretion, and subsequent activation of the IFN-I pathway. In MCF-7 breast cancer cells, activation of the IFN-I pathway resulted in increased expression of stem-like markers. Moreover, cell sorting of MCF-7 breast cancer cells using the stem marker CD44 revealed that $CD44^+$ cells had higher levels of STAT1 expression than $CD44^-$ cells. In addition, treatment with IFNα/β induced/increased ALDH1 activity and self-renewal capacity. To further confirm the role of IFN-I as a driver of stemness, IFN-β pre-treated cells were used in a limiting dilution assay (LDA), which revealed the ability of IFN-β to enhance tumorigenic potential *in vivo*. These findings are not limited to one cancer type, as the authors were able to show similar results for GBM and squamous cell carcinoma (SSC). Interestingly, knocking-down STAT1 resulted in abrogation of STAT2 and STAT3 phosphorylation, concomitant with a loss of IFN-I-induced stem-like properties, suggesting the involvement of STAT2 and STAT3 activation in mediating the observed CSCs promoting effects of IFN-I in a STAT1-dependent manner. Overall, this thorough study strongly suggests IFN-I as a cancer stemness driver in breast cancer, SCC and GBM, involving activation of STAT1, STAT2, and STAT3.

In line with this, IFN-β has also been linked to tumor stemness promotion in pancreatic ductal adenocarcinoma (PDAC). Sainz et al. (162) described an intimate communication between tumor-associated macrophages (TAMs) and pancreatic CSCs in primary tumor tissues and derived cultures. Interestingly, PDAC cells polarized resident TAMs toward an M2 phenotype, which in turn actively secreted high levels of ISG15, an interferon-stimulated gene. ISG15 can act as a free molecule—intracellularly or in the tumor milieu—and it can also conjugate to proteins as a ubiquitin-like modifier through a process known as ISGylation (163). In this work, TAM-secreted ISG15 was found to enhance the stem-like properties of PDAC CSCs *in vivo* and *in vitro*, promoting their self-renewal, tumorigenic, chemoresistant and migratory capacities, in addition to higher levels of intracellular ISGylation, which have also been related to CSC promotion in nasopharyngeal carcinoma (164). Strikingly, TAMs secreted ISG15 in response to IFN-β secretion by pancreatic CSCs, thus establishing an intricate communication between CSCs and TAMs that resulted in reinforcement of stem-like properties in pancreatic CSCs. The fact that tumor cells (or CSCs) can secrete IFNs is not a novel concept. In 2011 Tsai et al. (165) described that ZR-75-1 breast cancer cells secreted elevated levels of IFN-β, which in turn contributed to Ras transformation. In addition, sarcoma, melanoma and leukemia tumor cells have been described to secrete IFN-α in response to doxorubicin treatment (166). Moreover, inflammatory breast cancer (IBC) cells have been reported to secrete high levels of IFN-α to the TME milieu, which contributed to increase its pro-tumorigenic character (102, 167). In addition to an IFN-α-secreting phenotype, IBC cells showed an upregulation of the IFN-α signaling pathway. Interestingly, Monsurrò et al. (168) identified two molecular phenotypes of PDAC based on differential expression of ISGs; the "anti-viral state" phenotype was characterized by increased resistance to oncolytic viral infection and was associated with activation of hypoxia pathways and increase of HLA proteins expression.

Interferon Type II

Regarding IFN-II, a study by Ni et al. (169) investigated the impact of IFN-γ on a specific subpopulation of quiescent colon CSCs (i.e., Label-retaining cancer cells or LRCCs), isolated from primary colon tumors based on PKH26/67 high staining. This work revealed that IFN-γ selectively targeted LRCCs due to their overexpression of IFNGR, compared to non-LRCCs. The authors showed that IFN-γ treatment of LRCCs greatly inhibited their self-renewal and tumorigenic capacities and induced apoptosis, while non-LRCCs were less affected. Therefore, in this context and in this model system, IFN-γ was proposed as a selective anti-CSC agent.

Another relevant study by Song et al. (170) explored the connection between endogenous IFN-γ levels and tumor stemness in a cohort of non-small cell lung cancer (NSCLC), esophageal squamous cell carcinoma (ESCC), CRC and HCC patients. Strikingly, the study revealed that low-IFN-γ levels in tumor interstitial fluid (TIF) strongly correlated with poor prognosis, TNM tumor staging, brain metastasis and chemoresistance. In line with this, NSCLC, ESCC, CRC, and HCC patients with low TIF-IFN-γ levels showed higher CD133 and Vimentin expression, as well as increased tumor stemness-related and EMT-related gene expression. *In vitro* treatment of NSCLC cell lines with high and low doses of IFN-γ revealed that low dose treatments increased the self-renewal capacity and expression of stem-like makers. In line with this observation, *in vivo* treatment of NSCLC-derived cell lines with a low IFN-γ

dose resulted in higher frequency of $CD133^+$ cells compared with those treated with a high IFN-γ dose, indicating an enrichment in the CSC compartment due to low IFN-γ stimulation. Dissection of the signaling cascade behind these effects revealed that low IFN-γ treatment of NSCLC cell lines induced I-CAM expression, which activated the PI3K-Akt-Notch1 axis leading to increased stemness. On the other hand, high IFN-γ doses induced apoptosis via the JAK1/STAT1/caspases pathway. This work not only illustrates the complex regulation of IFN signaling, but it also describes the opposing effects that can be achieved with the very same molecule using different dosing strategies. In addition, the results of this study are likely applicable to the immunoediting process, in which infiltrated effector T-cells and NK cells initially produce high levels of IFN-γ in the TME, resulting in tumor cell apoptosis. However, this initial immune response wave can eventually lead to T-cell and NK cell exhaustion and dysfunctional activity (171, 172), thus decreasing IFN-γ production and generating an IFN-γ-low TME with tumor stemness promoting capacity. Importantly, such a scenario could also occur during the earlier stages of tumor development in so-called "cold tumors" that are poorly infiltrated with immune cells. Interestingly, however, this study may also help to resolve the contradictory pro- and anti-tumor effects described above for both IFN-I and IFN-II, which may be the result of the doses of IFNs used across the different studies.

Very recently, Matteucci et al. (173) described and reviewed the pivotal role of human endogenous retrovirus (HERVs) activation in the promotion and maintenance of pluripotency and stem-like properties in melanoma CSCs. The authors also highlight the correlation between HERVs activation and aggressiveness features across several types of cancer. In this line, in the same year Cañadas et al. (174) described a very interesting interplay between IFN-γ and a particular subtype of HERVs named Stimulated 3 prime antisense retroviral coding sequences (SPARCS), which are located in the 3′ untranslated region of IFN-γ-inducible genes. Strikingly, IFN-γ induces the activation of SPARCS-containing genes—many of which are involved in innate immune regulation—resulting in the promotion of a more aggressive mesenchymal-like state of SCLC cells and in the production of cytosolic dsRNA through the bi-directional transcription of target genes. In turn, dsRNA can be sensed via the RIG-I/MAVS or the cGAS/STING pathways, which induce the production of IFNs, thus creating a positive feedback loop. Of note, IFN-γ induced the overexpression of PD-L1, which correlated with high baseline expression of the stem-like marker CD44. Moreover, deletion of *MAVS* significantly reduced the tumorigenic capacity of SCLC tumor cells. In summary, this work highlights the role of IFN-γ in activating the transcription of SPARCS and its impact on SCLC cells phenotype and opens the door to considering IFN-γ-induced SPARCS activation as a regulator of stem-like features in SCLC tumor cells.

INVASION, MIGRATION, AND METASTASIS

Interferon Type I

In ovarian cancer, Li et al. observed that IFI27, an IFN-α inducible protein, was upregulated in patient tumor tissue samples, compared to their paired healthy controls, and correlated with poor disease-free survival. The authors subsequently found IFI27 to not only be a driver of stemness (175), but this IFN-induced protein could also promote EMT, resulting in increased migration and invasion. It is well-known that EMT is one of the driving biological processes of stemness in tumor cells (176, 177), and in this work the authors make a very unique connection between EMT induction by an IFN-α stimulated gene and acquisition of stem-like properties such as increased self-renewal and drug resistance. In accordance with this observation, Zhu et al. (178) also described IFN-α as a promoter of stemness in PDAC. In an attempt to unveil possible differences of the effects of IFN-α on CSCs and non-CSCs, two PDAC cell lines with opposing stem markers levels were used: MiaPaca (low levels) and Panc1 (high levels). The authors showed in their study how IFN-α treatment of both PDAC cell lines reduced cell viability and proliferation *in vitro*, while simultaneously increasing the expression of CSCs cell surface markers, suggesting IFN-α induces a CSC enrichment, likely via killing off non-CSCs. In order to confirm these results *in vivo*, an orthotopic PDAC mouse model was used. While administration of IFN-α to mice reduced tumor volume in comparison to the control group, CSCs markers were significantly upregulated, suggesting again an enrichment in CSCs. Along these lines, IFN-α-treated mice presented more colon metastases compared to the non-IFN-treated control group. In summary, these results suggest that IFN-α treatment of PDAC cells leads to elimination of the tumor bulk cells resulting in an enrichment of the CSC compartment, concomitant with a boost in metastatic spread. However, based on the concept of plasticity, it is also feasible that non-CSCs converted into CSCs, contributing to the enrichment of the CSC population.

Interferon Type II

In head and neck squamous cell carcinomas (HNSCC), as in many other cancer types, the CXCL12/CXCR4 axis is involved in metastatic dissemination (179). As metastasis formation is one of the hallmarks of CSCs, CXCR4 is often used as a stem-like marker for the identification of CSCs with enhanced metastatic capacity (180). In this respect, Katayama et al. (181) performed a study to determine the effects of IFN-γ on CXCR4 expression and function in several HNSCC cell lines. Histological analysis of primary tumors and metastases from a cohort of 56 patients revealed high levels of CXCR4 in tumor cells, but not in healthy head and neck tissue, which correlated with poor prognosis. In addition, CXCL12 expression was barely detectable in the primary tumor stromal tissue, but was strongly expressed in metastatic lymph node stroma, illustrating the CXCR4/CXCL12 axis as a highly plausible mechanism for metastatic spread in this cancer. In this study, the authors aimed to regulate CXCR4 levels in HNSCC cell lines using IFN-γ as an inhibitor, since IFN-γ had been previously shown to downregulate expression of CXCR4 in immune cells like neutrophils (182). Interestingly, they discovered that IFN-γ treatment induced a downregulation of CXCR4, and this downregulation translated into an inhibition in the migratory and invasive capacities of HNSCC cells, as well as CXCR4/CXCL12 axis-mediated cell proliferation. Thus, these

authors proposed IFN-γ as a modulator of CXCR4 functional expression and as an inhibitor of HNSCC cell migration induced by this receptor.

Interestingly, during the late 1980's, multiple studies explored the relationship between IFN-γ and metastasis in mice. Firstly, Taniguchi et al. (97) observed that treatment of H-2-deficient non-metastatic B16 melanoma cells with physiological doses of IFN-γ (1–10 U/ml) was sufficient to decrease cell growth *in vitro* and, surprisingly, to increase the lung-colonizing potential of these cells *in vivo*. Treatment with IFN-β was also able to induce the same metastatic phenotype, although a 1,000-fold higher concentration was required to observe similar effects. Investigating the mechanisms behind this IFN-γ-mediated or -enhanced metastasis, it appeared that IFN-γ induced a higher expression of surface H-2, that enabled tumor cells to resist NK-mediated killing. Other studies published in the very same year supported the main concept of Taniguchi et al.'s. work but in melanoma and colon cancer (183–186). More recent studies have also reported the capacity of IFN-γ to promote invasion and metastasis (187), and to act as a double-edge sword in cancer (188, 189). These results suggest that local endogenous IFN-γ released in the TME may play a pivotal role in modulating tumor cells' sensitivity to innate and adaptive immune cells and therefore in their capacity to colonize other organs and metastasize. Again, as shown by Song et al. above, the concentration of IFN-γ at a specific given time during the evolution of the tumor may be critical for IFN-γ to act as a pro- or anti-metastatic/invasive factor.

DORMANCY

Interferon Type I

It is generally recognized that tumor cell dormancy represents a major obstacle when it comes to effectively treating cancer, as dormant cells are more chemoresistant and upon treatment cessation, these cells can drive tumor relapse. In a recent study, Liu et al. (190) dissected the impact of IFN-β in melanoma CSCs, establishing a previously unknown association with dormancy. In this work, murine and human implanted tumors in mice were treated *in vivo* with IFN-β. Subsequent analysis of isolated single tumor cells revealed that IFN-β treated tumors had a higher proportion of G_0/G_1 cells, which were not senescent. In fact, sorting cells using the CSC cell surface marker CD133 revealed that while IFN-β treatment did not reduced the $CD133^+$ CSC compartment, IFN-β did induce cell cycle arrest in $CD133^+$ and not in $CD133^-$ cells, suggesting a specific effect of IFN-β on CSCs. Interestingly, both murine and human $CD133^+$ "tumor repopulating cell" (TRC)-derived tumors showed halted growth when treated with IFN-β and a quick re-growth after IFN-β withdrawal, indicating that IFN-β induces a reversible dormancy in melanoma cells. Further studies *in vitro* supported these findings. Specifically, IFN-β treatment of $CD133^+$ murine and human melanoma cells in soft 3D fibrin gels induced G_0/G_1 cell cycle arrest, expression of dormancy markers, decreased glucose consumption and higher resistance to chemotherapy, many of these features being hallmarks of CSCs. Consistently, IFN-β was not able to induce dormancy in 2D-cultured cells, which are conditions that favor cell differentiation over CSC enrichment. Moreover, knocking-down either STAT1 or STAT2 abolished the IFN-β-mediated quiescence induction in melanoma cells, confirming IFN-β as the driver of dormancy in these cells. Finally, a thorough study of the signaling pathway responsible for this effect underlined the IDO/Kyn/AhR cascade and serine-phosphorylation of STAT3 as the effectors, providing new insights into tumor dormancy mechanisms associated with IFNs.

Interferon Type II

A similar approach to the Liu et al. (190) study was conducted by the same group using IFN-γ (191), and similar results at the level of stemness promotion were obtained in murine TRCs (i.e., stem cell-like cancer cells that can repopulate tumors). Again, the authors showed that IFN-γ treatment resulted in IDO1/AhR-dependent p27 induction, that prevented STAT1 signaling, suppressing cell death and inducing tumor cell-dormancy in murine TRCs. Importantly, a similar effect with IFN-γ was also shown in human melanoma, breast cancer and HCC cell lines, again through the IDO/AhR/p27 pathway. While Liu et al. dissected the molecular signaling pathway behind IFN-γ-mediated tumor cell-dormancy, Farrar et al. (192) discovered in 1999 that IFN-γ produced by $CD8^+$ T cells played a major role in inducing tumor cell dormancy *in vivo*; however, the authors did not dissect the mechanism of action. In their study, a model of tumor dormancy was used, in which a murine B cell lymphoma (BCL_1) implanted in immunocompetent mice previously immunized with the BCL_1-derived Ig to orchestrate an anti-Id immune response could be induced into a dormant state. Adoptive transfer of Id-immune $CD8^+$ T cells into SCID mice administered with α-BCL_1-Ig, concomitant with α-IFN-γ antibodies, resulted in complete abrogation of the induction and maintenance of tumor dormancy. These results indicated that endogenous production of IFN-γ by $CD8^+$ T cells, in collaboration with humoral immunity, induced and maintained tumor cell dormancy *in vivo*. In line with this, Kmieciak et al. (193) reported 4 years later that $CD8^+$ T cell-produced IFN-γ was able to induce apoptosis in those tumor cells expressing high levels of IFNGR, while those expressing low levels entered into a quiescent state. In addition, relapsed tumor-cells presented increased expression of cell surface stem-like markers and higher tumorigenic capacity *in vivo*, thus connecting IFN-γ stimulation in a subset of tumor cells with a quiescent phenotype and a subsequent enrichment in the CSC compartment after tumor regrowth.

DISCUSSION

The regulation of IFN signaling has been extensively investigated, and yet there are still many aspects that are not fully understood and many questions remain unresolved. An example is the question of how IFN-α and -β are able to exert different effects on cells while signaling through the same receptor—IFNAR—via the JAK/STAT pathway. We now know that IFN stimulation and subsequent downstream effects are highly dependent on the cell type, IFN dose and the cell surface-receptor density in the stimulated cell. Likewise, factors behind the regulation of IFN

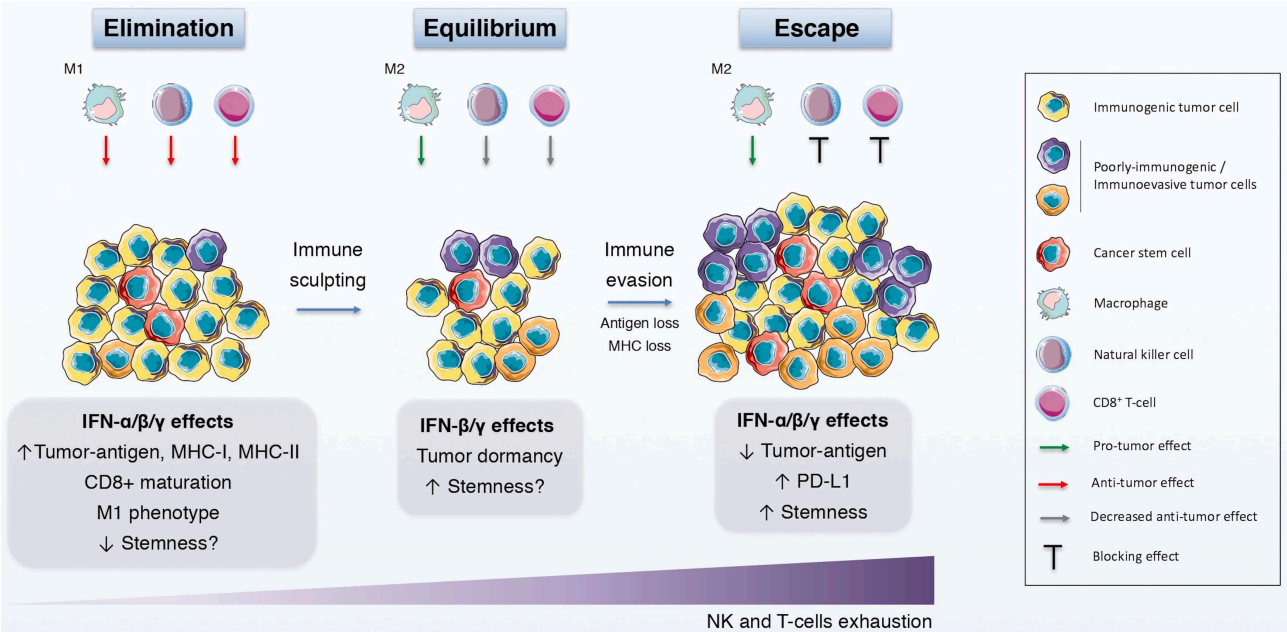


FIGURE 2 | IFNs in the immunoediting process. Schematic representation of the immunoediting process, which is divided in the elimination, equilibrium and escape phases, and how IFNs affects this process. During the elimination phase, both innate and adaptive immune systems identify and eliminate immunogenic tumor cells with the help of the anti-tumor effects of IFN. The immune pressure gives rise to the selection of poorly-immunogenic tumor cells and to a static phase, the equilibrium, in which the growth and elimination of tumor cells is balanced and the quiescence state is promoted, in part by IFNs. Finally, tumor evolution favors the induction and selection of immunoevasive features on tumor cells, some of which are elicited by IFNs, thus driving tumor survival and growth.

TABLE 1 | Anti- and pro-CSC effects of IFN-I and IFN-II.

Anti-CSC effects	Pro-CSC effects
IFN-I	
Decreased expression of stem-like markers and/or pluripotency genes (1–3)	Increased expression of stem-like markers and/or pluripotency genes (4–6)
Reduced self-renewal capacity (1, 2, 7–10)	Increased self-renewal capacity (4–6)
Reduced tumorigenic potential (1, 7, 9)	Increased tumorigenic potential (5, 6)
Reduced proliferation (7, 9, 10)	Chemoresistance (6)
Reduced metastatic potential (9)	Increased migratory/invasive and/or metastatic capacities (3, 6)
	Induction of dormancy (11)
IFN-II	
Reduced self-renewal capacity (14)	Increased self-renewal capacity (12, 15)
Reduced tumorigenic potential (14)	Increased tumorigenic potential (13)
Reduced migratory/invasive capacities (16)	Activation of EMT and/or migration/invasion (12, 15)
	Increased expression of stem-like markers and/or pluripotency genes (12, 13)
	Increased metastatic potential (15, 17–22)
	Chemoresistance (15)
	Induction of dormancy (23–25)

receptor presentation and IFN secretion levels are numerous and vary (2). This scenario highlights the importance of the cellular and environmental context in which a cell is stimulated by IFNs, and CSCs are no exception. Thus, more research is needed to fully characterize and dissect the factors that mediate the different responses of distinct CSCs to IFNs, described in this review. While we have put forth several possible explanations, including IFN dosing, more studies are still needed. Nevertheless, it is highly likely that what we will discover are cell-type specific effects. For example, regarding IFNs and dormancy, it is known that IFN-α is able to activate dormant hematopoietic stem cells (HSCs), inducing them to proliferate and making them more vulnerable to anti-cycling therapies such as 5-fluorouracil (194); however, while CSCs share many common features with normal stem cells, they also possess an aberrant malignant behavior based in part on a very different signaling circuitry. Thus, the very same stimulus can have completely different effects on normal- and cancer- stem cells. This is certainly the case with respect to the dormancy-specific studies described in this manuscript, which demonstrate that IFN-β and -γ are dormancy drivers (190–193). To complicate the matter further, acute exposure of HSCs to IFN-I has been shown to induce quiescence exit and promote proliferation; however, far from leading to HSC pool exhaustion, chronic exposure to IFN-I reestablished the HSC quiescent state and induced protection from the killing effects of IFN-I (195). These findings highlight the importance of advancing research focused on IFN pathway regulation, since IFNs (specially IFN-α) have been proposed as "awakening" agents for dormant CSCs. Despite these findings described for HSC, it is yet to be demonstrated whether acute and chronic exposure of other CSCs to IFN-I induces the same effects as those described

for HSCs, but caution should be taken when exploring the therapeutic effects of IFNs on CSCs, specifically at the level of dormancy.

Finally and more interestingly, the concept of immunoediting might prove beneficial to further explain the contradictory conclusions regarding the effect of IFNs on CSCs (196) (**Figure 2**) (**Table 1**). Briefly, cancer immunoediting refers to a complex interplay between tumor cells and the host immune system that can be divided into three phases: elimination (immunosurveillance), equilibrium (quiescent state) and escape (immunoevasion) [reviewed by McCoach and Bivona (197)]. Thus, depending on the molecular and functional traits of a CSC subset at a certain time during tumor progression, IFNs would be able to boost or shut down that subpopulation. Although knowledge of how CSCs participate in cancer immunoediting is now expanding (198, 199), less is known about the role of IFNs in that interplay. Exploring this field would surely contribute significantly to a better understanding of the dynamics and relationship that exists between IFNs and CSCs.

In conclusion, IFNs comprise a family of cytokines with pleiotropic effects, and among the many effects attributed to IFNs and their signaling pathways, growing evidence now validates a unique role for these cytokines in CSC biology. IFNs are able to display both pro- and anti-CSCs effects, depending on the context, including synergistic effects with other cytokines. For this reason, further research is needed in order to build a more comprehensive perspective of these contradictory roles with the hope of being able to exploit the anti-tumor effects of IFNs and at the same time downregulate their pro-CSCs capabilities as a means of targeting CSCs to improve cancer patient overall survival.

AUTHOR CONTRIBUTIONS

BS and LM-H developed the idea and edited the text. LM-H wrote the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Isaacs A, Lindenmann J, Andrewes CH. Virus interference. I. The interferon. *Proc R Soc Lond Ser B Biol Sci*. (1957) 147:258–67. doi: 10.1098/rspb.1957.0048
2. de Weerd NA, Nguyen T. The interferons and their receptors–distribution and regulation. *Immunol Cell Biol*. (2012) 90:483–91. doi: 10.1038/icb.2012.9
3. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its Receptor. *Annu Rev Immunol*. (1993) 11:571–611. doi: 10.1146/annurev.iy.11.040193.003035
4. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol*. (2003) 4:69–77. doi: 10.1038/ni875
5. Majoros A, Platanitis E, Kernbauer-Hölzl E, Rosebrock F, Müller M, Decker T. Canonical and non-canonical aspects of JAK–STAT signaling: lessons from interferons for cytokine responses. *Front Immunol*. (2017) 8:29. doi: 10.3389/fimmu.2017.00029
6. Hooks JJ, Jordan GW, Cupps T, Moutsopoulos HM, Fauci AS, Notkins AL. Multiple interferons in the circulation of patients with systemic lupus erythematosus and vasculitis. *Arthritis Rheum*. (1982) 25:396–400. doi: 10.1002/art.1780250406
7. Preble OT, Black RJ, Friedman RM, Klippel JH, Vilcek J. Systemic lupus erythematosus: presence in human serum of an unusual acid-labile leukocyte interferon. *Science*. (1982) 216:429–31. doi: 10.1126/science.6176024
8. Skurkovich SV, Eremkina EI. The probable role of interferon in allergy. *Ann Allergy*. (1975) 35:356–60.
9. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science*. (2001) 294:1540–3. doi: 10.1126/science.1064890
10. van der Pouw Kraan TCTM, Wijbrandts CA, van Baarsen LGM, Voskuyl AE, Rustenburg F, Baggen JM, et al. Rheumatoid arthritis subtypes identified by genomic profiling of peripheral blood cells: assignment of a type I interferon signature in a subpopulation of patients. *Ann Rheum Dis*. (2007) 66:1008–14. doi: 10.1136/ard.2006.063412
11. Castañeda-Delgado JE, Bastián-Hernandez Y, Macias-Segura N, Santiago-Algarra D, Castillo-Ortiz JD, Alemán-Navarro AL, et al. Type I interferon gene response is increased in early and established rheumatoid arthritis and correlates with autoantibody production. *Front Immunol*. (2017) 8:285. doi: 10.3389/fimmu.2017.00285
12. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev*. (2004) 202:8–32. doi: 10.1111/j.0105-2896.2004.00204.x
13. Uddin S, Majchrzak B, Woodson J, Arunkumar P, Alsayed Y, Pine R, et al. Activation of the p38 mitogen-activated protein kinase by type I interferons. *J Biol Chem*. (1999) 274:30127–31. doi: 10.1074/jbc.274.42.30127
14. David M, Petricoin E, Benjamin C, Pine R, Weber MJ, Larner AC. Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. *Science*. (1995) 269:1721–3. doi: 10.1126/science.7569900
15. Uddin S, Fish EN, Sher DA, Gardziola C, White MF, Platanias LC. Activation of the phosphatidylinositol 3-kinase serine kinase by IFN-alpha. *J Immunol*. (1997) 158:2390–7.
16. Uddin S, Yenush L, Sun XJ, Sweet ME, White MF, Platanias LC. Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3'-kinase. *J Biol Chem*. (1995) 270:15938–41. doi: 10.1074/jbc.270.27.15938
17. Kaur S, Uddin S, Platanias LC. The PI3' kinase pathway in interferon signaling. *J Interferon Cytokine Res*. (2005) 25:780–7. doi: 10.1089/jir.2005.25.780
18. Yang CH, Murti A, Pfeffer SR, Kim JG, Donner DB, Pfeffer LM. Interferon alpha /beta promotes cell survival by activating nuclear factor kappa B through phosphatidylinositol 3-kinase and Akt. *J Biol Chem*. (2001) 276:13756–61. doi: 10.1074/jbc.M011006200
19. Du Z, Wei L, Murti A, Pfeffer SR, Fan M, Yang CH, et al. Non-conventional signal transduction by type 1 interferons: the NF-kappaB pathway. *J Cell Biochem*. (2007) 102:1087–94. doi: 10.1002/jcb.21535
20. Cheon H, Stark GR. Unphosphorylated STAT1 prolongs the expression of interferon-induced immune regulatory genes. *Proc Natl Acad Sci USA*. (2009) 106:9373–8. doi: 10.1073/pnas.0903487106
21. Schneider WM, Chevillotte MD, Rice CM. Interferon-Stimulated genes: a complex web of host defenses. *Annu Rev Immunol*. (2014) 32:513–45. doi: 10.1146/annurev-immunol-032713-120231
22. Madera S, Rapp M, Firth MA, Beilke JN, Lanier LL, Sun JC. Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide. *J Exp Med*. (2016) 213:225–33. doi: 10.1084/jem.20150712
23. Biron CA, Sonnenfeld G, Welsh RM. Interferon induces natural killer cell blastogenesis *in vivo*. *J Leukoc Biol*. (1984) 35:31–7. doi: 10.1002/jlb.35.1.31
24. Martinez J, Huang X, Yang Y. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection *in vivo*. *J Immunol*. (2008) 180:1592–7. doi: 10.4049/jimmunol.180.3.1592
25. Le Bon A, Etchart N, Rossmann C, Ashton M, Hou S, Gewert D, et al. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat Immunol*. (2003) 4:1009–15. doi: 10.1038/ni978

26. Brinkmann V, Geiger T, Alkan S, Heusser CH. Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. *J Exp Med*. (1993) 178:1655–63. doi: 10.1084/jem.178.5.1655
27. Marrack P, Kappler J, Mitchell T. Type I interferons keep activated t cells alive. *J Exp Med*. (1999) 189:521–30. doi: 10.1084/jem.189.3.521
28. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med*. (2005) 202:637–50. doi: 10.1084/jem.20050821
29. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J Immunol*. (2005) 174:4465–9. doi: 10.4049/jimmunol.174.8.4465
30. Gessani S, Conti L, Cornò MD, Belardelli F. Type I Interferons as regulators of human antigen presenting cell functions. *Toxins*. (2014) 6:1696–723. doi: 10.3390/toxins6061696
31. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol*. (2005) 5:375. doi: 10.1038/nri1604
32. Nguyen H, Ramana CV, Bayes J, Stark GR. Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression. *J Biol Chem*. (2001) 276:33361–8. doi: 10.1074/jbc.M105070200
33. Rosner D, Stoneman V, Littlewood T, McCarthy N, Figg N, Wang Y, et al. Interferon-γ induces fas trafficking and sensitization to apoptosis in vascular smooth muscle cells via a PI3K- and Akt-dependent mechanism. *Am J Pathol*. (2006) 168:2054–63. doi: 10.2353/ajpath.2006.050473
34. Lin Y, Jamison S, Lin W. Interferon-γ activates nuclear factor-κ B in oligodendrocytes through a process mediated by the unfolded protein response. *PLoS ONE*. (2012) 7:e36408. doi: 10.1371/journal.pone.0036408
35. Gough DJ, Levy DE, Johnstone RW, Clarke CJ. IFNgamma signaling-does it mean JAK-STAT? *Cytokine Growth Factor Rev*. (2008) 19:383–94. doi: 10.1016/j.cytogfr.2008.08.004
36. Dallagi A, Girouard J, Hamelin-Morrissette J, Dadzie R, Laurent L, Vaillancourt C, et al. The activating effect of IFN-γ on monocytes/macrophages is regulated by the LIF–trophoblast–IL-10 axis via Stat1 inhibition and Stat3 activation. *Cell Mol Immunol*. (2015) 12:326–41. doi: 10.1038/cmi.2014.50
37. Wu C, Xue Y, Wang P, Lin L, Liu Q, Li N, et al. IFN-γ primes macrophage activation by increasing phosphatase and tensin homolog via downregulation of miR-3473b. *J Immunol*. (2014) 193:3036–44. doi: 10.4049/jimmunol.1302379
38. Herbst S, Schaible UE, Schneider BE. Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. *PLoS ONE*. (2011) 6:e19105. doi: 10.1371/journal.pone.0019105
39. Held TK, Weihua X, Yuan L, Kalvakolanu DV, Cross AS. Gamma interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1. *Infect Immun*. (1999) 67:206–12. doi: 10.1128/IAI.67.1.206-212.1999
40. Haring JS, Corbin GA, Harty JT. Dynamic regulation of IFN-γ signaling in antigen-specific CD8+ T cells responding to infection. *J Immunol*. (2005) 174:6791–802. doi: 10.4049/jimmunol.174.11.6791
41. Refaeli Y, Van Parijs L, Alexander SI, Abbas AK. Interferon γ is required for activation-induced death of T lymphocytes. *J Exp Med*. (2002) 196:999–1005. doi: 10.1084/jem.20020666
42. Whitmire JK, Tan JT, Whitton JL. Interferon-γ acts directly on CD8+ T cells to increase their abundance during virus infection. *J Exp Med*. (2005) 201:1053–9. doi: 10.1084/jem.20041463
43. Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med*. (1983) 158:670–89. doi: 10.1084/jem.158.3.670
44. MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science*. (2003) 302:654–9. doi: 10.1126/science.1088063
45. Karupiah G, Xie QW, Buller RM, Nathan C, Duarte C, MacMicking JD. Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. *Science*. (1993) 261:1445–8. doi: 10.1126/science.7690156
46. Collazo CM, Yap GS, Sempowski GD, Lusby KC, Tessarollo L, Woude GFV, et al. Inactivation of Lrg-47 and Irg-47 reveals a family of interferon γ-inducible genes with essential, pathogen-specific roles in resistance to infection. *J Exp Med*. (2001) 194:181–8. doi: 10.1084/jem.194.2.181
47. Gao PQ, Sims SH, Chang DC, Deisseroth AB. Interferon-gamma priming effects in the activation and deactivation of ISGF3 in K562 cells. *J Biol Chem*. (1993) 268:12380–7.
48. Yoshida A, Koide Y, Uchijima M, Yoshida TO. IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. *Biochem Biophys Res Commun*. (1994) 198:857–61. doi: 10.1006/bbrc.1994.1122
49. Gough DJ, Messina NL, Hii L, Gould JA, Sabapathy K, Robertson APS, et al. Functional crosstalk between type I and II Interferon through the regulated expression of STAT1. *PLoS Biol*. (2010) 8:e1000361. doi: 10.1371/journal.pbio.1000361
50. Hu X, Ivashkiv LB. Cross-regulation of signaling pathways by interferon-γ: implications for immune responses and autoimmune diseases. *Immunity*. (2009) 31:539–50. doi: 10.1016/j.immuni.2009.09.002
51. Zhang Y, Apilado R, Coleman J, Ben-Sasson S, Tsang S, Hu-Li J, et al. Interferon gamma stabilizes the T helper cell type 1 phenotype. *J Exp Med*. (2001) 194:165–72. doi: 10.1084/jem.194.2.165
52. Tau GZ, von der Weid T, Lu B, Cowan S, Kvatyuk M, Pernis A, et al. Interferon γ signaling alters the function of T helper type 1 cells. *J Exp Med*. (2000) 192:977–86. doi: 10.1084/jem.192.7.977
53. Wensky A, Marcondes MCG, Lafaille JJ. The role of IFN-γ in the production of Th2 subpopulations: implications for variable Th2-mediated pathologies in autoimmunity. *J Immunol*. (2001) 167:3074–81. doi: 10.4049/jimmunol.167.6.3074
54. Mohr E, Cunningham AF, Toellner K-M, Bobat S, Coughlan RE, Bird RA, et al. IFN-γ produced by CD8 T cells induces T-bet–dependent and –independent class switching in B cells in responses to alum-precipitated protein vaccine. *Proc Natl Acad Sci USA*. (2010) 107:17292–7. doi: 10.1073/pnas.1004879107
55. Bossie A, Vitetta ES. IFN-gamma enhances secretion of IgG2a from IgG2a-committed LPS-stimulated murine B cells: implications for the role of IFN-gamma in class switching. *Cell Immunol*. (1991) 135:95–104. doi: 10.1016/0008-8749(91)90257-C
56. Melero I, Rouzaut A, Motz GT, Coukos G. T-cell and NK-cell infiltration into solid tumors: a key limiting factor for efficacious cancer immunotherapy. *Cancer Discov*. (2014) 4:522–6. doi: 10.1158/2159-8290.CD-13-0985
57. Pak-Wittel MA, Yang L, Sojka DK, Rivenbark JG, Yokoyama WM. Interferon-γ mediates chemokine-dependent recruitment of natural killer cells during viral infection. *Proc Natl Acad Sci USA*. (2013) 110:E50–9. doi: 10.1073/pnas.1220456110
58. Aquino-López A, Senyukov VV, Vlasic Z, Kleinerman ES, Lee DA. Interferon gamma induces changes in Natural Killer (NK) cell ligand expression and alters NK Cell-mediated lysis of pediatric cancer cell lines. *Front Immunol*. (2017) 8:391. doi: 10.3389/fimmu.2017.00391
59. Chawla-Sarkar M, Leaman DW, Borden EC. Preferential induction of apoptosis by interferon (IFN)-beta compared with IFN-alpha2: correlation with TRAIL/Apo2L induction in melanoma cell lines. *Clin Cancer Res*. (2001) 7:1821–31. Available online at: https://clincancerres.aacrjournals.org/
60. Cheriyath V, Glaser KB, Waring JF, Baz R, Hussein MA, Borden EC. G1P3, an IFN-induced survival factor, antagonizes TRAIL-induced apoptosis in human myeloma cells. *J Clin Invest*. (2007) 117:3107–17. doi: 10.1172/JCI31122
61. Bernassola F, Scheuerpflug C, Herr I, Krammer PH, Debatin K-M, Melino G. Induction of apoptosis by IFNγ in human neuroblastoma cell lines through the CD95/CD95L autocrine circuit. *Cell Death Differ*. (1999) 6:652–60. doi: 10.1038/sj.cdd.4400537
62. Ugurel S, Seiter S, Rappl G, Stark A, Tilgen W, Reinhold U. Heterogenous susceptibility to CD95-induced apoptosis in melanoma cells correlates with bcl-2 and bcl-x expression and is sensitive to modulation by interferon-γ. *Int J Cancer*. (1999) 82:727–36. doi: 10.1002/(SICI)1097-0215(19990827)82:5<727::AID-IJC17>3.0.CO;2-E
63. Kotredes KP, Gamero AM. Interferons as inducers of apoptosis in malignant cells. *J Interferon Cytokine Res*. (2013) 33:162–70. doi: 10.1089/jir.2012.0110

64. Shang D, Yang P, Liu Y, Song J, Zhang F, Tian Y. Interferon-α induces G1 cell-cycle arrest in renal cell carcinoma cells via activation of Jak-Stat signaling. *Cancer Invest*. (2011) 29:347–52. doi: 10.3109/07357907.2011.568566
65. Bekisz J, Baron S, Balinsky C, Morrow A, Zoon KC. Antiproliferative properties of type I and type II interferon. *Pharmaceuticals*. (2010) 3:994–1015. doi: 10.3390/ph3040994
66. Rosewicz S, Detjen K, Scholz A, von Marschall Z. Interferon-alpha: regulatory effects on cell cycle and angiogenesis. *Neuroendocrinology*. (2004) 80(Suppl. 1):85–93. doi: 10.1159/000080748
67. Sangfelt O, Erickson S, Grander D. Mechanisms of interferon-induced cell cycle arrest. *Front Biosci*. (2000) 5:D479–87. doi: 10.2741/A527
68. von Marschall Z, Scholz A, Cramer T, Schäfer G, Schirner M, Oberg K, et al. Effects of interferon alpha on vascular endothelial growth factor gene transcription and tumor angiogenesis. *J Natl Cancer Inst*. (2003) 95:437–48. doi: 10.1093/jnci/95.6.437
69. Sato N, Nariuchi H, Tsuruoka N, Nishihara T, Beitz JG, Calabresi P, et al. Actions of TNF and IFN-gamma on angiogenesis *in vitro*. *J Invest Dermatol*. (1990) 95:85S—9S. doi: 10.1111/1523-1747.ep12874809
70. Indraccolo S. Interferon-alpha as angiogenesis inhibitor: learning from tumor models. *Autoimmunity*. (2010) 43:244–7. doi: 10.3109/08916930903510963
71. Takano S, Ishikawa E, Matsuda M, Yamamoto T, Matsumura A. Interferon-β inhibits glioma angiogenesis through downregulation of vascular endothelial growth factor and upregulation of interferon inducible protein 10. *Int J Oncol*. (2014) 45:1837–46. doi: 10.3892/ijo.2014.2620
72. Sun T, Yang Y, Luo X, Cheng Y, Zhang M, Wang K, et al. Inhibition of tumor angiogenesis by interferon-γ by suppression of tumor-associated macrophage differentiation. *Oncol Res*. (2014) 21:227–35. doi: 10.3727/096504014X13890370410285
73. Friesel R, Komoriya A, Maciag T. Inhibition of endothelial cell proliferation by gamma-interferon. *J Cell Biol*. (1987) 104:689–96. doi: 10.1083/jcb.104.3.689
74. Albini A, Marchisone C, Del Grosso F, Benelli R, Masiello L, Tacchetti C, et al. Inhibition of angiogenesis and vascular tumor growth by interferon-Producing cells. *Am J Pathol*. (2000) 156:1381–93. doi: 10.1016/S0002-9440(10)65007-9
75. Boyer CM, Dawson DV, Neal SE, Winchell LF, Leslie DS, Ring D, et al. Differential induction by interferons of major histocompatibility complex-encoded and non-major histocompatibility complex-encoded antigens in human breast and ovarian carcinoma cell lines. *Cancer Res*. (1989) 49:2928–34.
76. Greiner JW, Hand PH, Noguchi P, Fisher PB, Pestka S, Schlom J. Enhanced expression of surface tumor-associated antigens on human breast and colon tumor cells after recombinant human leukocyte alpha-interferon treatment. *Cancer Res*. (1984) 44:3208–14. doi: 10.1016/B978-0-08-031739-7.50204-4
77. Dolei A, Capobianchi MR, Ameglio F. Human interferon-gamma enhances the expression of class I and class II major histocompatibility complex products in neoplastic cells more effectively than interferon-alpha and interferon-beta. *Infect Immun*. (1983) 40:172–6. doi: 10.1128/IAI.40.1.172-176.1983
78. Schiavoni G, Mattei F, Gabriele L. Type I interferons as stimulators of DC-mediated cross-priming: impact on anti-tumor response. *Front Immunol*. (2013) 4:483. doi: 10.3389/fimmu.2013.00483
79. Curtsinger JM, Mescher MF. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol*. (2010) 22:333–40. doi: 10.1016/j.coi.2010.02.013
80. Fuertes MB, Kacha AK, Kline J, Woo S-R, Kranz DM, Murphy KM, et al. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. *J Exp Med*. (2011) 208:2005–16. doi: 10.1084/jem.20101159
81. Pace L, Vitale S, Dettori B, Palombi C, La Sorsa V, Belardelli F, et al. APC activation by IFN-alpha decreases regulatory T cell and enhances Th cell functions. *J Immunol*. (2010) 184:5969–79. doi: 10.4049/jimmunol.0900526
82. Srivastava S, Koch MA, Pepper M, Campbell DJ. Type I interferons directly inhibit regulatory T cells to allow optimal antiviral T cell responses during acute LCMV infection. *J Exp Med*. (2014) 211:961–74. doi: 10.1084/jem.20131556
83. Duluc D, Corvaisier M, Blanchard S, Catala L, Descamps P, Gamelin E, et al. Interferon-gamma reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. *Int J Cancer*. (2009) 125:367–73. doi: 10.1002/ijc.24401
84. Watanabe MAE, Oda JMM, Amarante MK, Cesar Voltarelli J. Regulatory T cells and breast cancer: implications for immunopathogenesis. *Cancer Metastasis Rev*. (2010) 29:569–79. doi: 10.1007/s10555-010-9247-y
85. Parker BS, Rautela J, Hertzog PJ. Antitumour actions of interferons: implications for cancer therapy. *Nat Rev Cancer*. (2016) 16:131–44. doi: 10.1038/nrc.2016.14
86. Yang CH, Murti A, Pfeffer SR, Basu L, Kim JG, Pfeffer LM. IFNα/β promotes cell survival by activating NF-κB. *Proc Natl Acad Sci USA*. (2000) 97:13631–6. doi: 10.1073/pnas.250477397
87. Pfeffer LM. The role of nuclear factor κB in the interferon response. *J Interferon Cytokine Res*. (2011) 31:553–9. doi: 10.1089/jir.2011.0028
88. Puthier D, Thabard W, Rapp M, Etrillard M, Harousseau J, Bataille R, et al. Interferon alpha extends the survival of human myeloma cells through an upregulation of the Mcl-1 anti-apoptotic molecule. *Br J Haematol*. (2001) 112:358–63. doi: 10.1046/j.1365-2141.2001.02575.x
89. Cheriyath V, Kuhns MA, Jacobs BS, Evangelista P, Elson P, Downs-Kelly E, et al. G1P3, an interferon- and estrogen-induced survival protein contributes to hyperplasia, tamoxifen resistance and poor outcomes in breast cancer. *Oncogene*. (2012) 31:2222–36. doi: 10.1038/onc.2011.393
90. Asao H, Fu X-Y. Interferon-γ has dual potentials in inhibiting or promoting cell proliferation. *J Biol Chem*. (2000) 275:867–74. doi: 10.1074/jbc.275.2.867
91. Yan J, Jiang Y, Lu J, Wu J, Zhang M. Inhibiting of proliferation, migration, and invasion in lung cancer induced by silencing interferon-induced transmembrane protein 1 (IFITM1). *BioMed Res Int*. (2019) 2019:9085435. doi: 10.1155/2019/9085435
92. Hatano H, Kudo Y, Ogawa I, Tsunematsu T, Kikuchi A, Abiko Y, et al. IFN-induced transmembrane protein 1 promotes invasion at early stage of head and neck cancer progression. *Clin Cancer Res*. (2008) 14:6097–105. doi: 10.1158/1078-0432.CCR-07-4761
93. Gomez D, Reich NC. Stimulation of primary human endothelial cell proliferation by IFN. *J Immunol*. (2003) 170:5373–81. doi: 10.4049/jimmunol.170.11.5373
94. Duarte CW, Willey CD, Zhi D, Cui X, Harris JJ, Vaughan LK, et al. Expression signature of IFN/STAT1 signaling genes predicts poor survival outcome in glioblastoma multiforme in a subtype-specific manner. *PLoS ONE*. (2012) 7:e29653. doi: 10.1371/journal.pone.0029653
95. Weichselbaum RR, Ishwaran H, Yoon T, Nuyten DSA, Baker SW, Khodarev N, et al. An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer. *Proc Natl Acad Sci USA*. (2008) 105:18490–5. doi: 10.1073/pnas.0809242105
96. Khodarev NN, Roach P, Pitroda SP, Golden DW, Bhayani M, Shao MY, et al. STAT1 pathway mediates amplification of metastatic potential and resistance to therapy. *PLoS ONE*. (2009) 4:e5821. doi: 10.1371/journal.pone.0005821
97. Taniguchi K, Petersson M, Höglund P, Kiessling R, Klein G, Kärre K. Interferon gamma induces lung colonization by intravenously inoculated B16 melanoma cells in parallel with enhanced expression of class I major histocompatibility complex antigens. *Proc Natl Acad Sci USA*. (1987) 84:3405–9. doi: 10.1073/pnas.84.10.3405
98. Beatty GL, Paterson Y. IFN-γ can promote tumor evasion of the immune system *in vivo* by down-regulating cellular levels of an endogenous tumor antigen. *J Immunol*. (2000) 165:5502–8. doi: 10.4049/jimmunol.165.10.5502
99. Le Poole IC, Riker AI, Quevedo ME, Stennett LS, Wang E, Marincola FM, et al. Interferon-gamma reduces melanosomal antigen expression and recognition of melanoma cells by cytotoxic T cells. *Am J Pathol*. (2002) 160:521–8. doi: 10.1016/S0002-9440(10)64871-7
100. Abiko K, Matsumura N, Hamanishi J, Horikawa N, Murakami R, Yamaguchi K, et al. IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. *Br J Cancer*. (2015) 112:1501–9. doi: 10.1038/bjc.2015.101
101. Karasar P, Esendagli G. T helper responses are maintained by basal-like breast cancer cells and confer to immune modulation via upregulation of PD-1 ligands. *Breast Cancer Res Treat*. (2014) 145:605–14. doi: 10.1007/s10549-014-2984-9

102. Provance OK, Lewis-Wambi J. Deciphering the role of interferon alpha signaling and microenvironment crosstalk in inflammatory breast cancer. *Breast Cancer Res*. (2019) 21:59. doi: 10.1186/s13058-019-1140-1
103. *FDA-Approved Cancer Immunotherapies and CRI's Impact*. Cancer Res Inst. Available online at: https://www.cancerresearch.org/blog/april-2015/fda-approved-cancer-immunotherapies-cris-impact (accessed October 10, 2019).
104. Talpaz M, Hehlmann R, Quintás-Cardama A, Mercer J, Cortes J. Re-emergence of interferon-α in the treatment of chronic myeloid leukemia. *Leukemia*. (2013) 27:803–12. doi: 10.1038/leu.2012.313
105. Vacchelli E, Aranda F, Obrist F, Eggermont A, Galon J, Cremer I, et al. Trial watch: immunostimulatory cytokines in cancer therapy. *Oncoimmunology*. (2014) 3:e29030. doi: 10.4161/onci.29030
106. Zitvogel L, Galluzzi L, Kepp O, Smyth MJ, Kroemer G. Type I interferons in anticancer immunity. *Nat Rev Immunol*. (2015) 15:405–14. doi: 10.1038/nri3845
107. Namikawa K, Tsutsumida A, Mizutani T, Shibata T, Takenouchi T, Yoshikawa S, et al. Randomized phase III trial of adjuvant therapy with locoregional interferon beta versus surgery alone in stage II/III cutaneous melanoma: Japan clinical oncology group study (JCOG1309, J-FERON). *Jpn J Clin Oncol*. (2017) 47:664–7. doi: 10.1093/jjco/hyx063
108. Nakamura Y, Tanaka K, Shibata T, Mizusawa J, Mizutani T, Fukuda H, et al. Confirmatory trial of non-amputative digit preservation surgery in subungual melanoma: JCOG1602 (J-NAIL study). *J Clin Oncol*. (2018) 36:TPS9607. doi: 10.1200/JCO.2018.36.15_suppl.TPS9607
109. Kawaji H, Tokuyama T, Yamasaki T, Amano S, Sakai N, Namba H. Interferon-β and temozolomide combination therapy for temozolomide monotherapy-refractory malignant gliomas. *Mol Clin Oncol*. (2015) 3:909–13. doi: 10.3892/mco.2015.542
110. Wakabayashi T, Natsume A, Mizusawa J, Katayama H, Fukuda H, Sumi M, et al. JCOG0911 INTEGRA study: a randomized screening phase II trial of interferonβ plus temozolomide in comparison with temozolomide alone for newly diagnosed glioblastoma. *J Neurooncol*. (2018) 138:627–36. doi: 10.1007/s11060-018-2831-7
111. Repetto L, Giannessi PG, Campora E, Pronzato P, Vigani A, Naso C, et al. Tamoxifen and interferon-beta for the treatment of metastatic breast cancer. *Breast Cancer Res Treat*. (1996) 39:235–8. doi: 10.1007/BF01806190
112. Windbichler GH, Hausmaninger H, Stummvoll W, Graf AH, Kainz C, Lahodny J, et al. Interferon-gamma in the first-line therapy of ovarian cancer: a randomized phase III trial. *Br J Cancer*. (2000) 82:1138–44. doi: 10.1054/bjoc.1999.1053
113. Giannopoulos A, Constantinides C, Fokaeas E, Stravodimos C, Giannopoulou M, Kyroudi A, et al. The immunomodulating effect of interferon-gamma intravesical instillations in preventing bladder cancer recurrence. *Clin Cancer Res*. (2003) 9:5550–8. Available online at: https://clincancerres.aacrjournals.org/
114. Creagan ET, Schaid DJ, Ahmann DL, Frytak S. Disseminated malignant melanoma and recombinant interferon: analysis of seven consecutive phase II investigations. *J Invest Dermatol*. (1990) 95:188S—92S. doi: 10.1111/1523-1747.ep12875512
115. Talpaz M, Kurzrock R, Kantarjian H, Rothberg J, Saks S, Evans L, et al. A phase II study alternating alpha-2a-interferon and gamma-interferon therapy in patients with chronic myelogenous leukemia. *Cancer*. (1991) 68:2125–30. doi: 10.1002/1097-0142(19911115)68:10<2125::AID-CNCR2820681006>3.0.CO;2-Q
116. Brown TD, Goodman PJ, Fleming T, Macdonald JS, O'Rourke T, Taylor SA, et al. Phase II trial of recombinant DNA gamma-interferon in advanced colorectal cancer: a southwest oncology group study. *J Immunother*. (1991) 10:379–82. doi: 10.1097/00002371-199110000-00011
117. Von Hoff DD, Fleming TR, Macdonald JS, Goodman PJ, Van Damme J, Brown TD, et al. Phase II evaluation of recombinant gamma-interferon in patients with advanced pancreatic carcinoma: a southwest oncology group study. *J Biol Response Mod*. (1990) 9:584–7.
118. Lollini PL, Bosco MC, Cavallo F, De Giovanni C, Giovarelli M, Landuzzi L, et al. Inhibition of tumor growth and enhancement of metastasis after transfection of the gamma-interferon gene. *Int J Cancer*. (1993) 55:320–9. doi: 10.1002/ijc.2910550224
119. Aul C, Gattermann N, Germing U, Heyll A. *Adverse Effects of Interferon Treatment*. In: Aul C, Schneider W, editors. *Interferons*. Heidelberg: Springer (1997). p. 250–66. doi: 10.1007/978-3-642-60411-9_15
120. Pinto EF, Andrade C. Interferon-related depression: a primer on mechanisms, treatment, and prevention of a common clinical problem. *Curr Neuropharmacol*. (2016) 14:743–8. doi: 10.2174/1570159X14666160106155129
121. Valle S, Martin-Hijano L, Alcalá S, Alonso-Nocelo M, Sainz Jr. B. The ever-evolving concept of the cancer stem cell in pancreatic cancer. *Cancers*. (2018) 10:33. doi: 10.3390/cancers10020033
122. Rich JN. Cancer stem cells: understanding tumor hierarchy and heterogeneity. *Medicine (Baltimore)*. (2016) 95 (1 Suppl. 1):S2–7. doi: 10.1097/MD.0000000000004764
123. Nguyen LV, Vanner R, Dirks P, Eaves CJ. Cancer stem cells: an evolving concept. *Nat Rev Cancer*. (2012) 12:133–43. doi: 10.1038/nrc3184
124. Ding B, Liu P, Liu W, Sun P, Wang C-L. Emerging roles of krüppel-like factor 4 in cancer and cancer stem cells. *Asian Pac J Cancer Prev*. (2015) 16:3629–33. doi: 10.7314/APJCP.2015.16.9.3629
125. Wang M-L, Chiou S-H, Wu C-W. Targeting cancer stem cells: emerging role of nanog transcription factor. *OncoTargets Ther*. (2013) 6:1207–20. doi: 10.2147/OTT.S38114
126. Rizzino A. Sox2 and Oct-3/4: a versatile pair of master regulators that orchestrate the self-renewal and pluripotency of embryonic stem cells by functioning as molecular rheostats. *Wiley Interdiscip Rev Syst Biol Med*. (2009) 1:228–36. doi: 10.1002/wsbm.12
127. Takeda K, Mizushima T, Yokoyama Y, Hirose H, Wu X, Qian Y, et al. Sox2 is associated with cancer stem-like properties in colorectal cancer. *Sci Rep*. (2018) 8:17639. doi: 10.1038/s41598-018-36251-0
128. Lonardo E, Hermann PC, Mueller M-T, Huber S, Balic A, Miranda-Lorenzo I, et al. Nodal/Activin signaling drives self-renewal and tumorigenicity of pancreatic cancer stem cells and provides a target for combined drug therapy. *Cell Stem Cell*. (2011) 9:433–46. doi: 10.1016/j.stem.2011.10.001
129. Karsten U, Goletz S. What makes cancer stem cell markers different? *Springerplus*. (2013) 2:301. doi: 10.1186/2193-1801-2-301
130. Li W, Ma H, Zhang J, Zhu L, Wang C, Yang Y. Unraveling the roles of CD44/CD24 and ALDH1 as cancer stem cell markers in tumorigenesis and metastasis. *Sci Rep*. (2017) 7:13856. doi: 10.1038/s41598-017-14364-2
131. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CHM, Jones DL, et al. Cancer stem cells–perspectives on current status and future directions: AACR workshop on cancer stem cells. *Cancer Res*. (2006) 66:9339–44. doi: 10.1158/0008-5472.CAN-06-3126
132. Prager BC, Xie Q, Bao S, Rich JN. Cancer stem cells: the architects of the tumor ecosystem. *Cell Stem Cell*. (2019) 24:41–53. doi: 10.1016/j.stem.2018.12.009
133. Grosse-Wilde A, Fouquier d'Hérouël A, McIntosh E, Ertaylan G, Skupin A, Kuestner RE, et al. Stemness of the hybrid epithelial/mesenchymal state in breast cancer and its association with poor survival. *PLoS ONE*. (2015) 10:e0126522. doi: 10.1371/journal.pone.0126522
134. Jolly MK, Boareto M, Huang B, Jia D, Lu M, Ben-Jacob E, et al. Implications of the hybrid epithelial/mesenchymal phenotype in metastasis. *Front Oncol*. (2015) 5:155. doi: 10.3389/fonc.2015.00155
135. Jolly MK, Somarelli JA, Sheth M, Biddle A, Tripathi SC, Armstrong AJ, et al. Hybrid epithelial/mesenchymal phenotypes promote metastasis and therapy resistance across carcinomas. *Pharmacol Ther*. (2019) 194:161–84. doi: 10.1016/j.pharmthera.2018.09.007
136. Chen W, Dong J, Haiech J, Kilhoffer M-C, Zeniou M. Cancer stem cell quiescence and plasticity as major challenges in cancer therapy. *Stem Cells Int*. (2016) 2016:1740936. doi: 10.1155/2016/1740936
137. Dembinski JL, Krauss S. Characterization and functional analysis of a slow cycling stem cell-like subpopulation in pancreas adenocarcinoma. *Clin Exp Metastasis*. (2009) 26:611–23. doi: 10.1007/s10585-009-9260-0
138. Zhao J. Cancer stem cells and chemoresistance: the smartest survives the raid. *Pharmacol Ther*. (2016) 160:145–58. doi: 10.1016/j.pharmthera.2016.02.008
139. Izumiya M, Kabashima A, Higuchi H, Igarashi T, Sakai G, Iizuka H, et al. Chemoresistance is associated with cancer stem cell-like properties and epithelial-to-mesenchymal transition in pancreatic cancer cells. *Anticancer Res*. (2012) 32:3847–53. Available online at: http://ar.iiarjournals.org/

140. Begicevic R-R, Falasca M. ABC transporters in cancer stem cells: beyond chemoresistance. *Int J Mol Sci*. (2017) 18:2362. doi: 10.3390/ijms18112362
141. Fletcher JI, Haber M, Henderson MJ, Norris MD. ABC transporters in cancer: more than just drug efflux pumps. *Nat Rev Cancer*. (2010) 10:147–56. doi: 10.1038/nrc2789
142. Abdullah LN, Chow EK-H. Mechanisms of chemoresistance in cancer stem cells. *Clin Transl Med*. (2013) 2:3. doi: 10.1186/2001-1326-2-3
143. Castiello L, Sestili P, Schiavoni G, Dattilo R, Monque DM, Ciaffoni F, et al. Disruption of IFN-I signaling promotes HER2/Neu tumor progression and breast cancer stem cells. *Cancer Immunol Res*. (2018) 6:658–70. doi: 10.1158/2326-6066.CIR-17-0675
144. Doherty MR, Cheon H, Junk DJ, Vinayak S, Varadan V, Telli ML, et al. Interferon-beta represses cancer stem cell properties in triple-negative breast cancer. *Proc Natl Acad Sci USA*. (2017) 114:13792–7. doi: 10.1073/pnas.1713728114
145. Yuki K, Natsume A, Yokoyama H, Kondo Y, Ohno M, Kato T, et al. Induction of oligodendrogenesis in glioblastoma-initiating cells by IFN-mediated activation of STAT3 signaling. *Cancer Lett*. (2009) 284:71–9. doi: 10.1016/j.canlet.2009.04.020
146. Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, et al. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science*. (1997) 278:477–83. doi: 10.1126/science.278.5337.477
147. Rajan P, McKay RDG. Multiple routes to astrocytic differentiation in the CNS. *J Neurosci*. (1998) 18:3620–9. doi: 10.1523/JNEUROSCI.18-10-03620.1998
148. Cimini A, Cerù MP. Emerging roles of peroxisome proliferator-activated receptors (PPARs) in the regulation of neural stem cells proliferation and differentiation. *Stem Cell Rev*. (2008) 4:293–303. doi: 10.1007/s12015-008-9024-2
149. Hackett AR, Lee D-H, Dawood A, Rodriguez M, Funk L, Tsoulfas P, et al. STAT3 and SOCS3 regulate NG2 cell proliferation and differentiation after contusive spinal cord injury. *Neurobiol Dis*. (2016) 89:10–22. doi: 10.1016/j.nbd.2016.01.017
150. Spiotto MT, Chung TD. STAT3 mediates IL-6-induced neuroendocrine differentiation in prostate cancer cells. *Prostate*. (2000) 42:186–95. doi: 10.1002/(SICI)1097-0045(20000215)42:3<186::AID-PROS4>3.0.CO;2-E
151. Marotta LLC, Almendro V, Marusyk A, Shipitsin M, Schemme J, Walker SR, et al. The JAK2/STAT3 signaling pathway is required for growth of $CD44^+CD24^-$ stem cell-like breast cancer cells in human tumors. *J Clin Invest*. (2011) 121:2723–35. doi: 10.1172/JCI44745
152. Chung SS, Aroh C, Vadgama JV. Constitutive activation of STAT3 signaling regulates hTERT and promotes stem cell-like traits in human breast cancer cells. *PLoS ONE*. (2013) 8:e83971. doi: 10.1371/journal.pone.0083971
153. Lin L, Fuchs J, Li C, Olson V, Bekaii-Saab T, Lin J. STAT3 signaling pathway is necessary for cell survival and tumorsphere forming capacity in $ALDH^+/CD133^+$ stem cell-like human colon cancer cells. *Biochem Biophys Res Commun*. (2011) 416:246–51. doi: 10.1016/j.bbrc.2011.10.112
154. Schroeder A, Herrmann A, Cherryholmes G, Kowolik C, Buettner R, Pal S, et al. Loss of androgen receptor expression promotes a stem-like cell phenotype in prostate cancer through STAT3 signaling. *Cancer Res*. (2014) 74:1227–37. doi: 10.1158/0008-5472.CAN-13-0594
155. Hajimoradi M, Hassan ZM, Ebrahimi M, Soleimani M, Bakhshi M, Firouzi J, et al. STAT3 is overactivated in gastric cancer stem-like cells. *Cell J Yakhteh*. (2017) 17:617–28. doi: 10.22074/cellj.2016.3834
156. Du Z, Cai C, Sims M, Boop FA, Davidoff AM, Pfeffer LM. The effects of type I interferon on glioblastoma cancer stem cells. *Biochem Biophys Res Commun*. (2017) 491:343–8. doi: 10.1016/j.bbrc.2017.07.098
157. Lykhova AA, Kudryavets YI, Strokovska LI, Bezdenezhnykh NA, Semesiuk NI, Adamenko IN, et al. Suppression of proliferation, tumorigenicity and metastasis of lung cancer cells after their transduction by interferon-beta gene in baculovirus vector. *Cytokine*. (2015) 71:318–26. doi: 10.1016/j.cyto.2014.10.029
158. Shimoda M, Ota M, Okada Y. Isolation of cancer stem cells by side population method. *Methods Mol Biol*. (2018) 1692:49–59. doi: 10.1007/978-1-4939-7401-6_5
159. Ma H, Jin S, Yang W, Tian Z, Liu S, Wang Y, et al. Interferon-α promotes the expression of cancer stem cell markers in oral squamous cell carcinoma. *J Cancer*. (2017) 8:2384–93. doi: 10.7150/jca.19486
160. Qadir AS, Ceppi P, Brockway S, Law C, Mu L, Khodarev NN, et al. CD95/Fas increases stemness in cancer cells by inducing a STAT1-dependent type I interferon response. *Cell Rep*. (2017) 18:2373–86. doi: 10.1016/j.celrep.2017.02.037
161. Ceppi P, Hadji A, Kohlhapp FJ, Pattanayak A, Hau A, Liu X, et al. CD95 and CD95L promote and protect cancer stem cells. *Nat Commun*. (2014) 5:5238. doi: 10.1038/ncomms6238
162. Sainz B, Martín B, Tatari M, Heeschen C, Guerra S. ISG15 is a critical microenvironmental factor for pancreatic cancer stem cells. *Cancer Res*. (2014) 74:7309–20. doi: 10.1158/0008-5472.CAN-14-1354
163. Zhang D, Zhang D-E. Interferon-stimulated gene 15 and the protein ISGylation system. *J Interferon Cytokine Res*. (2011) 31:119–30. doi: 10.1089/jir.2010.0110
164. Chen R-H, Du Y, Han P, Wang H-B, Liang F-Y, Feng G-K, et al. ISG15 predicts poor prognosis and promotes cancer stem cell phenotype in nasopharyngeal carcinoma. *Oncotarget*. (2016) 7:16910–22. doi: 10.18632/oncotarget.7626
165. Tsai Y-C, Pestka S, Wang L-H, Runnels LW, Wan S, Lyu YL, et al. Interferon-β signaling contributes to Ras transformation. *PLoS ONE*. (2011) 6:e24291. doi: 10.1371/journal.pone.0024291
166. Sistigu A, Yamazaki T, Vacchelli E, Chaba K, Enot DP, Adam J, et al. Cancer cell–autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nat Med*. (2014) 20:1301–9. doi: 10.1038/nm.3708
167. Ogony J, Choi HJ, Lui A, Cristofanilli M, Lewis-Wambi J. Interferon-induced transmembrane protein 1 (IFITM1) overexpression enhances the aggressive phenotype of SUM149 inflammatory breast cancer cells in a signal transducer and activator of transcription 2 (STAT2)-dependent manner. *Breast Cancer Res BCR*. (2016) 18:25. doi: 10.1186/s13058-016-0683-7
168. Monsurrò V, Beghelli S, Wang R, Barbi S, Coin S, Di Pasquale G, et al. Anti-viral state segregates two molecular phenotypes of pancreatic adenocarcinoma: potential relevance for adenoviral gene therapy. *J Transl Med*. (2010) 8:10. doi: 10.1186/1479-5876-8-10
169. Ni C, Wu P, Zhu X, Ye J, Zhang Z, Chen Z, et al. IFN-γ selectively exerts pro-apoptotic effects on tumor-initiating label-retaining colon cancer cells. *Cancer Lett*. (2013) 336:174–84. doi: 10.1016/j.canlet.2013.04.029
170. Song M, Ping Y, Zhang K, Yang L, Li F, Zhang C, et al. Low-dose IFN-γ induces tumor cell stemness in the tumor microenvironment of non-small cell lung cancer. *Cancer Res*. (2019) 79:canres.0596.2019 doi: 10.1158/0008-5472.CAN-19-0596
171. Bi J, Tian Z. NK cell exhaustion. *Front Immunol*. (2017) 8:760. doi: 10.3389/fimmu.2017.00760
172. Wherry EJ. T cell exhaustion. *Nat Immunol*. (2011) 12:492–9. doi: 10.1038/ni.2035
173. Matteucci C, Balestrieri E, Argaw-Denboba A, Sinibaldi-Vallebona P. Human endogenous retroviruses role in cancer cell stemness. *Semin Cancer Biol*. (2018) 53:17–30. doi: 10.1016/j.semcancer.2018.10.001
174. Cañadas I, Thummalapalli R, Kim JW, Kitajima S, Jenkins RW, Christensen CL, et al. Tumor innate immunity primed by specific interferon-stimulated endogenous retroviruses. *Nat Med*. (2018) 24:1143–50. doi: 10.1038/s41591-018-0116-5
175. Li S, Xie Y, Zhang W, Gao J, Wang M, Zheng G, et al. Interferon alpha-inducible protein 27 promotes epithelial-mesenchymal transition and induces ovarian tumorigenicity and stemness. *J Surg Res*. (2015) 193:255–64. doi: 10.1016/j.jss.2014.06.055
176. Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. (2008) 133:704–15. doi: 10.1016/j.cell.2008.03.027
177. Shibue T, Weinberg RA. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. *Nat Rev Clin Oncol*. (2017) 14:611–29. doi: 10.1038/nrclinonc.2017.44
178. Zhu Y, Karakhanova S, Huang X, Deng SP, Werner J, Bazhin AV. Influence of interferon-α on the expression of the cancer stem cell

markers in pancreatic carcinoma cells. *Exp Cell Res*. (2014) 324:146–56. doi: 10.1016/j.yexcr.2014.03.020
179. Clatot F, Cornic M, Berghian A, Marchand V, Choussy O, El Ouakif F, et al. CXCL12 and CXCR4, but not CXCR7, are primarily expressed by the stroma in head and neck squamous cell carcinoma. *Pathology*. (2015) 47:45–50. doi: 10.1097/PAT.0000000000000191
180. Eckert F, Schilbach K, Klumpp L, Bardoscia L, Sezgin EC, Schwab M, et al. Potential role of CXCR4 targeting in the context of radiotherapy and immunotherapy of cancer. *Front Immunol*. (2018) 9:3018. doi: 10.3389/fimmu.2018.03018
181. Katayama A, Ogino T, Bandoh N, Nonaka S, Harabuchi Y. Expression of CXCR4 and its down-regulation by IFN-γ in head and neck squamous cell carcinoma. *Clin Cancer Res*. (2005) 11:2937–46. doi: 10.1158/1078-0432.CCR-04-1470
182. Nagase H, Miyamasu M, Yamaguchi M, Imanishi M, Tsuno NH, Matsushima K, et al. Cytokine-mediated regulation of CXCR4 expression in human neutrophils. *J Leukoc Biol*. (2002) 71:711–7. Available online at: https://jlb.onlinelibrary.wiley.com/
183. Lollini PL, De Giovanni C, Del Re B, Nicoletti G, Prodi G, Nanni P. Interferon-mediated enhancement of metastasis. Are MHC antigens involved? *Clin Exp Metastasis*. (1987) 5:277–87. doi: 10.1007/BF00120723
184. Zöller M, Strubel A, Hämmerling G, Andrighetto G, Raz A, Ben-Ze'ev A. Interferon-gamma treatment of B16 melanoma cells: opposing effects for non-adaptive and adaptive immune defense and its reflection by metastatic spread. *Int J Cancer*. (1988) 41:256–66. doi: 10.1002/ijc.2910410217
185. McMillan TJ, Rao J, Everett CA, Hart IR. Interferon-induced alterations in metastatic capacity, class-1 antigen expression and natural killer cell sensitivity of melanoma cells. *Int J Cancer*. (1987) 40:659–63. doi: 10.1002/ijc.2910400515
186. Ramani P, Balkwill FR. Enhanced metastases of a mouse carcinoma after *in vitro* treatment with murine interferon gamma. *Int J Cancer*. (1987) 40:830–4. doi: 10.1002/ijc.2910400621
187. Xu Y-H, Li Z-L, Qiu S-F. IFN-γ induces gastric cancer cell proliferation and metastasis through upregulation of integrin β3-mediated NF-κB signaling. *Transl Oncol*. (2018) 11:182–92. doi: 10.1016/j.tranon.2017.11.008
188. Mojic M, Takeda K, Hayakawa Y. The dark side of IFN-γ: its role in promoting cancer immunoevasion. *Int J Mol Sci*. (2017) 19:89. doi: 10.3390/ijms19010089
189. Liu C, Gao AC. IFNγ, a double-edged sword in cancer immunity and metastasis. *Cancer Res*. (2019) 79:1032–3. doi: 10.1158/0008-5472.CAN-19-0083
190. Liu Y, Lv J, Liu J, Liang X, Jin X, Xie J, et al. STAT3/p53 pathway activation disrupts IFN-β–induced dormancy in tumor-repopulating cells. *J Clin Invest*. (2018) 128:1057–73. doi: 10.1172/JCI96329
191. Liu Y, Liang X, Yin X, Lv J, Tang K, Ma J, et al. Blockade of IDO-kynurenine-AhR metabolic circuitry abrogates IFN-γ-induced immunologic dormancy of tumor-repopulating cells. *Nat Commun*. (2017) 8:15207. doi: 10.1038/ncomms15207
192. Farrar JD, Katz KH, Windsor J, Thrush G, Scheuermann RH, Uhr JW, et al. Cancer dormancy. VII. a regulatory role for CD8+ T cells and IFN-γ in establishing and maintaining the tumor-dormant state. *J Immunol*. (1999) 162:2842–9.
193. Kmieciak M, Payne KK, Wang X-Y, Manjili MH. IFN-γ Rα is a key determinant of CD8+ T cell-mediated tumor elimination or tumor escape and relapse in FVB mouse. *PLoS ONE*. (2013) 8:e82544. doi: 10.1371/journal.pone.0082544
194. Essers MAG, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA, et al. IFNα activates dormant haematopoietic stem cells *in vivo*. *Nature*. (2009) 458:904–8. doi: 10.1038/nature07815
195. Pietras EM, Lakshminarasimhan R, Techner J-M, Fong S, Flach J, Binnewies M, et al. Re-entry into quiescence protects hematopoietic stem cells from the killing effect of chronic exposure to type I interferons. *J Exp Med*. (2014) 211:245–62. doi: 10.1084/jem.20131043
196. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*. (2006) 6:836–48. doi: 10.1038/nri1961
197. McCoach CE, Bivona TG. The evolving understanding of immunoediting and the clinical impact of immune escape. *J Thorac Dis*. (2018) 10:1248–52. doi: 10.21037/jtd.2018.03.60
198. Vahidian F, Duijf PHG, Safarzadeh E, Derakhshani A, Baghbanzadeh A, Baradaran B. Interactions between cancer stem cells, immune system and some environmental components: friends or foes? *Immunol Lett*. (2019) 208:19–29. doi: 10.1016/j.imlet.2019.03.004
199. Bhatia A, Kumar Y. Cancer stem cells and tumor immunoediting: putting two and two together. *Expert Rev Clin Immunol*. (2016) 12:605–7. doi: 10.1586/1744666X.2016.1159133

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 07 August 2020
doi: 10.3389/fonc.2020.01334

Phyto-Immunotherapy, a Complementary Therapeutic Option to Decrease Metastasis and Attack Breast Cancer Stem Cells

Susana Fiorentino, Claudia Urueña, Paola Lasso, Karol Prieto and Alfonso Barreto*

Grupo de Inmunobiología y Biología Celular, Pontificia Universidad Javeriana, Bogotá, Colombia

In this review, we report on the complexity of breast cancer stem cells as key cells in the emergence of a chemoresistant tumor phenotype, and as a result, the appearance of distant metastasis in breast cancer patients. The search for mechanisms that increase sensitivity to chemotherapy and also allow activation of the tumor-specific immune response is of high priority. As we observed throughout this review, natural products isolated or in standardized extracts, such as P2Et or others, could act synergistically, increasing tumor sensitivity to chemotherapy, recovering the tumor microenvironment, and participating in the induction of a specific immune response. This, in turn, would lead to the destruction of cancer stem cells and the decrease in metastasis.

Source of Data: Relevant studies were found using the following keywords or medical subject headings (MeSH) in PubMed, and Google Scholar: "immune response" and "polyphenols" and "natural products" and "BCSC" and "therapy" and "metabolism" and "immunogenic cell death." The focus was primarily on the most recent scientific publication.

Keywords: breast cancer stem cells, natural products, immune response, polyphenols, phytomedicine, phyto-immunotherapy, tumor metabolism

OPEN ACCESS

Edited by:
Maurizio Callari,
University of Cambridge,
United Kingdom

Reviewed by:
Asfar S. Azmi,
Wayne State University Karmanos Cancer Institute, United States
Erina Vlashi,
University of California, Los Angeles, United States

****Correspondence:***
Susana Fiorentino
susana.fiorentino@javeriana.edu.co

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *25 March 2020*
Accepted: *26 June 2020*
Published: *07 August 2020*

Citation:
Fiorentino S, Urueña C, Lasso P, Prieto K and Barreto A (2020) Phyto-Immunotherapy, a Complementary Therapeutic Option to Decrease Metastasis and Attack Breast Cancer Stem Cells. Front. Oncol. 10:1334. doi: 10.3389/fonc.2020.01334

INTRODUCTION

The antitumor activity of natural products derived from plants is estimated to occur through the combination of various phytochemicals acting synergistically, rather than by isolated molecules, which alone may have greater toxicity and not exert the same activity. Additionally, these mixtures have specificity against their molecular targets, which, although difficult to identify, are considered drugs in themselves. These molecular targets may be found in events related to the prevention of carcinogenesis, the destruction of tumor cells directly, the modulation of the tumor microenvironment, the activation of the specific anti-tumor immune response, the induction of epigenetic changes, or the improvement of quality of life of the patient (1, 2). This plethora of activities is particularly relevant in the control of metastasis, where the breast cancer stem cells (BCSCs), are the main ones involved. In this review, we will focus on the role of these complex extracts on BCSC control through the modulation of energetic metabolisms and immune system activation.

BREAST CANCER OVERVIEW

Breast cancer is the most frequent tumor in women worldwide, accounting for 2.1 million new cases estimated for 2018 and 626.679 associated deaths for the same year, according to Globocan (3). Approximately one in 4 of all new cancer cases diagnosed in women worldwide is breast cancer, being the most common in 154 of the 185 countries polled. Breast cancer is also the main cause of cancer death in women (15.0%), followed by lung cancer (13.8%) and colorectal cancer (9.5%), which are also the third and second most common type of cancer, respectively; cervical cancer ranks fourth in both incidence (6.6%) and mortality (7.5%) (3, 4).

Regarding the origin of tumor cells, there are two models: the clonal evolution model (in which mutations accumulate and epigenetic changes occur in tumor cells, increasing survival of the fittest and most adaptable cells), and the cancer stem cell model (in which only the precursor cancer cells initiate and sustain tumor progression). Cancer stem cells (CSC) can also evolve clonally, so the two models are not mutually exclusive (5). At the molecular level, there is evidence showing that breast cancer evolves along two divergent molecular pathways of progression, primarily related to estrogen receptor (ER) expression, tumor grade, and proliferation. In addition, the identification of breast cancer susceptibility genes has shed light on some aspects of the pathogenesis of sporadic and inherited breast cancer (6).

The presence of specific markers in breast cancer such as ER, progesterone receptor (PR) and epidermal growth factor receptor 2 (HER-2) is useful to define the type of treatment indicated and the prognosis of the disease (7). ER and PR were the first biological markers evaluated, representing 50–75% of ductal carcinoma *in situ* (DCIS) lesions. The expression of ER correlates with the degree of DCIS and invasive breast cancer (IBC), and makes this type of cancer more sensitive to endocrine treatment and chemotherapy. In contrast, ER negative tumors have a higher proliferation rate and therefore greater aggressiveness (8). HER-2 plays an important role in the activation of HER-dependent cell growth and is over-expressed in 20–25% of IBC and 10–25% of IBC ER+. Overexpression of this marker in IBC contributes to oncogenic transformation and is considered a marker of poor prognosis. On the other hand, triple-negative (TN) patients for these markers have a poor overall prognosis since they respond poorly to treatment, possibly due to the close relationship that tumor cells have with the microenvironment and the presence of a molecular phenotype similar to that of BCSCs, which favors relapse with distant metastases (9). Although chemotherapy is currently the main systemic treatment for these patients, they are highly resistant (10). Therefore, having therapies directed at CSCs will be essential to treat both metastasis and this highly TN aggressive tumor.

Cancer staging system from breast cancer is based on the guidelines of the American Joint Committee on Cancer (AJCC) (11). Different therapeutic strategies are currently used in breast cancer treatment. These therapeutic strategies include, but are not limited to, local interventions (surgery and/or radiotherapy) and systemic treatments (chemotherapy, hormonal therapy, or targeted therapies) as anthracycline or taxane chemotherapy. The therapeutic options are limited when the primary response to chemotherapy is low. Patients with tumor progression or resistance are treated with Capecitabine, Vinorelbine, Gemcitabine, or albumin-bound Paclitaxel, however, these modifications have a low response rate (20–30%) with an average duration of survival of fewer than 6 months. Breast cancer patients undergoing prolonged chemotherapy treatment frequently develop resistance to various structurally related compounds, known as cross-resistance or multi-drug resistance (12). There are countless biological reasons for chemotherapy failure, which are heightened by the intrinsic heterogeneity of breast cancer cells present in the tumor (13). The presence of CSCs, which can be discovered from the development of the primary tumor, but can also be selected by treatment, aggravates this panorama due to their greater resistance to chemotherapy and radiation, contributing to therapeutic failures. Some studies have shown that CSCs are responsible for tumor formation and progression due in part to its self-renewal characteristics (14) and the expression by themselves of some other key factors implied in metastatic progression in breast cancer (15–17).

The first CSCs of solid tumors were identified in breast cancer (18) and later they were isolated in other organs. Al-Hajj et al. were the first to describe this subpopulation with the potential to form tumors within immunodeficient non-obese diabetic mice (NOD) with severe combined immunodeficiency (SCID) (18). Different surface markers such as $CD44^{+}/CD24^{low}/EpCAM^{+}$ and lineage markers (negative for CD3, CD2, CD10, CD16, CD18, CD31, CD64, and CD140b) $CD133^{+}$, $CD49f^{+}$ and ALDH1 (19, 20), have been used for the BCSC identification from primary isolated tumors or metastases (18, 21, 22). Human BCSCs are identified by the ability to form spheres in low-adherence cultures, called mammospheres (14). Both normal and BCSC express the enzyme aldehyde dehydrogenase (ALDH) (22), however the expression of this ALDH seems to differentiate BCSC with epithelial phenotypic characteristics ($ALDH^{+}$) which are more proliferating, from BCSC with mesenchymal characteristics ($ALDH^{-}$) that have a greater invasive capacity but less proliferation. Furthermore, these cells demonstrate high plasticity that allows the transition between these two stages, thus increasing their aggressiveness (23). ALDH1 has also been widely used to identify CSCs, as well as the overexpression of ATP-binding cassette (ABC) transporters (24–26), belonging to the multidrug resistance proteins (MDR), in various types of cancer, including leukemias (27), colon (28), lung and pancreas (29, 30), among others. In fact, these MDR proteins are responsible for the expulsion of the Hoechst 33342 dye in the so-called "*side population*" (SP), which is also a phenotypic characteristic of CSC (31). CSCs can also be characterized by their ability to form spheres in culture (mammosphere assay for breast cancer), which is useful in enriching the BCSC population *in vitro* for in-depth study. Ponti and colleagues were the first to spread CSC from three breast cancer lesions and from an established breast carcinoma cell line, which grew *in vitro* in non-adherent cultures (14). This method allows for studying the properties of BCSCs as

well as designing therapeutic strategies that target this type of cell (30).

We have recently shown in our laboratory, in a cohort of 78 patients with breast cancer, that those classified as Luminal B and TN who received neoadjuvant therapy before surgery, present an increase in the frequency of BCSC with a $CD44^+/CD24^-/EPCAM^+/CD49f^+$ phenotype together with the expression of ALDH compared to healthy donor tissue. Additionally, the correlation between ALDH expression and the breast cancer resistance protein (BCRP) was only observed in TN patients (unpublished data). An increase in the $CD44^+/CD24^{low}$ population has been observed in patients who have undergone anthracycline/cyclophosphamide or taxane chemotherapy, regardless of molecular subtype (32). In another study, an increase in the proportion of ALDH-1 cells was demonstrated in a cohort of 52 patients who had previously received neoadjuvant chemotherapy and had no complete pathological response. However, this increase was not observed when using the $CD44^+/CD24^-$ markers via immunohistochemistry (33). These results show that although chemotherapy is the first therapeutic option, BCSC enrichment is a risk factor that favors the appearance of metastasis a posteriori, and in many cases, the death of patients (34).

In a recent study, we also showed that the standardized extracts from *Caesalpinia spinosa* (P2Et) and *Petiveria alliacea* (Anamu), both of which present antitumor activity *in vitro* and *in vivo*, previously reported by our group (35–41), decreases the viability of CSC-enriched human tumor cell mammospheres, induces immunogenic cell death (ICD), in a better way than doxorubicin (42), and slows tumor appearance *in vivo* in immunodeficient mice (unpublished data). Furthermore, in a murine model of breast cancer, P2Et acts synergistically with doxorubicin reducing tumor growth, possibly due to its ability to inhibit the function of the Pgp multidrug resistant pump (39). These results may be promising, even more so when several studies have shown that chemotherapy can increase the population of the stem phenotype, and that co-treatment with plant extracts might increase sensitivity to therapy.

METABOLISM OF BREAST CANCER STEM CELLS

Cells under normal conditions have a low replication rate and use mitochondrial metabolism as an energy source, generating ATP through the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS). By contrast, tumor cells need to increase the biosynthesis of macromolecules due to their high replication rates, for which they increase glucose uptake and use glycolysis as the main energy source, producing an increase in lactate levels even in the presence of oxygen. This process of increasing aerobic glycolysis is known as the Warburg effect (43). Although CSCs have been reported to increase the expression of markers that promote proliferation, differentiation, and metastasis, it is unclear how their metabolism works, in part because of the senescent characteristics they present. Initially, in CSCs generated from breast cancer lines, it was observed that they presented a higher glucose uptake with an increase in glycolysis, which in turn increased the synthesis of macromolecules and reactive oxygen species (44). Additionally, glucose availability has been shown to induce expression of specific genes in CSCs associated with glycolytic metabolisms such as c-Myc and Glut-1 (45). Likewise, in colon cancer and glioma cells, it was found that CD44 regulates the phosphorylation of pyruvate kinase M2 (PKM2), a key enzyme in the glycolysis of tumor cells, and thereby increases glycolytic metabolism, promoting an increase in glucose uptake and glutathione production to decrease the generation of reactive oxygen species (ROS) in hypoxic cells (46). These findings have suggested that low oxygen stresses and glycolytic metabolism are essential for the maintenance of CSCs in their undifferentiated state. In fact, hypoxia, in addition to increasing glycolysis, has also been considered a fundamental factor for CSC self-renewal, since it increases the activity of factors such as Oct4, c-Myc, and Nanog, important in maintaining the stem phenotype (47).

Contrasting with these assertions, it has been described that in embryonic stem cells, hypoxia increases metabolic plasticity, changing its glycolytic metabolism to a more oxidative metabolism, characterized by an increase in mitochondrial mass and the production of ROS, which favors differentiation (43, 48). Additionally, the increase in OXPHOS has also been associated with the induction of pluripotency, suggesting that the state of metabolic plasticity is necessary for the maintenance of pluripotent capacity in stem cells (49). These changes can also occur in CSCs, for which the microenvironment seems to play a fundamental role both in the generation and maintenance of CSCs and in their metabolic plasticity. The generation of metabolites such as lactate and free fatty acids by highly proliferating cells serves as fuel for anabolic cells, which would give an advantage at times when low availability of nutrients occurs, such as during chemotherapy (49). It has also been described that CSCs resistant to therapy have an increase in mitochondrial mass, which suggests an increase OXPHOS metabolism (50). This is related to a recent work showing that 4T1 cells with higher metastatic potential are metabolically more active compared to less metastatic cells. Additionally, it was observed that the metastases generated by 4T1 cells had different metabolisms depending on where they were established. This suggests that metabolic plasticity is necessary for CSCs to easily adapt to the microenvironment in which they are found and to give rise to highly proliferating cells (51).

This evidence suggest that CSC population may require a double-hit treatment, initially inhibiting glycolysis, which would result in increased phosphorylative activity, for which specific therapy for mitochondria, such as mitocanes (52–54) would then be used. This sequential double molecular target treatment would allow more effective attack on CSCs, reducing their plasticity and therefore their survival (55). In this sense, we previously showed that a *Petiveria alliacea* extract induces apoptosis of the murine breast cancer 4T1 cells through caspase-3 activation, DNA fragmentation without mitochondria membrane depolarization, and decreases in cell colony growth capacity. Changes in glycolytic enzyme expression, including reduction of PKM2, lead to a decrease in glucose uptake and lactate production,

related with tumor regression in BALB/c mice transplanted with GFP-tagged 4T1 cells (37). Later we were able to show that *P. alliacea* extract entails the reduction in β-F1-ATPase expression, glycolytic flux triggering diminished intracellular ATP levels, mitochondrial basal respiration and oxygen consumption. Consequently, a decline in cell proliferation was observed in conventional and 3D breast cancer cells culture. Treatment of BALB/c mice transplanted with the murine breast cancer TS/A tumor showed that *P. alliacea* extract decreases the primary tumor growth and increases survival (56). It is interesting that we also observed antitumor activity on this mice breast cancer model with the P2Et extract (40, 41), even though the mechanisms of action of the two extracts, as well as their molecular composition, are diametrically opposed. In the case of *P. alliacea* extract, some of the main compounds are dibenzyl sulfide, 4-ethyl petiveral and glutamyl-S-benzyl cysteine, lignoceric acid and myricitrine. Additionally, in all the cell models studied, the anamu extract has a strong intracellular pro-oxidant capacity that is more or less intense depending on the cell type. In contrast, P2Et, mainly composed of derivatives of gallic acid induces cell death through mitochondrial depolarization, reticulum stress via PERK and increase of intracellular Ca^{++} without a remarkable increase in ROS (57). Unlike *P. alliacea* extract, P2Et has an important antioxidant capacity (39), however, both induce the expression of immunogenic death markers, possibly using different pathways.

BCSCs have shown plasticity that allows them to transition between a proliferative state that has been called epithelial-like (E) with high ALDH expression, and a quiescent, invasive mesenchymal-like state (M) characterized by $CD44^{+}/CD24^{-}$ phenotype. The balance between these two stages is modulated by the microenvironment (23). The participation of metabolism or oxidative stress in this phenomenon is not well-understood, but it has recently been shown that the M and E stages of the BCSC are related to different metabolic pathways and show marked differences in terms of sensitivity to glycolysis inhibitors or of redox metabolism. Metabolic or oxidative stress generated by 2-Deoxy-D-glucose (2-DG), H_2O_2 or hypoxia promotes the transition from a ROS^{lo}-M-BCSC state to a ROS^{hi}-E-state. This transition is reversed by N-acetylcysteine and mediated by activation of the AMPK-HIF-1α axis. E-BCSCs show a robust antioxidant response mediated by NRF2, making them vulnerable to ROS-induced differentiation and cytotoxicity resulting from NRF2 suppression.

In this sense, the activity of 2DG on TN breast cancer (TNBC) cell lines, the parental Hs578T, and it's more aggressive variant Hs578Ts (i) 8 was recently evaluated. Treatment with this glycolysis inhibitor showed inhibition of migration, invasion, and decreased ability to resist the anoikis of the more aggressive subtype Hs578Ts (i) 8, above the parental line. The aggressive line has a more glycolytic phenotype due to mitochondrial dysfunction and also has a higher proportion of cells with a CSC phenotype (58). Given the above, inhibition of glycolysis in conjunction with other metabolic modulators could be a useful therapy in the elimination of BCSC (59). Finding specific compounds targeting tumor metabolism has not been easy, but standardized complex extracts might be part of the solution, as we have been seeing it in our laboratory.

Other targets, such as phosphoglycerate dehydrogenase (PHGDH), are required for redox homeostasis, maintenance of BCSC, and lung metastases (60). It also participates in the resistance to sorafenib in advanced cellular hepatocarcinoma (61). This enzyme is a target of Ixocarpalactone A, obtained from diet tomatillo (*Physalis ixocarpa*) (62). Other compounds derived from natural products, such as turmeric, have been extensively studied, and their activity on the inhibition of different signaling pathways characteristic of BCSCs has been observed, as well as their ability to reverse multi-resistance to drugs through the inhibition of drug resistance pumps, decrease in the number of mammals *in vitro*, and increased awareness of chemotherapy treatment, among others (63).

Indeed, natural products such as phenolic compounds, isoprenoids and alkaloids target cellular metabolism (64). They have also been implicated in the inhibition of glycolysis, glucose transporters, and glycolytic enzymes such as GLUT1-4, Hexokinasa1-2, Pyruvate kinase M2, and lactate dehydrogenase (65). A study in mice showed that the content of ROS in stem cells of epithelial origin $CD24^{med}/CD49f^{high}/Lin^{-}$ with high capacity for renewal is lower than its $CD24^{high}/CD49f^{low}/Lin^{-}$ counterpart. In this same work, it was shown that the expression of genes coding for antioxidant enzymes in human BSCS obtained from primary tumors is higher than in their non-tumor counterpart, which was related to a lower content of ROS and a higher radioresistance (66). Given the complexity of these cells and the important influence of the tumor microenvironment in the generation and maintenance of the resistant and highly tumorigenic phenotype of CSCs, in general, therapy could be aimed at recovering the normality of the tumor microenvironment, which could allow CSCs to recover its normal phenotype as well as the sensitivity to chemotherapy and to the immune response (67, 68). Likewise, it is important to consider a mixed therapy that contemplates both the inhibition of glycolysis and the reduction of phosphorylation metabolism, in order to cover the tumor in all its heterogeneity.

Another important factor associated with the tumor microenvironment is the role tumor stromal cells play, for example as cancer-associated fibroblasts (CAF), which may be derived from tissue-resident fibroblasts or from mesenchymal cells recruited during growth-induced chronic inflammation tumors, in the maintenance of CSCs. CAFs, in addition to providing metabolism-derived by-products that support the growth of tumor cells (69), produce a good amount of cytokines that help promote the phenotype "stemness" in CSCs. For example, they secrete IL-6 that helps generate the mesenchymal epithelial transition phenotype involved with CSCs, or IL-8 that primarily regulates epithelial-like phenotype (E) with high ALDH expression (70). Therefore, CAFs become an alternative target for natural products due to their innate anti-oxidant activity, which could reverse the protumoral phenotype of these cells, leading them to reconstruct a normal microenvironment.

NATURAL PRODUCTS IN THE CONTROL OF BCSC AND THE INDUCTION OF IMMUNE RESPONSE

Natural products can have antitumor activity through the elimination of the tumor cells themselves or indirectly through the activation of the antitumor immune response, as we will see later. Although its role on the BCSC is beginning to be understood, there is evidence about their role in controlling metastases or improving survival in patients with breast cancer (71), which means that they indirectly act by decreasing the tumorigenicity of BCSCs.

Plant extracts can act on multiple pathways that participate in the maintenance of BCSC (72), regulating tumor metabolism (64) or even by acting on the tumor microenvironment (73–75). There is a close relationship between the tumor microenvironment and tumor metabolism. This is reflected in the most important genetic alterations of CSCs, which are related to changes in tumor metabolism, such as by OCT4, KLF4, SOX2, and MYC. Meanwhile, NOTCH, WNT/β-catenin, PI3K/Akt, PTEN, NF-κB, KRAS, HIF, TP53, and other oncogenic pathways are related to the maintenance of the stemness capacity of CSCs, which could be a consequence of the first wave of genetic alterations (49).

PIK3CA mutations are found in patients with positive lymph nodes and subsequently manifest the mutation in the BCSCs present in the residual disease. Neoadjuvant therapy does not decrease cells with PIK3CA mutations, which appear to be more resistant to chemotherapy (76). In fact, tumors in which the BCSCs present defects in the signaling pathway PI3K/Akt are more predisposed to present nodal metastases. This marker is of utmost importance, and there are currently clinical studies evaluating the activity of some inhibitors of this pathway (77). Recently, it was reported that a group of compounds derived from pyrrolo pyrimidines have activity on PI3K and could act particularly on BCSCs (78). Another study found that fisetin, a dietary flavonoid, alone or in combination with 5-FU, affects tumorigenesis in the mammalian intestine. Treatment of cells with mutations in PIK3CA with fisetin and 5-FU decreases the expression of PI3K, the phosphorylation of AKT, mTOR, its target proteins, the constituents of the mTOR signaling complex and increases the phosphorylation of AMPKα, therefore possibly have a direct role on the BCSC (79).

On the other hand, hypoxia-inducible factor (HIF) is important in the selection and generation of BCSC, but also in the modulation of inflammatory response. In cancer, it has a primary role in PKM2 activation and aldolase A synthesis which in tumor cells play a fundamental role in the maintenance of glycolytic metabolism. HIF-1α is over expressed in several types of tumors, such as breast cancer, and conditional deletion of HIF-1α leads to a primary tumor decrease and metastasis, related with a reduction in BCSC frequency (80). HIF-1α can also regulate the interaction of BCSC with the microenvironment, placing it in a key position in maintenance of the tumor stem phenotype and suppression of immune response (81). Some natural compounds, such as diallyl trisulfides, reduce the expression of HIF-1α in breast cancer cell line (MDA-MB-231) inhibiting hypoxia-induced breast cancer metastasis (82). HIF inhibitors in clinical studies have shown modest results and high toxicity (83), therefore, HIF inhibition should be approached in a more holistic way, modulating for example the external factors that lead to HIF-1α activation in the tumor context.

Genetic signatures in BCSC responsible for self-renewal, such as c-KIT, TGF-β, the alpha 6 subunit of the integrin, STAT3, together with the Wnt/β-Catenin pathway which is also altered in TNBC (84, 85), are targets of Piperines (86). More generally, a recent study reviews how natural products can inhibit several signaling pathways involved in TNBC tumorigenesis or induce apoptosis through the inhibition of survival pathways activated by intrinsic tumorous cell disorders (87). This opens the door to continue studying how to use these natural products, preferably in combination with conventional chemotherapy that can modulate the signaling pathways that favor the maintenance of BCSCs.

In an *in vivo* model of murine breast cancer, called 4T1-H17, enriched with CSC-ALDH$^+$, we showed that the anti-tumor immune response was the main element capable of controlling tumor progression and metastasis. Animals were vaccinated with 4T1-H17 cells previously treated with doxorubicin, a known ICD inducer (88), and fewer mice were found to develop primary tumors and macrometastasis, while inducing a multifunctional response of CD4$^+$ and CD8$^+$ T cells, suggesting that this treatment improved the control of highly metastatic and resistant 4T1-H17 tumor cells (89). A recent study showed that the cytotoxic T lymphocytes (CTL) response induced by autologous dendritic cells activated with antigens derived from BCSC significantly inhibits the proliferation of stem cells *in vitro* and decreases tumor size when treating transplanted mice with 4T1 breast cancer (90). Likewise, immunodominant epitopes derived from ALDH have been used to generate CD8$^+$ T cells that specifically recognize and lyse human tumor cells of the breast, pancreas, head, and neck with elevated levels of ALDH1A1 (91, 92). The percentages of ALDH1A1 high cells decreased by 60–89% as a result of ALDH1A1-specific CD8$^+$ T cells-mediated toxicity *in vitro*. In preclinical models using human tumor xenografts in immunodeficient mice, ALDH-specific CD8$^+$ T cells inhibited the growth of xenografts and metastases, and prolonged survival after adopted (92). These studies show that CSCs are sensitive to T cell-mediated death.

P2Et has *in vitro* and *in vivo* activity against tumors generated by conventional 4T1 cells, but in the case of the 4T1-H17 line, despite having shown *in vitro* activity, both in 2D and 3D models, it did not show activity *in vivo* (89). The molecular mechanism by which P2Et acts is related to its ability to induce mitochondria-dependent apoptosis, with caspase activation, unfolded protein response (UPR) activated through PERK and cytoplasmic calcium increase, and its high intra and extracellular antioxidant capacity (57). The reason why P2Et does not present activity *in vivo,* despite inhibiting the formation of spheres *in vitro*, remains unknown. We can rule out the issue of bioavailability since it presents *in vivo* activity against 4T1 and B16-F10 cells (36, 40, 41), which is why the interaction of the tumor with the microenvironment must play a fundamental role. However, this question must be addressed in a tumor model in which the

microenvironment is modified as the tumor develops, since it is this communication over time that generates this favorable space for the tumor growth and it is there where we can see if the P2Et extract (as well as other plant extracts) really plays a synergistic role in tumor therapy. This will also allow us to evaluate if this extract really favors the generation of an adaptive immune response.

The role of the microenvironment in the response of tumors to treatment with cellular stress modulators or antioxidants is evidenced in the works of Sayin, and Le Gal, where it is observed that antioxidants accelerate tumor progression and metastasis in a melanoma model and a transgenic model of lung cancer. This effect is related to an increase in glutathione synthesis induced by antioxidants such as N-acetyl cysteine (NAC) and Trolox (93, 94). In this regard, we recently showed that preventive treatment with P2Et of healthy BALB/c or C57BL/6 mice promotes tumor growth and death of these animals when 4T1 and B16-F10 tumors are subsequently transplanted, respectively. These facts are related to the generation of a pro-inflammatory environment in the treated animals, related to a preactivation of the immune response, which was clearly evidenced by a significant increase in plasma IL-6 (38) in contrast to previously observed experiments, where the P2Et decreased markers of poor prognosis such as IL-6 in the 4T1 model (41). A systematic review of the effect of antioxidants on the immune system showed that they significantly decreased tumor necrosis factor-alpha (TNF-α) production only in individuals who had a pro-inflammatory base condition, but no change in normal individuals (95). On the other hand, several natural products have been used as photosensitizer that when accumulated in tumors are activated by a light source during photodynamic therapy, increasing ROS and inducing cell death in tumor cells (96). Particularly, hyperacin, a natural product obtained from *Hypericum perforatum*, is directly accumulated in ER and after Dynamic Photo Therapy (PDT) it favors ROS production, ER stress response, calreticulin surface exposure and ICD (97). Typically, ROS production is associated with induction of ICD; however, it should be modulated as ROS produced in cancer cells can impact TME mediating immunosuppression via tolerogenic myeloid cells (98).

Microenvironment factors produced during tumorigenesis or even due to the inadequate activation of the immune response, favor the maintenance of CSCs. IL-6 induces the conversion of non-BCSC to BCSC by activation of OCT-4 transcription through STAT3. Likewise, STAT3 binds to the SOX2 and MYC promoter, increasing tumorigenicity, the efficiency of sphere formation, and ALDH activity. By decreasing HIF activation, reducing ROS due to the antioxidant activity of natural products, STAT3 activation is then decreased, minimizing the tumorigenic capacity of BCSCs. The decrease in IL-6 production could lead to a decrease in the conversion of non-BCSC to BCSC. Inhibition of downstream signaling by the IL-6 receptor has been shown to inhibit the growth of TNBCs, but not of its non-TNBC counterpart (99).

In previous studies, we observed that P2Et decreases the number of $ALDH^+$ cells *in vitro* (39), although *in vivo* it presents a reduced activity to this $ALDH^+$ population. However, we observed that treatment with P2Et delays the appearance of the tumor in a model of orthotopic transplantation of tumor cells of TNBC enriched in tumor cells with CSC phenotype (unpublished data). Our recent results are in line with a recent review showing that the antitumor effects of 10 Chinese plants and their bioactive compounds have immunostimulatory and cytotoxic activity against breast cancer cells, further reducing metastasis and improving the quality of life of patients. Among them are *Angelica sinensis* (AS, Dang Gui in Chinese), *Panax notoginseng* (PN, San Qi in Chinese), *Scutellaria barbata* and *Oldenlandia diffusa*, Licorice (Gan Cao in Chinese) and *Radix Salvia miltiorrhiza* (SM, Dan Shen in Chinese) (100). All this suggests the presence of a complex network of cellular interactions, which should be analyzed taking into account multiple variables that so far do not seem to have been taken into account.

An example of this complexity is the fact that MAPKs, essential in inflammation and cancer control, regulate cellular activities involved in tumor progressions such as proliferation, apoptosis, and escape of the immune response. Inhibitors of this route have been associated with adverse effects; however, in the context of prevention and treatment, it has been suggested that some dietary factors, such as virgin olive oil or nutraceuticals containing them, may interact with this route, being adjuvants of antitumor therapy (101).

Medicinal fungi such as *Ganoderma lucidum*, which has been widely studied and is called the immortality fungus, are also among the activators of the immune system. Its main components are the polysaccharides obtained from the aqueous extract, as well as the triterpenes obtained from the extract with organic solvents, which together seem to have direct antitumor activity and even more importantly, an activating activity of the immune response. The vast majority of studies found in the literature evaluate antitumor activity *in vitro* on tumor cells, showing some of the mechanisms involved, which may give evidence of their direct activity. However, few studies measure the activation of the immune response to the tumor, which can only be evaluated in *in vivo* experiments, in animal models with a complete immune system, or controlled clinical studies in cancer patients (102).

Regarding the activity of *G. lucidum* on BCSC, it was found that it decreases the viability of human tumor cells of TN phenotype, it reduces the gene and protein expression mainly of STAT3, as well as the phosphorylation of the protein, it slightly reduces the activity of ALDH1, and it decreases the formation of mammospheres. It has also been observed that some isolated Ganoderma compounds can inhibit the *in vitro* growth of quiescent cells with a low proliferation rate, at high concentrations, and *in vitro* (103).

When looking for clinical studies in NCBI with the keyword Ganoderma and cancer, we found only 3 studies, of which one of them was on head and neck cancer with no results (NCT02238587), another in prostate cancer that is ongoing (NCT03589781) and another in cancer in children (NCT00575926). It is possible that, not only for Ganoderma but for other natural products, the low availability *in vivo*, together with the lack of knowledge of the dose required to induce

death, which is specific for each type of tumor, may explain why many of the clinical studies carried out so far have not given satisfactory results.

Although there is extensive literature on the role of natural products in the anti-tumor response, few studies have been carried out where the activity of plants in inducing tumor death is related to the activation of an appropriate anti-tumor immune response. The mechanism involved would be the induction of ICD in a tumor setting where the patient still has a functional immune response. Recently, we and others have shown that natural products can be the missing link by attacking tumor cells directly, improving the tumor microenvironment, and inducing activation of the immune response by mechanisms different from those previously reported for some chemotherapeutics that have the same activity (57).

Taking all the above, we could then think that BCSCs could be eliminated through the immune response generated *in situ* when chemotherapy-sensitive primary tumor cells die by mechanisms such as ICD. This mechanism allows the activation of dendritic cells as a consequence of the release of danger signals from dead cells. The processing and subsequent presentation of the tumor antigens released by the dead cells would allow the activation of $CD8^+$ T cells lymphocytes by cross-priming mechanisms (104), which would ultimately attack the BCSC resistance to chemotherapy (**Figure 1A**). This can occur on the condition that $CD8^+$ T cells recognize shared tumor antigens between chemotherapy-sensitive tumor cells and BCSCs, and also that BCSCs present these antigens in the context of MHC class I. This concept of endogenous or *in situ* vaccination should be explored in greater depth as a strategy to decrease metastasis and improve patient survival. The generation of this immune response *in situ* must also contemplate the reduction in immunosuppressive factors, that come from both the tumor cells themselves and the tumor microenvironment, that are released in the tumor microenvironment, and can be transformed by the presence of the tumor. The decrease in the deleterious inflammation, the ROS produced by cellular stress or even the regulation of tumor metabolism, could act as co-adjuvants, facilitating the effector action of the immune response. As presented in this review, natural products isolated or in standardized extracts, such as P2Et or others, could act synergistically, increasing tumor sensitivity to chemotherapy, recovering the tumor microenvironment, and participating in the induction of an immune specific response, which in the end would lead to the destruction of CSCs and the decrease in metastasis (**Figure 1B**).

EVIDENCE OF IMMUNE RESPONSE AGAINST CANCER STEM CELLS

Although not all antigens that are over-expressed in CSCs are targets of immunotherapy, ALDH1A1 (92), survivin (105, 106), livina (107), and Bcl-2 (108), among others, have been reported to induce a specific immune response against CSCs. CSCs have been reported to renew after activation by Notch and amplification by Hedgehog and Notch signals of β-catenin (Wnt) (109). When CSCs renew or become inactive, Notch and Numb are degraded to peptides by the proteasome and are presented by HLA I molecules from tumor cells or T cells (110). Thus, the first report demonstrating that CTL can recognize and eliminate CSC populations was using CSC-enriched MCF-7 breast cancer and SK-OV-3 ovarian cancer cells ($CD44^+/CD24^{lo}/CD133^+$) after treatment with 5-fluorouracil and paclitaxel. These cells were incubated with peripheral blood mononuclear cells previously activated with natural immunogenic peptides of the protein Notch-1 (2, 111) and Numb-1 (41, 86–93), finding a specific decrease in the CSC population in both models (110). Over-expressed enzymes that participate in metabolic pathways are considered a good antigenic target for the development of immunotherapies. For example, as previously shown, the enzyme ALDH1A1 has been shown to be an attractive target for the induction of adaptive immune responses against cancer (91, 92).

On the other hand, to consider the possibility of generating long-term protection, the immune compartment of T cells must be evaluated. An efficient way of inducing the generation of effector T cells against tumors in patients is through so-called vaccination *in situ,* where, by means of the intratumoral administration of different types of immunomodulators, the response of T cells is specifically induced or amplified in each patient (112). One of the *in situ* vaccination strategies that have strongly attracted attention in recent years is the induction of ICD by antitumor drugs such as anthracyclines. In ICD, a cellular stress response is induced prior to death by apoptosis accompanied by the generation of various danger signals or damage-associated molecular patterns (DAMPs), which ultimately promote an appropriate effector response by T cells (88). It is proposed that induction of the anti-tumor immune response through increased immunovigilance mechanisms can prevent the re-emergence of tumors from therapy-resistant cells such as CSCs. In this immunomodulatory microenvironment and in the presence of appropriate stimulation, $CD8^+$ T cells proliferate and differentiate into CTL. Activated CTLs acquire the ability to produce cytokines, such as interferon gamma (IFN-γ) and TNF-α, and cytotoxic molecules, such as perforin and granzymes (113). Helper $CD4^+$ T cells also play an important role in the development of anti-tumor immunity by improving clonal expansion of CTLs at the tumor site, preventing activation-induced cell death, and promoting the generation and maintenance of memory CTL (114).

However, it has been described that the immuno-evasive and immunosuppressive properties of CSCs can be an obstacle to inducing an effector immune response that can eradicate them. BCSCs express low levels of MHC I molecules (115), suggesting that these cells may evade the response of CTL. Furthermore, CSCs have a high expression of PD-L1, which is why they can inhibit the cytotoxic functions of T cells and, therefore, present a lower susceptibility to death (111). Still, various studies have shown that CSCs could be immunogenic in certain settings. Currently, different clinical studies have been developed that use dendritic cells loaded with CSC or mRNA lysates to vaccinate patients with lung (116), pancreas (117), glioblastoma (118), and breast cancer (119) among others. The results showed that vaccination induces a measurable

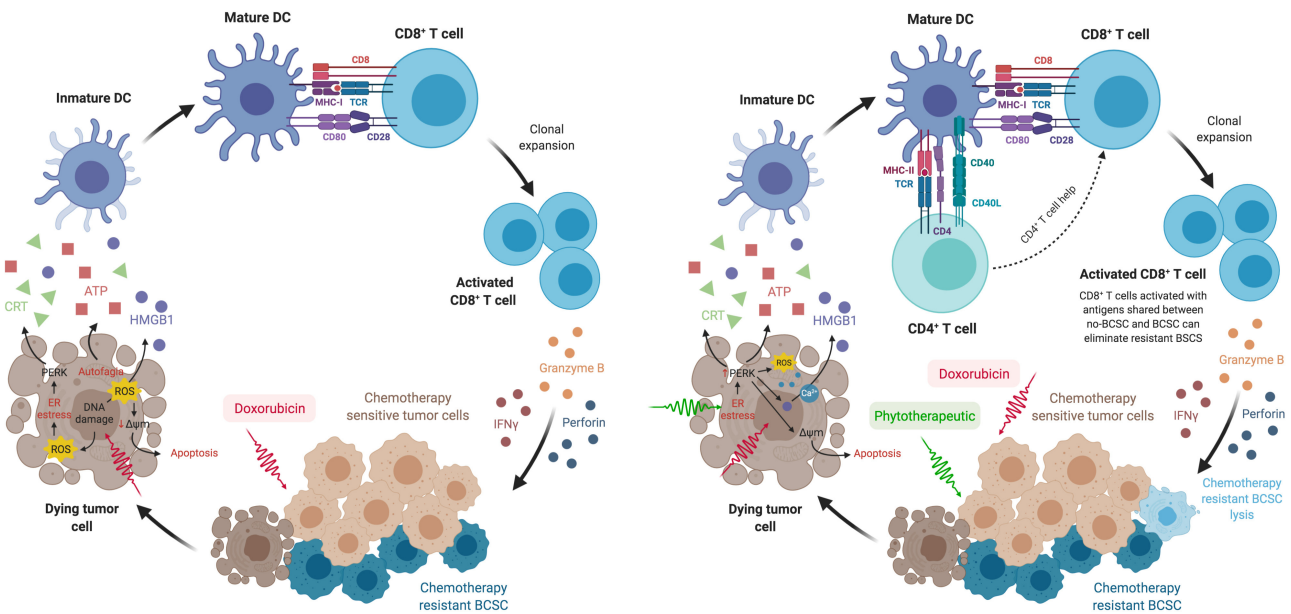


FIGURE 1 | (A) BCSCs could be eliminated through the immune response generated *in situ* when chemotherapy-sensitive tumor cells die from mechanisms such as immunogenic cell death. This mechanism allows the activation of dendritic cells as a consequence of the release of danger signals from dead cells, such as calreticulin exposure on their surface, secretion of ATP, and HMGB1 release. The processing and presentation of tumor antigens released by the dead cells would allow the activation of $CD8^+$ T cells by cross-priming mechanisms, which would ultimately attack the BCSC resistant to chemotherapy. **(B)** The combination of chemotherapy with phytotherapeutics or standardized extracts, such as P2Et, could act synergistically increasing tumor sensitivity to chemotherapy, recovering the tumor microenvironment and participating in the induction of an immune response not only of $CD8^+$ T cells but also of $CD4^+$ T cells, which in turn would lead to a better response of cytotoxic T cells that would attack BCSCs by cross-priming mechanisms, achieving lysis of BCSC and thus reducing metastasis. This figure was created using BioRender (https://biorender.com/) .

and specific anti-tumor immune response without strong side effects, suggesting that patients might benefit from anti-CSC vaccination. Treatment with standardized extracts from plants (phytomedicines), which have a large number of molecules with synergistic and sometimes antagonistic activities, which give them unique characteristics, could have multiple benefits. On the one hand, it could decrease the tumor mass by acting directly on the tumor, modulating the tumor microenvironment allowing its reversion to a normal metabolic stage, and as a consequence, favoring the activation of the anti-tumor immune response by a mechanism that could be considered as vaccination.

AUTHOR CONTRIBUTIONS

SF, CU, PL, KP, and AB reviewed the literature and wrote the manuscript. PL prepared figure. SF contributed to the planning of the content and the critical evaluation of the text. All authors approved the final version of the manuscript.

FUNDING

Funding of some of the results presented here was provided by the Sistema General de Regalías (BPIN: 2013000100196; contract number 1027-1-2015) and Programa Colombia Científica-Ministerio de Ciencia y Tecnología- Pontificia Universidad Javeriana. Convocatoria Ecosistema Científico (Contract No. FP44842-221-2018) and Pontificia Universidad Javeriana. PL was funded by the Departamento Administrativo de Ciencia, Tecnología e Innovación COLCIENCIAS, convocatoria 811-2018 (No. 335-2019; C160I003100000880) and Vicerrectoría de Investigaciones, Pontificia Universidad Javeriana (PUJ-04298-19, contrato de recuperación contingente No. 80740-335-2019), Bogotá, Colombia.

ACKNOWLEDGMENTS

The authors would like to thank Pontificia Universidad Javeriana and the Colombian Environmental Ministry for allowing the use of genetic resources and derived products (agreement number 1470 of 02/08/2018; No. 220, 2018).

REFERENCES

1. Kapinova A, Kubatka P, Golubnitschaja O, Kello M, Zubor P, Solar P, et al. Dietary phytochemicals in breast cancer research: anticancer effects and potential utility for effective chemoprevention. *Environ Health Prev Med.* (2018) 23:1–18. doi: 10.1186/s12199-018-0724-1
2. Xiang Y, Guo Z, Zhu P, Chen J, Huang Y. Traditional Chinese medicine as a cancer treatment: modern perspectives of ancient but advanced science. *Cancer Med.* (2019) 8:1958–75. doi: 10.1002/cam4.2108
3. WHO. *The Global Cancer Observatory*. (2018). Available online at: http://gco.iarc.fr (accessed July 20, 2020).
4. Fondo Colombiano de Enfermedades de Alto Costo. *Situación del cáncer en la población adulta atendida en el SGSSS de Colombia 2018* (2019). Available online at: https://cuentadealtocosto.org/site/cancer/
5. Bombonati A, Sgroi DC. The molecular pathology of breast cancer progression. *J Pathol.* (2011) 223:307–17. doi: 10.1002/path.2808
6. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast cancer. *Nat Rev Dis Primers.* (2019) 5:66. doi: 10.1038/s41572-019-0111-2
7. Bertos NR, Park M. Breast cancer - one term, many entities? *J Clin Invest.* (2011) 121:3789–96. doi: 10.1172/JCI57100
8. Wiechmann L, Kuerer HM. The molecular journey from ductal carcinoma *in situ* to invasive breast cancer. *Cancer.* (2008) 112:2130–42. doi: 10.1002/cncr.23430
9. Bareche Y, Buisseret L, Gruosso T, Girard E, Venet D, Dupont F, et al. Unraveling triple-negative breast cancer tumor microenvironment heterogeneity: towards an optimized treatment approach. *JNCI.* (2019) 112:708–19. doi: 10.1093/jnci/djz208
10. Carey L, Winer E, Viale G, Cameron D, Gianni L. Triple-negative breast cancer: disease entity or title of convenience? *Nat Rev Clin Oncol.* (2010) 7:683–92. doi: 10.1038/nrclinonc.2010.154
11. Giuliano AE, Connolly JL, Edge SB, Mittendorf EA, Rugo HS, Solin LJ, et al. Breast cancer-Major changes in the American joint committee on cancer eighth edition cancer staging manual. *CA Cancer J Clin.* (2017) 67:290–303. doi: 10.3322/caac.21393
12. Rivera E, Gomez H. Chemotherapy resistance in metastatic breast cancer: the evolving role of ixabepilone. *Breast Cancer Res.* (2010) 12(Suppl 2):S2. doi: 10.1186/bcr2573
13. Mego M, Mani SA, Cristofanilli M. Molecular mechanisms of metastasis in breast cancer–clinical applications. *Nat Rev Clin Oncol.* (2010) 7:693–701. doi: 10.1038/nrclinonc.2010.171
14. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, et al. Isolation and *in vitro* propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res.* (2005) 65:5506–11. doi: 10.1158/0008-5472.CAN-05-0626
15. Liu H, Patel MR, Prescher JA, Patsialou A, Qian D, Lin J, et al. Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. *Proc Natl Acad Sci USA.* (2010) 107:18115–20. doi: 10.1073/pnas.1006732107
16. Morrison BJ, Schmidt CW, Lakhani SR, Reynolds BA, Lopez JA. Breast cancer stem cells: implications for therapy of breast cancer. *Breast Cancer Res.* (2008) 10:210. doi: 10.1186/bcr2111
17. Dey P, Rathod M, De A. Targeting stem cells in the realm of drug-resistant breast cancer. *Breast Cancer.* (2019) 11:115–35. doi: 10.2147/BCTT.S189224
18. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA.* (2003) 100:3983–8. doi: 10.1073/pnas.0530291100
19. Covert H, Mellor LF, Wolf CL, Ankenbrandt N, Emathinger JM, Tawara K, et al. OSM-induced CD44 contributes to breast cancer metastatic potential through cell detachment but not epithelial-mesenchymal transition. *Cancer Manag Res.* (2019) 11:7721–737. doi: 10.2147/CMAR.S208721
20. Rangaswami H, Bulbule A, Kundu GC. Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol.* (2006) 16:79–87. doi: 10.1016/j.tcb.2005.12.005
21. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev.* (2003) 17:1253–70. doi: 10.1101/gad.1061803
22. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell.* (2007) 1:555–67. doi: 10.1016/j.stem.2007.08.014
23. Liu S, Cong Y, Wang D, Sun Y, Deng L, Liu Y, et al. Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. *Stem Cell Rep.* (2014) 2:78–91. doi: 10.1016/j.stemcr.2013.11.009
24. An Y, Ongkeko WM. ABCG2: the key to chemoresistance in cancer stem cells? *Expert Opin Drug Metab Toxicol.* (2009) 5:1529–42. doi: 10.1517/17425250903228834
25. Moitra K, Lou H, Dean M. Multidrug efflux pumps and cancer stem cells: insights into multidrug resistance and therapeutic development. *Clin Pharmacol Ther.* (2011) 89:491–502. doi: 10.1038/clpt.2011.14
26. Zinzi L, Contino M, Cantore M, Capparelli E, Leopoldo M, Colabufo NA. ABC transporters in CSCs membranes as a novel target for treating tumor relapse. *Front Pharmacol.* (2014) 5:163. doi: 10.3389/fphar.2014.00163
27. Cheung AM, Wan TS, Leung JC, Chan LY, Huang H, Kwong YL, et al. Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engrafting potential. *Leukemia.* (2007) 21:1423–30. doi: 10.1038/sj.leu.2404721
28. Carpentino JE, Hynes MJ, Appelman HD, Zheng T, Steindler DA, Scott EW, et al. Aldehyde dehydrogenase-expressing colon stem cells contribute to tumorigenesis in the transition from colitis to cancer. *Cancer Res.* (2009) 69:8208–15. doi: 10.1158/0008-5472.CAN-09-1132
29. Medema JP. Cancer stem cells: the challenges ahead. *Nat Cell Biol.* (2013) 15:338–44. doi: 10.1038/ncb2717
30. Carrasco E, Alvarez PJ, Prados J, Melguizo C, Rama AR, Aranega A, et al. Cancer stem cells and their implication in breast cancer. *Eur J Clin Invest.* (2014) 44:678–87. doi: 10.1111/eci.12276
31. Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res.* (2007) 67:4827–33. doi: 10.1158/0008-5472.CAN-06-3557
32. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu M-F, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst.* (2008) 100:672–9. doi: 10.1093/jnci/djn123
33. Tanei T, Morimoto K, Shimazu K, Kim SJ, Tanji Y, Taguchi T, et al. Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. *Clin Cancer Res.* (2009) 15:4234–41. doi: 10.1158/1078-0432.CCR-08-1479
34. Samanta D, Park Y, Ni X, Li H, Zahnow CA, Gabrielson E, et al. Chemotherapy induces enrichment of CD47⁺/CD73⁺/PDL1⁺ immune evasive triple-negative breast cancer cells. *Proc Natl Acad Sci USA.* (2018) 115:E1239–48. doi: 10.1073/pnas.1718197115
35. Castañeda DM, Pombo LM, Uruena CP, Hernandez JF, Fiorentino S. A gallotannin-rich fraction from *Caesalpinia spinosa* (Molina) kuntze displays cytotoxic activity and raises sensitivity to doxorubicin in a leukemia cell line. *BMC Complement Altern Med.* (2012) 12:38. doi: 10.1186/1472-6882-12-38
36. Gomez-Cadena A, Uruena C, Prieto K, Martinez-Usatorre A, Donda A, Barreto A, et al. Immune-system-dependent anti-tumor activity of a plant-derived polyphenol rich fraction in a melanoma mouse model. *Cell Death Dis.* (2016) 7:e2243. doi: 10.1038/cddis.2016.134
37. Hernandez JF, Uruena CP, Cifuentes MC, Sandoval TA, Pombo LM, Castaneda D, et al. A petiveria alliacea standardized fraction induces breast adenocarcinoma cell death by modulating glycolytic metabolism. *J Ethnopharmacol.* (2014) 153:641–9. doi: 10.1016/j.jep.2014.03.013
38. Lasso P, Gomez-Cadena A, Urueña C, Donda A, Martinez-Usatorre A, Barreto A, et al. Prophylactic vs. therapeutic treatment with P2Et polyphenol-rich extract has opposite effects on tumor growth. *Front Oncol.* (2018) 8:356. doi: 10.3389/fonc.2018.00356
39. Sandoval TA, Uruena CP, Llano M, Gomez-Cadena A, Hernandez JF, Sequeda LG, et al. Standardized extract from *Caesalpinia spinosa* is cytotoxic

over cancer stem cells and enhance anticancer activity of doxorubicin. *Am J Chin Med.* (2016) 44:1693–717. doi: 10.1142/S0192415X16500956
40. Urueña C, Gomez A, Sandoval T, Hernandez J, Li S, Barreto A, et al. Multifunctional T Lymphocytes generated after therapy with an antitumor gallotanin-rich normalized fraction are related to primary tumor size reduction in a breast cancer model. *Integr Cancer Ther.* (2015) 14:468–83. doi: 10.1177/1534735415596425
41. Urueña C, Mancipe J, Hernandez J, Castaneda D, Pombo L, Gomez A, et al. Gallotannin-rich *Caesalpinia spinosa* fraction decreases the primary tumor and factors associated with poor prognosis in a murine breast cancer model. *BMC Complement Altern Med.* (2013) 13:74. doi: 10.1186/1472-6882-13-74
42. Tesniere A, Apetoh L, Ghiringhelli F, Joza N, Panaretakis T, Kepp O, et al. Immunogenic cancer cell death: a key-lock paradigm. *Curr Opin Immunol.* (2008) 20:504–11. doi: 10.1016/j.coi.2008.05.007
43. Jia D, Lu M, Jung KH, Park JH, Yu L, Onuchic JN, et al. Elucidating cancer metabolic plasticity by coupling gene regulation with metabolic pathways. *Proc Natl Acad Sci USA.* (2019) 116:3909–18. doi: 10.1073/pnas.1816391116
44. Dong C, Yuan T, Wu Y, Wang Y, Fan TW, Miriyala S, et al. Loss of FBP1 by snail-mediated repression provides metabolic advantages in basal-like breast cancer. *Cancer Cell.* (2013) 23:316–31. doi: 10.1016/j.ccr.2013.01.022
45. Liu PP, Liao J, Tang ZJ, Wu WJ, Yang J, Zeng ZL, et al. Metabolic regulation of cancer cell side population by glucose through activation of the Akt pathway. *Cell Death Differ.* (2014) 21:124–35. doi: 10.1038/cdd.2013.131
46. Tamada M, Nagano O, Tateyama S, Ohmura M, Yae T, Ishimoto T, et al. Modulation of glucose metabolism by CD44 contributes to antioxidant status and drug resistance in cancer cells. *Cancer Res.* (2012) 72:1438–48. doi: 10.1158/0008-5472.CAN-11-3024
47. Li Z, Rich JN. Hypoxia and hypoxia inducible factors in cancer stem cell maintenance. *Curr Top Microbiol Immunol.* (2010) 345:21–30. doi: 10.1007/82_2010_75
48. Cho YM, Kwon S, Pak YK, Seol HW, Choi YM, Park DJ, et al. Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochem Biophys Res Commun.* (2006) 348:1472–8. doi: 10.1016/j.bbrc.2006.08.020
49. Chae YC, Kim JH. Cancer stem cell metabolism: target for cancer therapy. *BMB Rep.* (2018) 51:319–26. doi: 10.5483/BMBRep.2018.51.7.112
50. Farnie G, Sotgia F, Lisanti MP. High mitochondrial mass identifies a sub-population of stem-like cancer cells that are chemo-resistant. *Oncotarget.* (2015) 6:30472–86. doi: 10.18632/oncotarget.5401
51. Dupuy F, Tabaries S, Andrzejewski S, Dong Z, Blagih J, Annis MG, et al. PDK1-dependent metabolic reprogramming dictates metastatic potential in breast cancer. *Cell Metab.* (2015) 22:577–89. doi: 10.1016/j.cmet.2015.08.007
52. Mani S, Taneja N, Jain S, Singh M. Anticancerous plant compounds affecting the power house of cancerous cells: a possible herbal mitocan. In. Akhtar MS, Swamy MK, editors. *Anticancer Plants: Mechanisms and Molecular Interactions: Volume 4. Ed.* Singapore: Springer Singapore (2018). p. 227–9.
53. Cui Q, Wen S, Huang P. Targeting cancer cell mitochondria as a therapeutic approach: recent updates. *Future Med Chem.* (2017) 9:929–49. doi: 10.4155/fmc-2017-0011
54. Skoda J, Borankova K, Jansson PJ, Huang L-H, Veselska R, Richardson DR. Pharmacological targeting of mitochondria in cancer stem cells: an ancient organelle at the crossroad of novel anti-cancer therapies. *Pharmacol Res.* (2019) 139:298–313. doi: 10.1016/j.phrs.2018.11.020
55. De Francesco EM, Sotgia F, Lisanti MP. Cancer stem cells (CSCs): metabolic strategies for their identification and eradication. *Biochem J.* (2018) 475:1611–34. doi: 10.1042/BCJ20170164
56. Hernández J, Urueña C, Sandoval T, Cifuentes M, Formentini L, Cuezva J, et al. A cytotoxic petiveria alliacea dry extract induces ATP depletion and decreases β-F1-ATPase expression in breast cancer cells and promotes survival in tumor-bearing mice. *Rev Brasil Farmacog.* (2017) 27:306–14. doi: 10.1016/j.bjp.2016.09.008
57. Prieto K, Cao Y, Mohamed E, Trillo-Tinoco J, Sierra RA, Uruena C, et al. Polyphenol-rich extract induces apoptosis with immunogenic markers in melanoma cells through the ER stress-associated kinase PERK. *Cell Death Discov.* (2019) 5:134. doi: 10.1038/s41420-019-0214-2
58. O'Neill S, Porter RK, McNamee N, Martinez VG, L. O'Driscoll: 2-Deoxy-D-Glucose inhibits aggressive triple-negative breast cancer cells by targeting glycolysis and the cancer stem cell phenotype. *Sci Rep.* (2019) 9:3788. doi: 10.1038/s41598-019-39789-9
59. Luo M, Shang L, Brooks MD, Jiagge E, Zhu Y, Buschhaus JM, et al. Targeting breast cancer stem cell state equilibrium through modulation of redox signaling. *Cell Metab.* (2018) 28:69–86.e6. doi: 10.1016/j.cmet.2018.06.006
60. Samanta D, Park Y, Andrabi SA, Shelton LM, Gilkes DM, Semenza GL. PHGDH expression is required for mitochondrial redox homeostasis, breast cancer stem cell maintenance, lung metastasis. *Cancer Res.* (2016) 76:4430–42. doi: 10.1158/0008-5472.CAN-16-0530
61. Wei L, Lee D, Law C-T, Zhang MS, Shen JD, Chin W-C, et al. Genome-wide CRISPR/Cas9 library screening identified PHGDH as a critical driver for Sorafenib resistance in HCC. *Nat Commun.* (2019) 10:1–13. doi: 10.1038/s41467-019-12606-7
62. Zheng M, Guo J, Xu J, Yang K, Tang R, Gu X, et al. Ixocarpalactone A from dietary tomatillo inhibits pancreatic cancer growth by targeting PHGDH. *Food Func.* (2019) 10:3386–95. doi: 10.1039/C9FO00394K
63. Banik U, Parasuraman S, Adhikary AK, Othman NH. Curcumin: the spicy modulator of breast carcinogenesis. *J Exp Clin Cancer Res.* (2017) 36:98. doi: 10.1186/s13046-017-0566-5
64. Guerra AR, Duarte MF, Duarte IF. Targeting tumor metabolism with plant-derived natural products: emerging trends in cancer therapy. *J Agric Food Chem.* (2018) 66:10663–85. doi: 10.1021/acs.jafc.8b04104
65. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. *Nat Rev Drug Discov.* (2011) 10:671–84. doi: 10.1038/nrd3504
66. Diehn M, Cho RW, Lobo NA, Kalisky T, Dorie MJ, Kulp AN, et al. Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature.* (2009) 458:780–3. doi: 10.1038/nature07733
67. Batlle E, Clevers H. Cancer stem cells revisited. *Nat Med.* (2017) 23:1124–34. doi: 10.1038/nm.4409
68. Bhat V, Allan AL, Raouf A. Role of the microenvironment in regulating normal and cancer stem cell activity: implications for breast cancer progression and therapy response. *Cancers.* (2019) 11:1240. doi: 10.3390/cancers11091240
69. Martinez-Outschoorn UE, Lisanti MP, Sotgia F. Catabolic cancer-associated fibroblasts transfer energy and biomass to anabolic cancer cells, fueling tumor growth. *Sem Cancer Biol.* Elsevier. (2014) 25:47–60. doi: 10.1016/j.semcancer.2014.01.005
70. Chan T-S, Shaked Y, Tsai KK. Targeting the interplay between cancer fibroblasts, mesenchymal stem cells, and cancer stem cells in desmoplastic cancers. *Front Oncol.* (2019) 9:688. doi: 10.3389/fonc.2019.00688
71. Zhou Q, Bennett LL, Zhou S. Multifaceted ability of naturally occurring polyphenols against metastatic cancer. *Clin Exp Pharmacol Physiol.* (2016) 43:394–409. doi: 10.1111/1440-1681.12546
72. Abdal Dayem, Choi HY, Yang G-M, Kim K, Saha SK, Cho S.-G. The anti-cancer effect of polyphenols against breast cancer and cancer stem cells: molecular mechanisms. *Nutrients.* (2016) 8:581. doi: 10.3390/nu8090581
73. Kapinova A, Kubatka P, Liskova A, Baranenko D, Kruzliak P, Matta M, et al. Controlling metastatic cancer: the role of phytochemicals in cell signaling. *J Cancer Res Clin Oncol.* (2019) 145:1087–109. doi: 10.1007/s00432-019-02892-5
74. Pistollato F, Giampieri F, Battino M. The use of plant-derived bioactive compounds to target cancer stem cells and modulate tumor microenvironment. *Food Chem Toxicol.* (2015) 75:58–70. doi: 10.1016/j.fct.2014.11.004
75. Hermawan A, Putri H. Current report of natural product development against breast cancer stem cells. *Int J Biochem Cell Biol.* (2018) 104:114–32. doi: 10.1016/j.biocel.2018.09.012
76. Pommier SJ, Morgan RE, Limbach KE, Jackson CM, Naik AM, Peckham JL, et al. Surgical resection of breast cancers: molecular analysis of cancer stem cells in residual disease. *Surgery.* (2019) 165:1008–13. doi: 10.1016/j.surg.2019.01.001
77. Donovan CA, Pommier RF, Schillace R, O'Neill S, Muller P, Alabran JL, et al. Correlation of breast cancer axillary lymph node metastases with stem cell mutations. *JAMA Surg.* (2013) 148:873–8. doi: 10.1001/jamasurg.2013.3028
78. Daniilides K, Lougiakis N, Evangelidis T, Kostakis IK, Pouli N, Marakos P, et al. Discovery of new aminosubstituted pyrrolopyrimidines with antiproliferative activity against breast cancer cells and investigation of their

effect towards the PI3Kα enzyme. *Anticancer Agents Med Chem.* (2017) 17:990–1002. doi: 10.2174/1871520616666161207143450
79. Khan N, Jajeh F, Eberhardt EL, Miller DD, Albrecht DM, Van Doorn R, et al. Fisetin and 5-fluorouracil: effective combination for PIK3CA-mutant colorectal cancer. *Int J Cancer.* (2019) 145:3022–32. doi: 10.1002/ijc.32367
80. Schwab LP, Peacock DL, Majumdar D, Ingels JF, Jensen LC, Smith KD, et al. Hypoxia-inducible factor 1alpha promotes primary tumor growth and tumor-initiating cell activity in breast cancer. *Breast Cancer Res.* (2012) 14:R6. doi: 10.1186/bcr3087
81. Peng G, Liu Y. Hypoxia-inducible factors in cancer stem cells and inflammation. *Trends Pharmacol Sci.* (2015) 36:374–83. doi: 10.1016/j.tips.2015.03.003
82. Wei Z, Shan Y, Tao L, Liu Y, Zhu Z, Liu Z, et al. Diallyl trisulfides, a natural histone deacetylase inhibitor, attenuate HIF-1alpha synthesis, and decreases breast cancer metastasis. *Mol Carcinog.* (2017) 56:2317–31. doi: 10.1002/mc.22686
83. Fallah J, Rini BI. HIF inhibitors: status of current clinical development. *Curr Oncol Rep.* (2019) 21:6. doi: 10.1007/s11912-019-0752-z
84. Na JI, Shin JW, Choi HR, Kwon SH, Park KC. Resveratrol as a multifunctional topical hypopigmenting agent. *Int J Mol Sci.* (2019) 20:956. doi: 10.3390/ijms20040956
85. Pohl S-G, Brook N, Agostino M, Arfuso F, Kumar AP, Dharmarajan A. Wnt signaling in triple-negative breast cancer. *Oncogenesis.* (2017) 6:e310. doi: 10.1038/oncsis.2017.14
86. Rather RA, Bhagat M. Cancer chemoprevention and piperine: molecular mechanisms and therapeutic opportunities. *Front Cell Dev Biol.* (2018) 6:10. doi: 10.3389/fcell.2018.00010
87. Varghese E, Samuel SM, Abotaleb M, Cheema S, Mamtani R, Büsselberg D. The "yin and yang" of natural compounds in anticancer therapy of triple-negative breast cancers. *Cancers.* (2018) 10:346. doi: 10.3390/cancers10100346
88. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med.* (2005) 202:1691–701. doi: 10.1084/jem.20050915
89. Lasso P, Llano Murcia M, Sandoval TA, Uruena C, Barreto A, Fiorentino S. Breast tumor cells highly resistant to drugs are controlled only by the immune response induced in an immunocompetent mouse model. *Integr Cancer Ther.* (2019) 18:1534735419848047. doi: 10.1177/1534735419848047
90. Nguyen ST, Nguyen HL, Pham VQ, Nguyen GT, Tran CD, Phan NK, et al. Targeting specificity of dendritic cells on breast cancer stem cells: *in vitro* and *in vivo* evaluations. *Onco Targets Ther.* (2015) 8:323–34. doi: 10.2147/OTT.S77554
91. Visus C, Ito D, Amoscato A, Maciejewska-Franczak M, Abdelsalem A, Dhir R, et al. Identification of human aldehyde dehydrogenase 1 family member A1 as a novel CD8+ T-cell-defined tumor antigen in squamous cell carcinoma of the head and neck. *Cancer Res.* (2007) 67:10538–45. doi: 10.1158/0008-5472.CAN-07-1346
92. Visus C, Wang Y, Lozano-Leon A, Ferris RL, Silver S, Szczepanski MJ, et al. Targeting ALDH(bright) human carcinoma-initiating cells with ALDH1A1-specific CD8(+) T cells. *Clin Cancer Res.* (2011) 17:6174–84. doi: 10.1158/1078-0432.CCR-11-1111
93. Le Gal K, Ibrahim MX, Wiel C, Sayin VI, Akula MK, Karlsson C, et al. Antioxidants can increase melanoma metastasis in mice. *Sci Transl Med.* (2015) 7:308re8. doi: 10.1126/scitranslmed.aad3740
94. Sayin VI, Ibrahim MX, Larsson E, Nilsson JA, Lindahl P, Bergo MO. Antioxidants accelerate lung cancer progression in mice. *Sci Transl Med.* (2014) 6:221ra15. doi: 10.1126/scitranslmed.3007653
95. Peluso I, Miglio C, Morabito G, Ioannone F, Serafini M. Flavonoids and immune function in human: a systematic review. *Crit Rev Food Sci Nutr.* (2015) 55:383–95. doi: 10.1080/10408398.2012.656770
96. Mansoori B, Mohammadi A, Amin Doustvandi M, Mohammadnejad F, Kamari F, Gjerstorff MF, et al. Photodynamic therapy for cancer: role of natural products. *Photodiag Photodyn Ther.* (2019) 26:395–404. doi: 10.1016/j.pdpdt.2019.04.033
97. Garg AD, Nowis D, Golab J, Agostinis P. Photodynamic therapy: illuminating the road from cell death towards anti-tumour immunity. *Apoptosis.* (2010) 15:1050–71. doi: 10.1007/s10495-010-0479-7
98. Cubillos-Ruiz JR, Mohamed E, Rodriguez PC. Unfolding anti-tumor immunity: ER stress responses sculpt tolerogenic myeloid cells in cancer. *J Immunother Cancer.* (2017) 5:5. doi: 10.1186/s40425-016-0203-4
99. Hartman ZC, Poage GM, Den Hollander P, Tsimelzon A, Hill J, Panupinthu N, et al. Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. *Cancer Res.* (2013) 73:3470–80. doi: 10.1158/0008-5472.CAN-12-4524-T
100. Liu W, Yang B, Yang L, Kaur J, Jessop C, Fadhil R, et al. Therapeutic effects of ten commonly used chinese herbs and their bioactive compounds on cancers. *Evid Based Complement Alternat Med.* (2019) 2019:6057837. doi: 10.1155/2019/6057837
101. Peluso I, Yarla NS, Ambra R, Pastore G, Perry G. MAPK signalling pathway in cancers: Olive products as cancer preventive and therapeutic agents. *Sem Cancer Biol.* (2019) 56:185–95. doi: 10.1016/j.semcancer.2017.09.002
102. Chen W. New awareness on mushrooms and cancer: focusing on ganoderma lucidum and breast cancer. *Curr Top Nutrac Res.* (2011) 9:155–66.
103. Zhang J, Si J, Gan L, Di C, Xie Y, Sun C, et al. Research progress on therapeutic targeting of quiescent cancer cells. *Artif Cells Nanomed Biotechnol.* (2019) 47:2810–9. doi: 10.1080/21691401.2019.1638793
104. Wolfers J, Lozier A, Raposo G, Regnault A, Théry C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med.* (2001) 7:297–303. doi: 10.1038/85438
105. Hirohashi Y, Torigoe T, Inoda S, Kobayasi J, Nakatsugawa M, Mori T, et al. The functioning antigens: beyond just as the immunological targets. *Cancer Sci.* (2009) 100:798–806. doi: 10.1111/j.1349-7006.2009.01137.x
106. Idenoue S, Hirohashi Y, Torigoe T, Sato Y, Tamura Y, Hariu H, et al. A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins. *Clin Cancer Res.* (2005) 11:1474–82. doi: 10.1158/1078-0432.CCR-03-0817
107. Hariu H, Hirohashi Y, Torigoe T, Asanuma H, Hariu M, Tamura Y, et al. Aberrant expression and potency as a cancer immunotherapy target of inhibitor of apoptosis protein family, Livin/ML-IAP in lung cancer. *Clin Cancer Res.* (2005) 11:1000–9. Available online at: https://clincancerres.aacrjournals.org/content/11/3/1000.full-text.pdf
108. Madjd Z, Mehrjerdi AZ, Sharifi AM, Molanaei S, Shahzadi SZ, Asadi-Lari M. CD44+ cancer cells express higher levels of the anti-apoptotic protein Bcl-2 in breast tumours. *Cancer Immun.* (2009) 9:4. Available online at: https://cancerimmunolres.aacrjournals.org/content/canimmarch/9/1/4.full-text.pdf
109. Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, et al. Notch signalling regulates stem cell numbers *in vitro* and *in vivo*. *Nature.* (2006) 442:823–6. doi: 10.1038/nature04940
110. Mine T, Matsueda S, Li Y, Tokumitsu H, Gao H, Danes C, et al. Breast cancer cells expressing stem cell markers CD44+ CD24 lo are eliminated by numb-1 peptide-activated T cells. *Cancer Immunol Immunother.* (2009) 58:1185–94. doi: 10.1007/s00262-008-0623-1
111. Wu Y, Chen M, Wu P, Chen C, Xu ZP, Gu W. Increased PD-L1 expression in breast and colon cancer stem cells. *Clin Exp Pharmacol Physiol.* (2017) 44:602–4. doi: 10.1111/1440-1681.12732
112. Hammerich L, Binder A, Brody JD. *In situ* vaccination: cancer immunotherapy both personalized and off-the-shelf. *Mol Oncol.* (2015) 9:1966–81. doi: 10.1016/j.molonc.2015.10.016
113. Oehen S, Brduscha-Riem K. Differentiation of naive CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. *J Immunol.* (1998) 161:5338–46.
114. Kennedy R, Celis E. Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunol Rev.* (2008) 222:129–44. doi: 10.1111/j.1600-065X.2008.00616.x
115. Tallerico R, Conti L, Lanzardo S, Sottile R, Garofalo C, Wagner AK, et al. NK cells control breast cancer and related cancer stem cell hematological spread. *Oncoimmunology.* (2017) 6:e1284718. doi: 10.1080/2162402X.2017.1284718
116. Lin M, Li SY, Xu KC, Liu ZP, Mu F, Yuan YY, et al. Safety and efficacy study of lung cancer stem cell vaccine. *Immunol Res.* (2015) 62:16–22. doi: 10.1007/s12026-015-8631-7

117. Lin M, Yuan YY, Liu SP, Shi JJ, Long XA, Niu LZ, et al. Prospective study of the safety and efficacy of a pancreatic cancer stem cell vaccine. *J Cancer Res Clin Oncol*. (2015) 141:1827–33. doi: 10.1007/s00432-015-1968-4
118. Vik-Mo EO, Nyakas M, Mikkelsen BV, Moe MC, Due-Tonnesen P, Suso EM, et al. Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma. *Cancer Immunol Immunother*. (2013) 62:1499–509. doi: 10.1007/s00262-013-1453-3
119. Garcia-Cremades M, Solans BP, Hughes E, Ernest JP, Wallender E, Savic RM. Assessing the impact of the addition of dendritic cell vaccination to neoadjuvant chemotherapy in breast cancer patients: amodel-based characterization approach. *Br J Clin Pharmacol*. (2019) 85:1670–83. doi: 10.1111/bcp.13947

Conflict of Interest: SF and CU are inventors of a granted patent related to P2Et.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 10 September 2020
doi: 10.3389/fimmu.2020.01820

Galectin-3 in Prostate Cancer Stem-Like Cells Is Immunosuppressive and Drives Early Metastasis

Sara Caputo[1,2,3†], Matteo Grioni[1,2], Chiara S. Brambillasca[1,2†], Antonella Monno[4], Arianna Brevi[1,2], Massimo Freschi[2,5], Ignazio S. Piras[6], Angela R. Elia[1,2†], Valentina Pieri[7], Tania Baccega[3,8], Angelo Lombardo[3,8], Rossella Galli[7], Alberto Briganti[2,3,9], Claudio Doglioni[2,3,5], Elena Jachetti[1,2†] and Matteo Bellone[1,2]*

[1] Cellular Immunology Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milan, Italy, [2] NET-IMPACT, IRCCS San Raffaele Scientific Institute, Milan, Italy, [3] Vita-Salute San Raffaele University, Milan, Italy, [4] Innate Immunity and Tissue Remodeling Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milan, Italy, [5] Unit of Pathology, IRCCS San Raffaele Scientific Institute, Milan, Italy, [6] Neurogenomics Division, Center for Rare Childhood Disorders (C4RCD), Translational Genomics Research Institute, Phoenix, AZ, United States, [7] Neural Stem Cell Biology Unit, Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy, [8] San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy, [9] Unit of Urology and URI, Division of Oncology, IRCCS Ospedale San Raffaele, Milan, Italy

Galectin-3 (Gal-3) is an extracellular matrix glycan-binding protein with several immunosuppressive and pro-tumor functions. The role of Galectin-3 in cancer stem-like cells (CSCs) is poorly investigated. Here, we show that prostate CSCs also colonizing prostate-draining lymph nodes of transgenic adenocarcinoma of the mouse prostate (TRAMP) mice overexpress Gal-3. Gal-3 contributes to prostate CSC-mediated immune suppression because either Gal-3 silencing in CSCs, or co-culture of CSCs and T cells in the presence of the Gal-3 inhibitor N-Acetyl-D-lactosamine rescued T cell proliferation. N-Acetyl-D-lactosamine also rescued the proliferation of T cells in prostate-draining lymph nodes of TRAMP mice affected by prostate intraepithelial neoplasia. Additionally, Gal-3 impacted prostate CSC tumorigenic and metastatic potential *in vivo*, as Gal-3 silencing in prostate CSCs reduced both primary tumor growth and secondary invasion. Gal-3 was also found expressed in more differentiated prostate cancer cells, but with different intracellular distribution as compared to CSCs, which suggests different functions of Gal-3 in the two cell populations. In fact, the prevalent nuclear and cytoplasmic distribution of Gal-3 in prostate CSCs made them less susceptible to apoptosis, when compared to more differentiated prostate cancer cells, in which Gal-3 was predominantly intra-cytoplasmic. Finally, we found Gal-3 expressed in human and mouse prostate intraepithelial neoplasia lesions and in metastatic lymph nodes. All together, these findings identify Gal-3 as a key molecule and a potential therapeutic target already in the early phases of prostate cancer progression and metastasis.

Keywords: prostate cancer, cancer stem cell, Galectin-3, immunosuppression, immune surveillance, prostate intraepithelial neoplasia, metastasis, T lymphocytes

OPEN ACCESS

Edited by:
Ira Ida Skvortsova, Innsbruck Medical University, Austria

Reviewed by:
Ying Ma, University of Texas MD Anderson Cancer Center, United States
Nahum Puebla-Osorio, University of Texas MD Anderson Cancer Center, United States

***Correspondence:**
Matteo Bellone
bellone.matteo@hsr.it

†Present address:
Sara Caputo, L-Nutra Italia SRL, Milan, Italy
Chiara S. Brambillasca, Department of Molecular Pathology, The Netherlands Cancer Institute, Amsterdam, Netherlands
Angela R. Elia, Institute of Oncology Research, Bellinzona, Switzerland
Elena Jachetti, Molecular Immunology Unit, Department of Research, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *05 March 2020*
Accepted: *07 July 2020*
Published: *10 September 2020*

Citation:
Caputo S, Grioni M, Brambillasca CS, Monno A, Brevi A, Freschi M, Piras IS, Elia AR, Pieri V, Baccega T, Lombardo A, Galli R, Briganti A, Doglioni C, Jachetti E and Bellone M (2020) Galectin-3 in Prostate Cancer Stem-Like Cells Is Immunosuppressive and Drives Early Metastasis. Front. Immunol. 11:1820. doi: 10.3389/fimmu.2020.01820

INTRODUCTION

Cancer is a multifactorial disease in which genetic and environmental factors concomitantly and progressively lead to neoplastic transformation and tumor development (1). According to the hierarchical model of cancer evolution, cancer stem-like cells (CSCs) represent the subpopulation of cancer cells within the tumor bulk that are endowed with tumorigenic potential, thus driving tumor growth and metastasis (2, 3). Nevertheless, cancer cells are not solitary entities, as they are embedded within the tumor microenvironment, which is composed of several players among which stromal cells (4), immune cells (5), and non-cellular components such as collagen fibers and glycosylated molecules, constituting the extracellular matrix (6, 7). A constant crosstalk between cancer cells and the tumor microenvironment ensures tumor development and progression.

Galectin-3 (Gal-3) is an extracellular matrix glycan-binding protein whose function spans several biological processes, including immune modulation, chemoattraction, cell adhesion, activation, differentiation, and apoptosis (8). Gal-3 is involved in several pathological events, and the function of Gal-3 depends on its location within the cell. When expressed on the plasma membrane or secreted, Gal-3 interacts with glycoconjugates, i.e., carbohydrate structures linked to lipids, proteins and peptides, thus mediating cell-cell, and cell-matrix interactions. In the cytoplasm, Gal-3 participates to the regulation of cell growth, cell cycle progression, and may inhibit apoptosis. Conversely, when localized into the nucleus, Gal-3 is pro-apoptotic. Given its pleiotropic effects, Gal-3 has been referred to as: "the guardian of the tumor microenvironment" (9).

Gal-3 is variably expressed in tumors, where it favors malignant transformation, invasion, and metastasis, and can also exert immunosuppressive functions (10, 11).

Gal-3 has been extensively investigated in prostate cancer (12). The healthy human prostate epithelium shows moderate immunostaining for Gal-3 that is localized both in the nucleus and the cytoplasm (13–16). In prostate intraepithelial neoplasia (PIN) lesions, Gal-3 expression is mainly cytoplasmic, more heterogeneous and more intense than in non-tumoral epithelium, but with a lower percentage of positive cells (14). Gal-3 expression is further reduced in prostate adenocarcinoma (13, 15), likely due to promoter methylation (17). Indeed, Gal-3 appears to be cleaved upon disease progression (18). However, in one report of 145 prostate cancer patients subjected to radical prostatectomy, the intensity of Gal-3 expression in carcinoma cells significantly associated with prostate specific antigen (PSA) relapse in univariate analysis, and exclusive cytoplasmic localization of Gal-3 was an independent prognostic indicator of biochemical progression after radical prostatectomy (14). Thus, localization within some cancer cells rather then percentage of tumor cells expressing Gal-3 seems relevant in prostate cancer. Additionally, Gal-3 favors prostate cancer metastasis, and oral administration of modified citrus pectin reduced the number of lung metastases in rats (19). Gal-3 has also been suggested as complementary diagnostic marker to PSA blood test, as serum concentration of Gal-3 was found increased in metastatic prostate cancer patients when compared to healthy subjects (20). Intriguingly, Gal-3 has also been proposed as potential biomarker at early clinical stages of prostate cancer (21).

Although little is known about Gal-3 function in CSCs, Gal-3 expression has been reported in CSCs from ovarian, gastrointestinal, kidney, and lung tumors (22–26). CSCs have been implicated in the development and progression of primary lesions and metastases (27). How precociously CSCs invade sites of prospective clinical metastasis still needs to be defined. Two general paradigms explain the process of systemic cancer progression (28). The linear progression model establishes that tumor ontogeny fully occurs in the primary tumor, and identifies metastasis as a late event that follows the development of a large tumor bulk. Consequently, metastases and primary tumor share genetic similarities. Conversely, the parallel progression model posits that tumor cells leave the primary lesion before acquisition of full malignant phenotype, and migrate to secondary sites where they acquire additional genetic hits. Thus, great genetic and epigenetic disparities characterize primary tumor cells and metastasis founders. We have previously reported that CSCs obtained from mouse PIN lesions [hereafter named as TPIN-SCs; ref. (29)], and concomitantly, from histopathology negative prostate draining lymph nodes were phenotypically and functionally identical, thus suggesting a common origin (30), and demonstrating that lymph node invasion may already occur at the early stage of PIN in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice (31). Thus, our results support the hypothesis that prostate cancer adheres to the parallel progression model of metastasis.

Because Gal-3 is a key molecule involved in several aspects of tumor progression and metastasis (10), and our previous data suggested that TPIN-SCs over-express the Gal-3 transcript (29, 30), here we further investigated the expression of Gal-3 in TPIN-SCs, and asked if Gal-3 expressed by prostate CSCs plays a relevant role in the neoplastic process. Our findings demonstrate that already at the stage of mouse PIN, Gal-3 has a pivotal role in balancing tumorigenic, metastatic, and immunosuppressive abilities in CSCs.

MATERIALS AND METHODS

Mice

C57BL/6, C57BL/6-Tg(TcraTcrb)425Cbn/Crl, C57BL/6-Tg(TcraTcrb)1100Mjb/Crl (Charles River, Calco, Italy), and B6.129S7-Rag1tm1Mom/J mice (32) were housed in a pathogen-free animal facility. The latter two mouse strains were crossed to obtain RAG-$1^{-/-}$ OT1 mice. Heterozygous TRAMP mice (31) were generated as described (33). NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ mice are also known as NOD scid gamma (NSG; Charles River, Calco, Italy). Animals were treated in accordance with the European Community guidelines and with the approval of the Institutional Ethical Committee.

Cell Culture

TPIN071122 and TNE070116 cells and the newly obtained TPIN1323 CSCs were cultured in NeuroCult™ NS-A Basal Medium (STEMCELL TECHNOLOGIES) supplemented with

heparin, EGF, and bFGF according to the manufacturing instructions, as described previously (29). Murine splenocytes were cultured in T cell medium (TCM), composed by RPMI (Lonza), with 10% fetal bovine serum (FBS; Invitrogen, Milan, Italy), 2 mM L-glutamine, 150 U/ml streptomycin and 200 U/ml penicillin (Cambrex, Milan, Italy), 10 mM Hepes, 10 mM Sodium Pyruvate and 5 μM β-mercaptoetanol (Gibco-Invitrogen, Milan, Italy). TRAMP-C2 cells (34) were cultured in DMEM (Lonza), with 10% FBS (Invitrogen). Unless specified, all chemical reagents were from Sigma-Aldrich (St. Louis, MO, USA). Peptides were kindly provided by R. Longhi (CNR, Milan, Italy). Human Du145 (35) and PC-3 cells (36) were cultured in RPMI (Lonza), with 10% FBS (Invitrogen).

Proliferation Assays

Splenocytes were labeled with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester [CFSE, ref. (37)], and activated *in vitro* with anti-CD3 and anti-CD28 beads (Invitrogen) and IL-2 (R&D Systems, Minneapolis, MN, USA) according to the manufacturer instructions. When needed, irradiated (50 Gy) prostate CSC were added in co-culture at CSC:splenocyte ratio that corresponds to 1:10. When indicated, activated splenocytes were treated with 5 mM N-Acetyl-D-lactosamine (LacNac; Merck Life Science, Milan, Italy) 30 min before the addition of prostate CSCs. CFSE-labeled splenocytes from transgenic Rag-$1^{-/-}$ OTI mice were co-cultured with irradiated CSCs in the presence of the synthetic peptide $OVA_{257-264}$ (1 ng/ml) and 3.5 ng/ml IL-12 (R&D Systems) as previously described (38). After 4 or 3 days, respectively, cells were analyzed by FACS. Prostate-draining lymph nodes from TRAMP or WT mice were labeled with CFSE (30), cultured with or without 5 mM LacNAc, and analyzed after 3 days by FACS.

Gal-3 Silencing

TPIN071122 cells were stably infected with Gal-3 shRNA Lentiviral Particles or with control shRNA Lentiviral Particles (Sigma) at 10 MOI, according to the manifacturer's protocol, to generate TPIN-SCshGal3#5 and TPIN-SCshScram, respectively. Briefly, 5×10^4 cells/well were plated in a mixture of medium and Polybrene (Sigma). At day 2 lentiviral particles were added. At day 4 after infection, 2 μg/ml of puromycin dihydrochloride (Sigma) were added to select cells that had integrated the lentiviral particles.

Tumor Challenge

2×10^6 TPIN-SCshScram or TPIN-SCshGal3#5 were diluted 1:1 in Matrigel™ High Concentration (BD-Biosciences, Milan, Italy; 354248) and injected subcutaneously in male NSG recipients. 2×10^6 TRAMP-C2 cells were injected subcutaneously in male C57BL/6N recipients. Mice were monitored twice weekly. Mice were sacrificed if the tumor became ulcerated. Tumor size was evaluated by measuring two perpendicular diameters and height by a caliper. Because tumors grew homogeneously as ellipsoid shaped masses, their dimension was calculated applying the ellipsoid volume formula: $4/3\pi abc$, where a is height/2, b is width/2 and c is depth/2. To appreciate metastatic dissemination, the primary tumor was surgically resected when it achieved $\geq$ 80 mm^2 (major diameter $\times$ minor diameter) (39). Mice were sacrificed when lymph node metastases were palpable, and $\sim$1 month after surgery. Mice with no evidence of lymph node metastasis were killed 2 months after surgery.

Flow Cytometry

Single cell suspensions were obtained from cell cultures, incubated 10 min with FcR blocker (BD-Biosciences), labeled for 15 min at 4°C with fluorochrome-conjugated monoclonal antibodies or isotype controls (all from BD-Biosciences or BioLegend), and acquired by BD FACSCanto™ as previously described (40). Dead cells were excluded by gating on 7AAD staining or based on physical parameters. For apoptosis test, samples were stained in Annexin V binding buffer (BD). Data were analyzed using FlowJo software.

Western Blotting

Each cell pellet was homogenized in 10× volume of RIPA lysis buffer (10 mM Tris-Cl pH 7.2, 150 mM NaCl, 1 mM EDTA pH 8) with 1% Triton X-10/0.1% deoxycholate, 0.1% SDS, and protease and phosphatase inhibitor mixture (Roche). Samples were then diluted in Laemmli's SDS sample buffer. Proteins were separated by electrophoresis on 10% polyacrylamide gels according to the TGX Stain-Free FastCast Acrylamide kit protocol (Bio-Rad), and transferred onto Trans-Blot nitrocellulose membranes (Bio-Rad) according to the Trans-Blot Turbo Transfer System kit protocol (Bio-Rad). Ponceau staining (Sigma-Aldrich) was performed to confirm that the samples were loaded equally. The membranes were blocked in 5% non-fat dry milk in TBS-T (pH 7.4, with 0.1% Tween-20) for 1 h at room temperature. Primary antibodies were diluted in 3% BSA (Sigma-Aldrich) in TBS-T [mouse anti-calnexin 1:3,000 (Genetex) rat anti-mouse/human Gal-3 (E-Bioscience; 1:1,000)], and the membranes were incubated overnight at 4°C. The primary antibody was removed, and the blots were washed in TBS-T and then incubated for 45 min at room temperature in HRP-conjugated secondary antibodies [anti-mouse (Biorad) anti-rat (Amersham)]. Reactive proteins were visualized using a Clarity Western ECL substrate kit (Bio-Rad), and exposure was performed using UVItec (Cambridge MINI HD). Images were acquired by NineAlliance software.

Gal-3 Knocking Out

Gal3- and Cd44-KO cell lines were generated using CRISPR/Cas9 technology. sgRNA targeting the coding sequence of lagls3 (CTCAAGGATATCCGGGTGCA) or Cd44 (GATGTAACCTGCCGCTACGC) were cloned into a modified version of the lentiCRISPR lentiviral vector plasmid (Zhang lab, Addgene #52961). This third-generation lentiviral vector backbone was generated by replacing both the existing promoter with a Spleen Focus Forming Virus (SFFV) promoter (41) and the puromycin cassette with an enhanced Green Fluorescent Protein (eGFP) selection transgene, and by inserting a loxP site in the 3′-self-inactivating Long Terminal Repeat. A scramble sequence against the murine genome (GATCGGCAAGGTGTGGGTCG) was used as negative control. Vesicular stomatitis virus glycoprotein G pseudotyped lentiviral vectors stocks were prepared as previously described

(42). 10^5 TPIN1323 were brought at single-cell suspension and then transduced at multiplicity of infection of 5. Transduction medium was changed after 24 h and cells were expanded. Fifteen days after LV transduction, eGFP positive cells were sorted at BD FACSAria III (BD Bioscience), obtaining up to 98% cell purityImaging flow cytometry. Knock-out of Gal3 and Cd44 were evaluated by Surveyor Assay (41) and flow cytometry, respectively.

Imaging Flow Cytometry

The cells were resuspended at 40×10^6 cells/ml incubated 10 min with FcR blocker (BD-Biosciences), labeled for 30 min at 4°C with fluorochrome-conjugated monoclonal mouse/human Gal-3 antibodies (1:25; E-Bioscience) just before acquisition. For surface staining, fresh cells were also stained with 7AAD to exclude dead cells just before acquisition. For intracellular staining, cells were fixed with 2% PFA and permeabilized with Triton-X (0.1% in PBS), before incubation with the desired antibodies. Gal-3 antibody (1:100). Fresh samples were imaged by ImageStream IS100 Imaging Flow Cytometer (Amnis, Merck) using a 40× objective. The excitation laser powers used were as follow: 488 (150 mW) and 658 (90 mW). Fixed cells were instead imaged by ImageStream X MarkII Imaging Flow Cytometer using a 40× objective. The excitation laser powers used were as follow: 405 (10 mW), 488 (200 mW), and 642 (20 mW). At least 10,000 events were collected in each sample, and single stained controls were acquired with identical laser settings to create compensation matrix. Data analysis was performed using the IDEAS software (Amnis). First of all, cells were gated for cells in focus using the gradient root mean square feature and then single cells were identified using area and aspect ratio features on the brightfield image. In fixed samples we evaluated the intracellular localization of Gal-3 protein. Single cells were gated for Dapi and Gal-3 double positivity, and nuclear localization of Gal-3 was assessed by Similarity feature in the nuclear region. Similarity feature is the log transformed Pearson's Correlation Coefficient and is a measure of the degree to which two images are linearly correlated within a masked region (IDEAS software). Thus, when the intensity of Gal-3 in the nuclear region is high and Dapi staining is high, there is a linear correlation between the two images and the similarity feature has a high positive value.

Real-Time PCR

Total RNA from CSCs was extracted using the RNeasy Plus Mini kit (Qiagen, Chatsworth, CA, USA). cDNA was obtained from 1,000 ng of RNA using the M-MLV-Reverse Transcriptase kit (Promega, Madison, WI, USA). Real-Time PCR was performed in a total volume of 20 μL using the Taqman® Universal PCR Master Mix (Applied Biosystems, Monza, Italy), 2 μL of cDNA (prediluted 1:1) and specific probes for Gal-3 or L-19 (Applied Biosystems, Italy). Values were normalized to internal control (L-19) using the ΔCT method.

Immunohistochemistry and Immunofluorescence of Human and Mouse Samples

After institutional review board approval, a cohort of nine patients with pelvic node-positive prostate cancer treated with radical prostatectomy and extended pelvic lymph node dissection were randomly selected from our prospectively collected data-base. Human and TRAMP prostate or lymph node specimens were embedded in paraffin. Five micrometer sections were stained with Mayer- Hematoxylin and Eosin (BioOptica, Milan, Italy) and evaluated by an expert pathologist (33). Alternatively, after re-hydratation, antigen retrieval in 10 mM citric acid and blocking with 5% NGS, slides were incubated with the anti mouse/human Gal-3 (1:200; E-Bioscience) overnight at 4°C. A biotinylated secondary antibody was used 1:250 for 1 h at room temperature. Colorimetric revelation was made with Novared chromogen (Vector Labs, Burlingame, CA, USA). Slides were finally contrasted with Mayer-Hematoxylin (BioOptica), mounted with cover glass and examined under microscope (Carl Zeiss, Axioscope 40FL, Varese, Italy). Prostate CSCs or prostate cancer cells were plated on a matrigel-coated glass slide overnight, fixed with 4% PFA and permeabilized with PBS containing 0.1% Triton-X. After blocking with Triton X-100 0.1 and 5% NGS for 1 h at room temperature, cells were incubated with rat anti-mouse/human Gal-3 (E-Bioscience; 1:200) for 2 h at room temperature, and then with anti-rat Alexa 546 secondary antibody (E-bioscience; 1:200). Nuclei were stained with 0.1 μg/mL DAPI and slides were examined under TCS SP2 confocal microscope (Leica, Milan, Italy). For Oct-4 staining prostate CSCs were plated on a matrigel-coated glass slide overnight, fixed with 4% PFA and permeabilized with PBS containing 0.1% Triton-X and 2% BSA. After blocking with Triton X-100 0.1 and 5% NGS for 1 h at room temperature, cells were incubated with rat anti-mouse/human Oct-3/4 (Santa Cruz Biotechnology; 1:20) and rabbit anti-GFP (Invitrogen; 1:250) for 2 h at room temperature, and then with PE anti-mouse secondary antibody (Santa Cruz Biotechnology; 1:200) and anti-rabbit Alexa 488 secondary antibody (Life Technologies; 1:500), respectively. Nuclei were stained with 0.1 μg/mL DAPI and slides were examined under TCS SP2 confocal microscope (Leica, Milan, Italy).

IncuCyte

Cell and spheroid proliferations were assessed using Incucyte (Sartorius Essen Biosciences, AnnArbor, MI) over 88 h in culture, with image capture every 4 h. Cell and spheroid proliferations were measured and reported as mean area confluence percentage, IncuCyte Image Analysis Software. Spheroids identification was allowed by setting a minimum dimension in order to distinguish them from single cells.

Microarray-Based Gene Expression Profiling

Total RNA extracted using the RNeasy Micro and Mini kit (Qiagen, Chatsworth, CA, USA) was analyzed with Affimetrix Mouse Gene 1.0 ST Array as previously described (29).

Statistical Analyses

Statistical analyses were performed using the Student's *T*, One-Way Anova followed by Tukey's tests, or Fisher exact test. Values were considered statically significant for $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$.

RESULTS

Gal-3 Favors Proliferation, Spheroid Formation, Tumorigenicity, and Metastatic Potential of TPIN-SCs

We have previously reported that TPIN-SCs, despite their highly metastatic behavior (43), can be targeted both by innate and adaptive immune cells (44), but are also endowed with immunosuppressive activities (45). In particular, we have found that TPIN-SCs use the extracellular matrix protein Tenascin-C to dampen T cell activation (30, 46), a mechanism that might favor their metastatic propensity. However, Tenascin-C silencing in TPIN-SC did not completely abolish their immunosuppressive activity, thus suggesting that additional molecules were involved in CSCs-mediated immunosuppression (30). In search for additional mechanisms favoring CSC aggressiveness, we mined data from transcriptomic analyses of TPIN-SCs and CSCs derived from neuroendocrine tumors (TNE-SCs; **Figure 1A**) (29, 30), the latter being devoid of immunosuppressive activity (30). We found that lgals3, the gene coding for Gal-3, was over-expressed in TPIN-SCs (**Figure 1B**). Gal-3 expression in TPIN-SCs was confirmed by real time PCR (**Figure 1C**), flow cytometry (**Figure 1D**), and western blot (**Figure 1E**).

To assess the role of Gal-3 in CSC biology, we infected TPIN-SCs with lentiviral vectors encoding either a Gal-3-specific or a scrambled short hairpin RNA (shGal-3#5 and shScram, respectively). qPCR, flow cytometry and western blot analyses (**Figures 1C–E**) confirmed Gal-3 silencing in prostate CSCs. Strikingly, Gal-3 silencing in TPIN-SCs reduced cell confluence (**Figures 2A,B**) and spheroid confluence and area (**Figures 2A,D,F**). These results, although consistent with the hypothesis that Gal-3 regulates proliferation and sphere formation in CSCs, were not conclusive because Gal-3 expression in TPIN-SCshGal-3#5 cells was not totally abolished (**Figure 1**). Thus, we generated TPIN-SCkoGal-3 cells by knocking out lgals3 in TPIN-SCs (**Supplementary Figure 1**), and we compared their proliferation and sphere formation capacity with TPIN-SCkoScram (**Supplementary Figure 1**). As shown in **Figure 2C**, TPIN-SCkoGal-3 proliferated less than TPIN-SCkoScram, thus confirming that Gal-3 is important for TPIN-SC proliferation. At difference with TPIN-SCshGal-3#5 cells, TPIN-SCkoGal-3 did not show a reduced spheroid confluence and area (**Figures 2E,G**). Indeed, spheroid confluence was higher in TPIN-SCkoGal-3 than in TPIN-SCkoScram. A direct comparison between TPIN-SCshGal-3#5 and TPIN-SCkoGal-3 cannot be done because they have been derived from two different CSCs lines. Nevertheless, data reported in **Figure 2** suggest that Gal-3 dynamically regulate both cell proliferation and cell adhesion. Indeed Gal-3 contributes to cell-cell and cell-matrix interactions (8). Thus, it can be hypothesized that in the absence of endogenous Gal-3, less proliferating CSCs remain in clusters. CSCs might also compensate lack of Gal-3 by upregulating expression of other molecules involved in spheroid formation.

To investigate the tumorigenic and metastatic potential of prostate CSCs in a context devoid of the potentially confounding effects of the immune system, immunodeficient NSG mice were challenged with TPIN-SCs either silenced or not for Gal-3. TPIN-SCshScram and TPIN-SCshGal-3#5 generated tumors in 100% of NSG mice, but tumor growth was delayed in mice challenged with TPIN-SCshGal-3#5 (**Figure 3A**), and at day 44, the tumor dimension was reduced in mice challenged with TPIN-SCshGal-3#5 when compared to mice challenged with TPIN-SCshScram (**Figure 3B**). These data demonstrate that Gal-3 has a relevant tumor-cell intrinsic effect on prostate cancer progression.

To investigate the metastatic potential of TPIN-SCs, the primary subcutaneous lesion was surgically resected when the tumor mass reached an area of $\geq$80 mm^2, and mice were monitored thereafter for metastasis occurrence. The primary lesion was excised to allow metastases to show up before mice had to be culled due to primary lesion overgrowth. Gal-3 silencing in TPIN-SCs reduced their metastatic potential. Indeed, metastases were found in 56% of the mice injected with TPIN-SCshScram, and only in 36% of TPIN-SCshGal-3#5-challenged mice (**Figure 3C**). Tumor-draining lymph nodes were the preferred site of invasion upon subcutaneous injection. Interestingly, Gal-3 silencing in TPIN-SCs significantly reduced their tropism for lymph node invasion, and metastatic lymph nodes were found in 24% of the mice injected with TPIN-SCshGal-3#5, and 50% of mice challenged with TPIN-SCshScram (**Figure 3D**). Lack of Gal-3 did not significantly impact metastatic appearance in the lungs (**Figure 3E**), kidneys (**Figure 3F**), and liver (**Figure 3G**). Altogether, these findings suggest that Gal-3 has a relevant tumorigenic and metastatic role in prostate CSCs.

Localization of Gal-3 in Prostate CSCs and in TRAMP-C2 Prostate Cancer Cells

Because subcellular Gal-3 localization correlates with its function in tumor cells (10), we analyzed prostate CSCs by flow cytometry, immunofluorescence, and imageStream technology, which combines flow cytometry with the detail imagery of microscopy. TPIN-SCs expressed Gal-3 at the cell surface (**Figure 4A**) and in the intracellular space (**Figures 4B,C**). Approximately 50% of the TPIN-SCs were Gal-3 positive both in the cytoplasm and in the nucleus, whereas the remaining 50% showed a preferential cytoplasmic localization (**Figures 4C,D**). Also TRAMP-C2 cells, a line of more differentiated prostate cancer cells obtained from a TRAMP adenocarcinoma (34), expressed Gal-3 (**Figures 4A–C**). However, the majority of TRAMP-C2 cells showed a preferential cytoplasmic localization of Gal-3 (**Figures 4B–D**). Thus, Gal-3 is differently distributed in TPIN-SCs and more differentiated prostate cancer cells.

It has been recently reported that Gal-3 promotes lung cancer stemness via the EGFR/c-Myc/Sox-2 pathway (24), and Oct4, a stemness-related transcription factor (47), favors Gal-3 expression, thus establishing a positive regulatory loop in lung CSCs (24). To investigate whether a different intracellular expression of Gal-3 associates with different functional states (i.e., stem cells and their differentiated progeny), we transduced prostaspheres generated from TPIN-SCs, which contained both CSCs and committed more differentiated precursors, with a lentiviral vector carrying the GFP sequence under the control

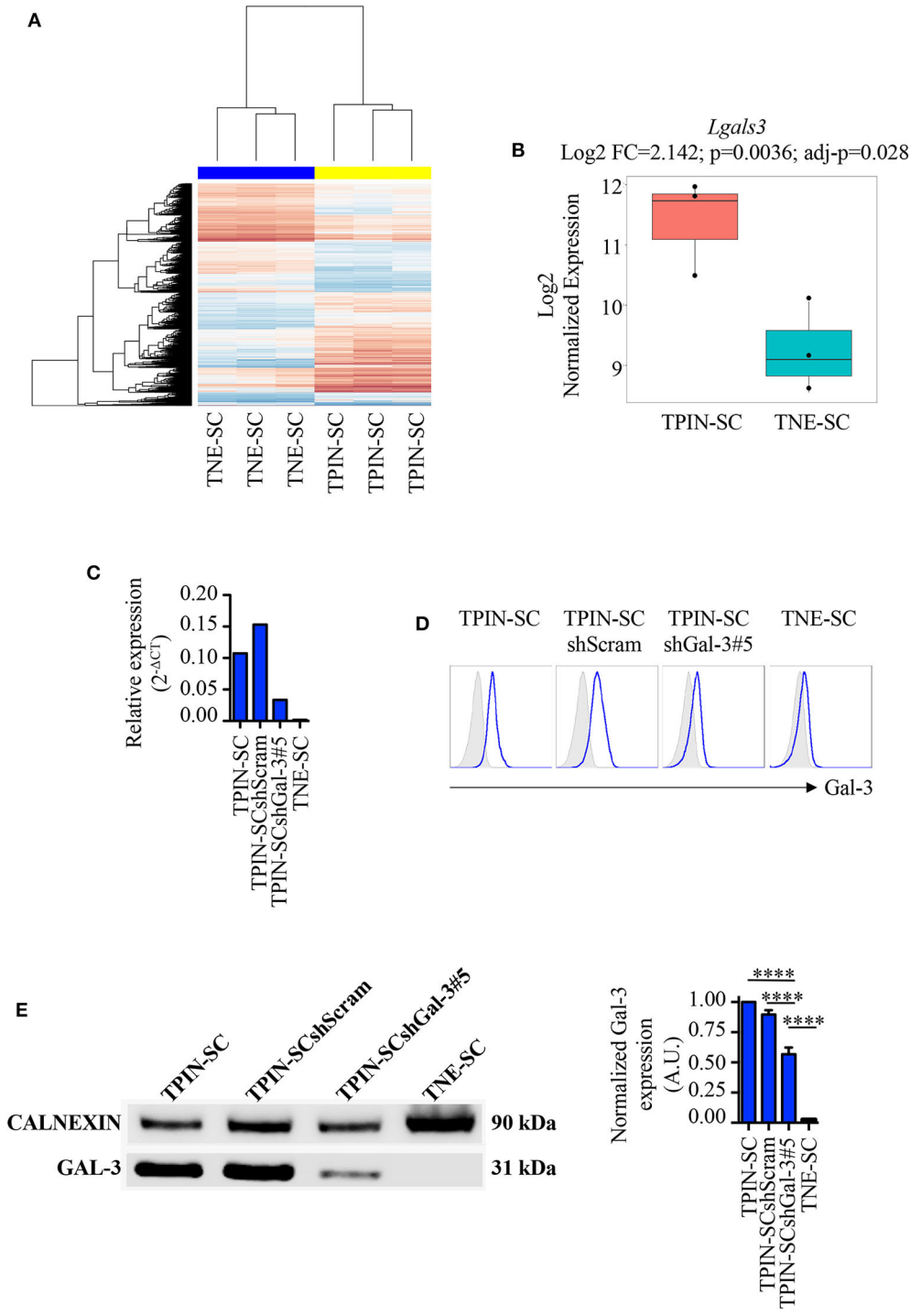


FIGURE 1 | Gal-3 is overexpressed in TPIN-SCs, and can be silenced by shRNA technology. Gene expression analysis of CSCs with Affimetrix Mouse Gene 1.0 ST Array. **(A)** Heatmap reports the global gene expression data in TPIN-SCs vs. TNE-SCs. Differentially expressed genes with a $p < 0.05$ are red (upregulated) or blue-colored (downregulated). **(B)** Boxplot reports Lgals3 expression in TPIN-SCs vs. TNE-SCs (Log2 FC = 2.142, $p = 0.0036$, *adj-p* = 0.028). Gal-3 silencing in TPIN-SCs was attempted with five different shRNA sequences. We obtained substantial inhibition of Gal-3 expression in all TPIN-SCs infected with viruses encoding Gal-3 specific shRNA (not shown). We selected TPIN-SCshGal-3#5 for our experiments. Expression of Gal-3 in the indicated cells was assessed by real-time PCR **(C)**, flow cytometry **(D)** and Western blot **(E)** analysis. **(C)** Relative expression of Gal-3 in the indicated cells was assessed by real-time PCR. **(D)** Fresh samples for cell surface detection of Gal-3 were stained with anti-Gal-3 antibody and 7AAD. The plots report representative histograms of Gal-3 staining (blue lines), gray histograms: isotype control. TNE-SCs were used as negative control for Gal-3 expression. The panel is representative of at least three independent experiments. **(E)** Western blotting analysis of total Gal-3 expression in the indicated cell lines and relative quantification. The Western blot is representative of two independent experiments, performed each time on biological duplicates. The graph is a pool of four independent blots. Statistical analysis was performed using the Anova Test. ****$p < 0.0001$.

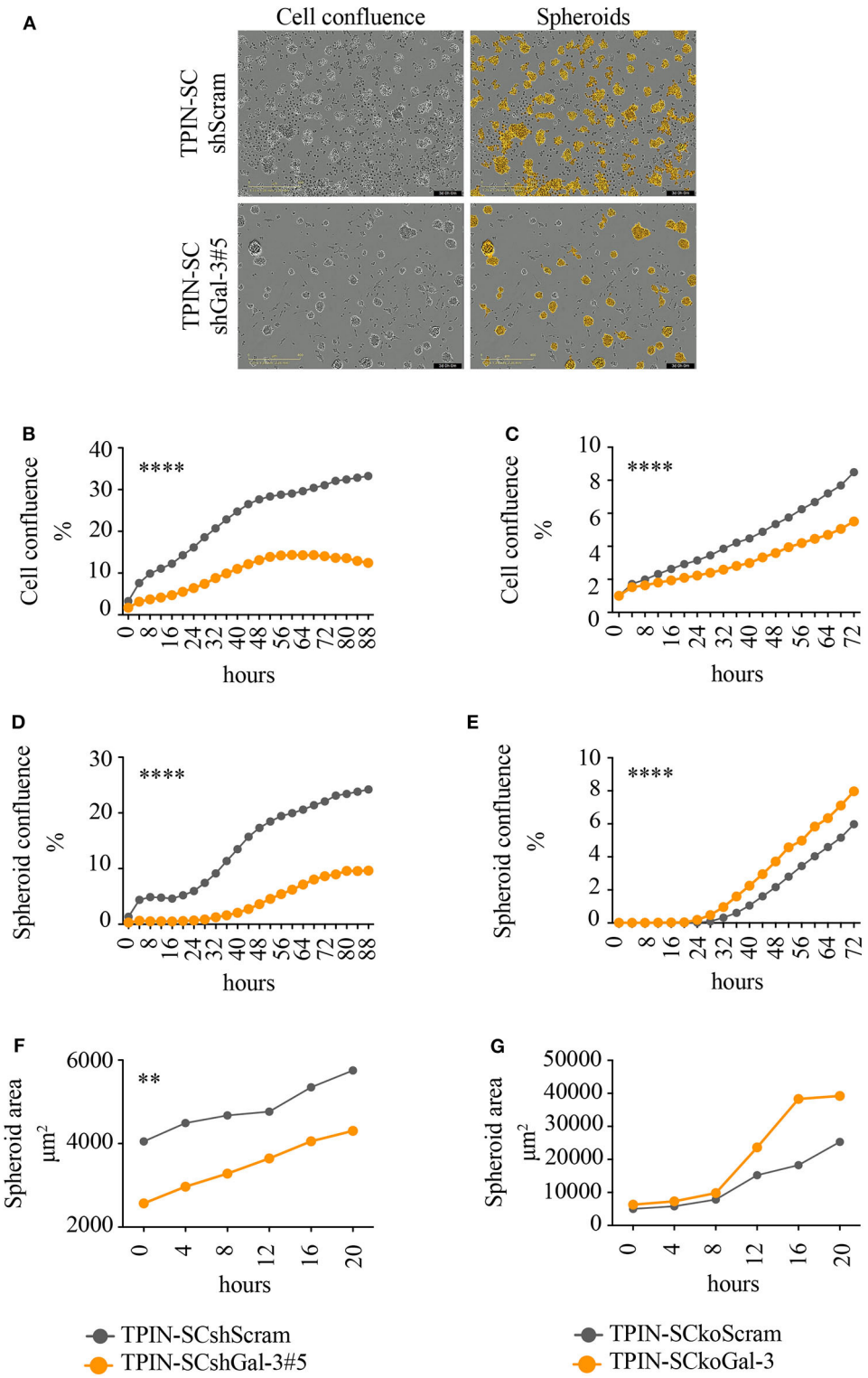


FIGURE 2 | Gal-3 impacts TPIN-SC proliferation, and sphere formation, tumorigenicity, and metastatic potential. TPIN-SCshScram or TPIN-SCshGal-3#5 were analyzed by IncuCyte for 88 h consecutively **(A,B,D,F)**, and TPIN-SCkoGal-3 and TPIN-SCkoScram were analyzed 72 h consecutively **(C,E,G)**, with image captured every 4 h. **(A)** Representative images of cell confluence and spheroids at day 3. Orange: mask to identify spheroids; objective: 10×; scale: 400 µm. **(B,C)** Cell proliferation was measured and reported as mean cell confluence percentage. **(D,E)** Spheroid proliferation was measured and reported as mean spheroid confluence percentage. **(F,G)** Spheroids dimension at day 3 was measured and reported as mean spheroid area (μm^2). Statistical analyses were performed using the Student *T*-test. $^{**}p < 0.01$; $^{****}p < 0.0001$.

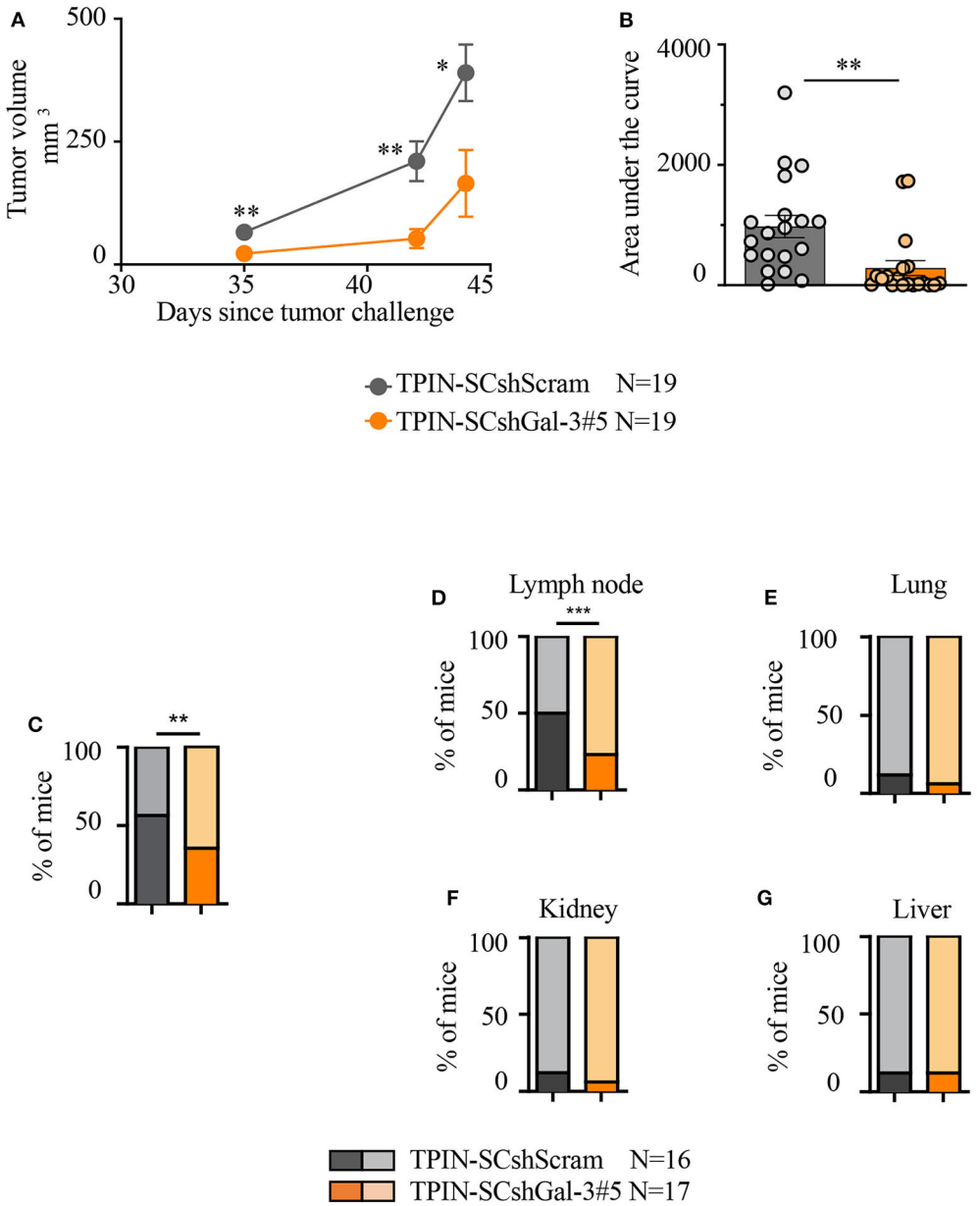


FIGURE 3 | Gal-3 impacts TPIN-SC tumorigenicity and metastatic potential. **(A)** Immunodeficient NSG mice received 2×10^6 TPIN-SCshScram or TPIN-SCshGal-3#5 (19 mice/group). The graph reports tumor growth (mm^3) progression volume. Average ± SEM of tumor volume. **(B)** The graph reports tumor progression expressed as area under the curve at day 44. Data are reported as a percentage ± SEM. Statistical analysis was performed using the Student *T*-test. Data represent a pool of four independent experiments. **(C)** When tumor area achieved ≥80 mm^2, the primary tumors were surgically resected to monitor metastatic spreading. The graph reports TPIN-SCshScram or TPIN-SCshGal-3#5 metastatic ability. **(D–G)** The graphs report the percentage of lymph node **(D)**, lung **(E)**, kidney **(F)**, liver **(G)**, metastatic spreading. Dark colors indicate metastasis-bearing mice, whereas light colors indicate metastasis-free mice. TPIN-SCshScram: $n = 16$ mice; TPIN-SCshGal-3#5: $n = 17$ mice. Statistical analysis was performed using the Fisher Exact Test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

of the Oct4 promoter (48). Thus, only $Oct4^+$ cells expressed GFP. GFP^+ cells were sorted by flow cytometry to obtain a pure population of GFP^+ TPIN-SCs. Upon *in vitro* culture, GFP^+ TPIN-SCs progressively gave rise to ∼20% GFP^- TPIN-SCs, thus suggesting that the CSC core of GFP^+ TPIN-SCs autonomously and progressively re-establishes the heterogeneity found in prostaspheres, allowing some CSCs to differentiate by decreasing Oct4 expression (**Supplementary Figures 2A,B**). We focused on $Oct4^+$ TPIN-SCs, as they likely constitute highly undifferentiated CSCs. By ImageStream technology, we found that $Oct4^+$ TPIN-SCs had a pattern of intracellular Gal-3 distribution comparable to TPIN-SCs (**Figures 4C,D**). We conclude that TPIN-SCs are composed of CSCs that *in vitro* spontaneously generate a population of committed more differentiated precursors. Gal-3

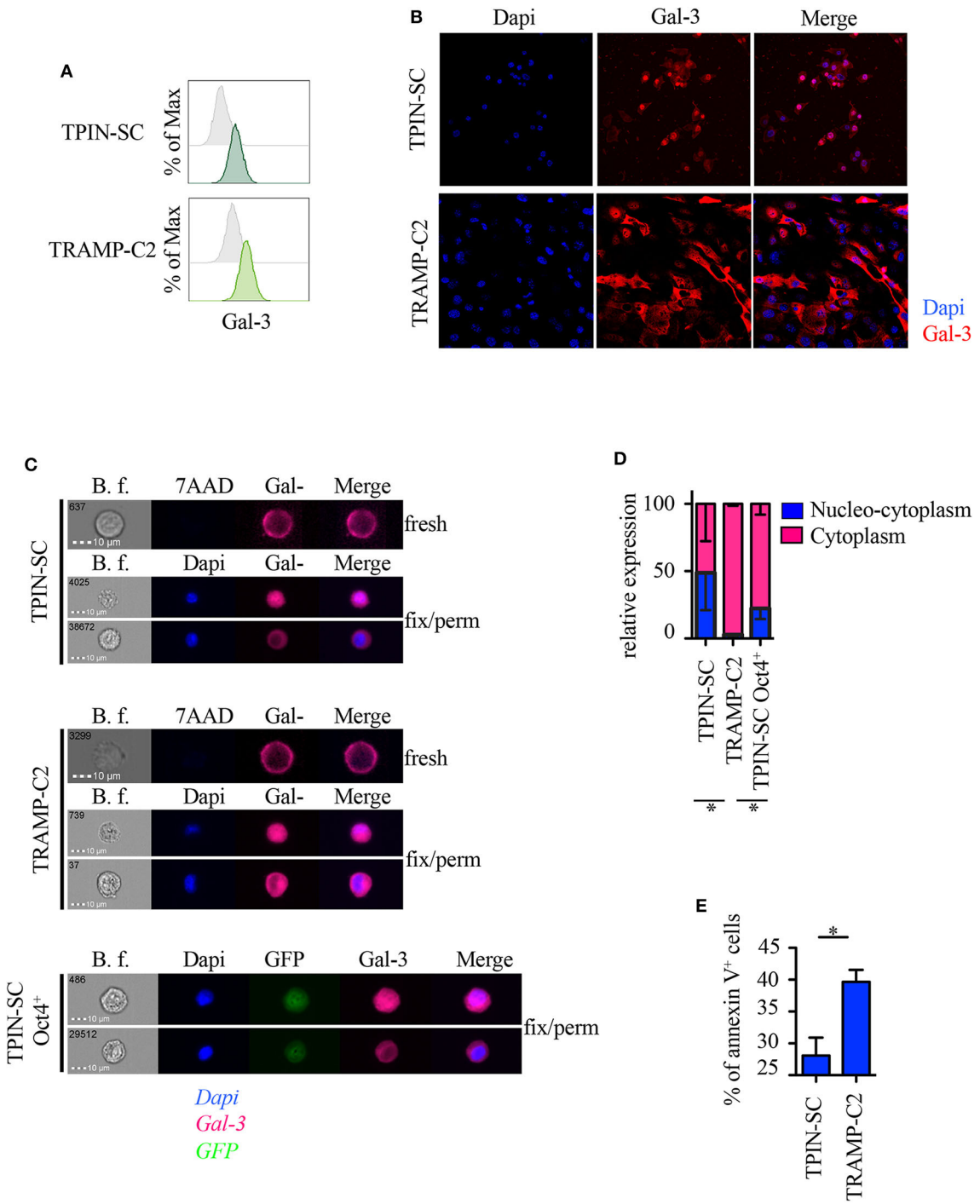


FIGURE 4 | Gal-3 is differently distributed in prostate CSCs and in more differentiated cancer cells. Expression of Gal-3 in the indicated mouse cells was assessed by flow cytometry analysis **(A)**, immunofluorescence **(B)** and ImageStream technology **(C)**. Fresh samples for cell surface detection of Gal-3 **(A,C)** were stained with 7AAD, while fixed and permeabilized samples for intracellular detection of Gal-3 were stained with Dapi **(B,C)**. In fresh samples for cell surface detection of Gal-3, dead cells were excluded by 7AAD positivity. Since we analyzed live cells only (thus, 7AAD negative), **(C)** does not show any 7AAD positivity. Cells were also stained with anti-Gal-3 antibodies. **(A)** Representative histograms of Gal-3 staining (green lines). Gray histograms: unstained. **(B)** Representative confocal images of Gal-3 staining. Red: Gal-3; Blue: Dapi. Magnification 63×. Images were optimized for brightness/contrast using imageJ. **(C)** Representative ImageStream images of Gal-3 staining. Magenta, Gal-3; Blue, Dapi; Green, GFP. **(D)** Quantification of Gal-3 intracellular distribution by ImageStream technology. Data are reported as relative expression of Gal-3 among the indicated cell lines; the blue bar reports the percentage of nucleo-cytoplasmic distribution of Gal-3, the magenta bar reports the percentage of mainly cytoplasmic distribution of Gal-3. Statistical analysis was performed using Student *T*-test. The panel is a pool of three independent experiments. **(E)** Quantification of Annexin V^+ cells analyzed by flow cytometry in the reported cell lines. Statistical analysis was performed using Student *T*-test. The panel reports one experiment representative of three independent experiments. *$p < 0.05$.

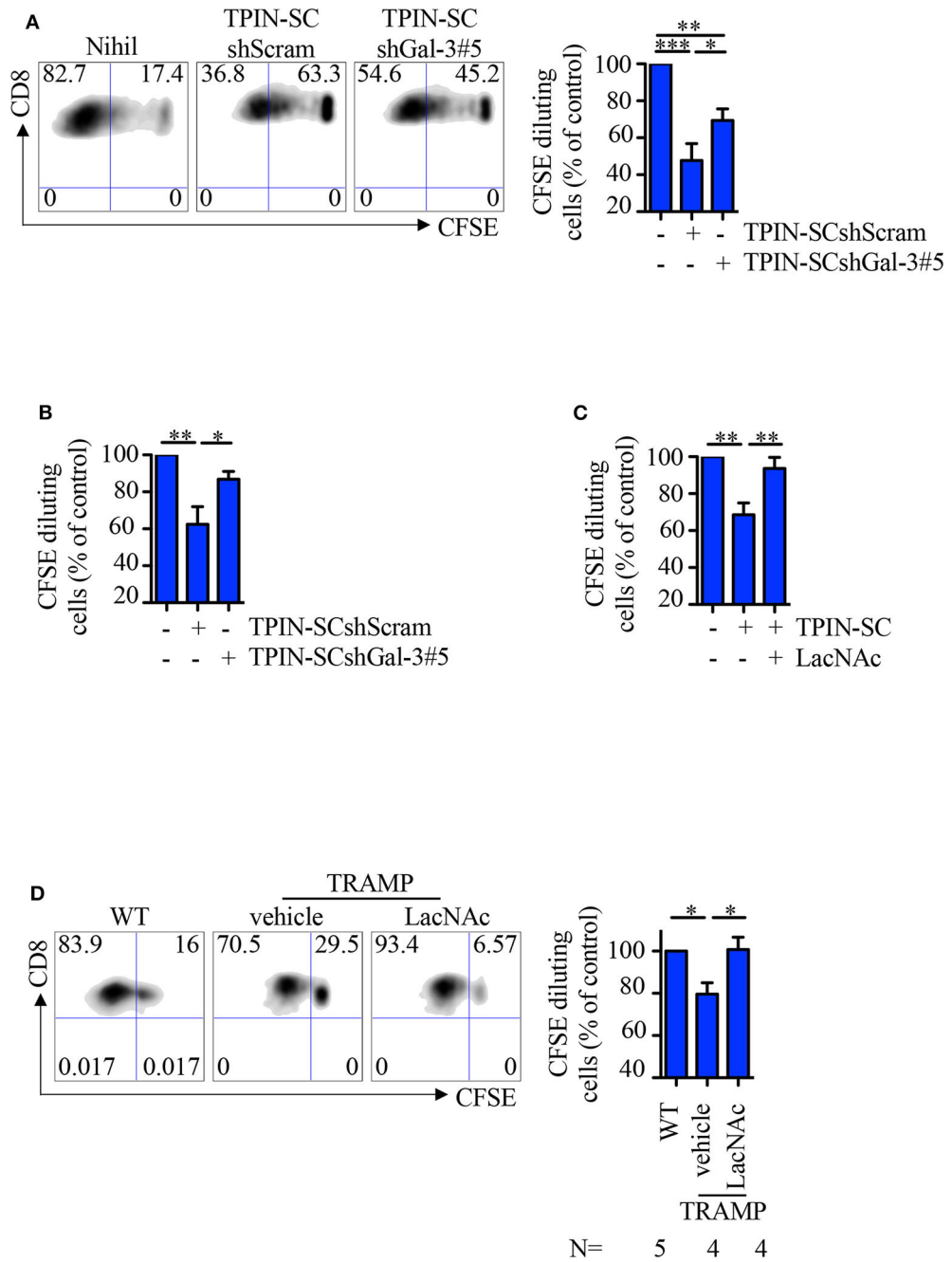


FIGURE 5 | TPIN-SC use Gal-3 to dampen T cell proliferation. Naïve **(A,C)** or RAG-OT1 **(B)** splenocytes were labeled with CFSE, and activated with anti-CD3 and anti-CD28 beads **(A,C)** or OVA **(B)**, respectively, in the presence or absence of irradiated TPIN-SC cells **(C)**, or TPIN-SC infected with lentiviral vectors encoding Gal-3-specific shRNA [TPIN-SCshGal-3#5 **(A,B)**] or unspecific [TPIN-SCshScram **(A,B)**]. Where indicated, 5 mM LacNAc was added to the culture **(C)**. Representative dot plots of CFSE dilution for each experimental condition **(A)** and quantification of CFSE dilution reported as percentage of CD8 proliferating T cells at day 4 **(A,C)** or day 3 **(B)**. Values were normalized to the positive control (splenocytes activated with anti-CD3 and anti-CD28 beads or OVA). Statistical analysis was performed using Anova followed by Tukey's test or Student *T*-test. Graph **(A)** is a pool of nine independent experiments; graph **(B)** is a pool of four independent experiments; graph **(C)** is a pool of six independent experiments. **(D)** CFSE dilution of CD8 T cells from prostate-draining lymph nodes of 12/13-week-old TRAMP and wild type (WT) mice at day 3 of stimulation with anti-CD3/CD28 beads. Cells from TRAMP-derived prostate-draining lymph nodes were subdivided in two parts, one of which was also incubated with 5 mM LacNAc. Representative dot plots of CFSE dilution for each experimental condition, and quantification of the percentage of CD8 proliferating T cells at day 3. Each panel is representative of at least two independent experiments. Values were normalized to the positive control (WT). Statistical analysis was performed using Anova followed by Tukey's test. The graph is a pool of four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p<0.001$.

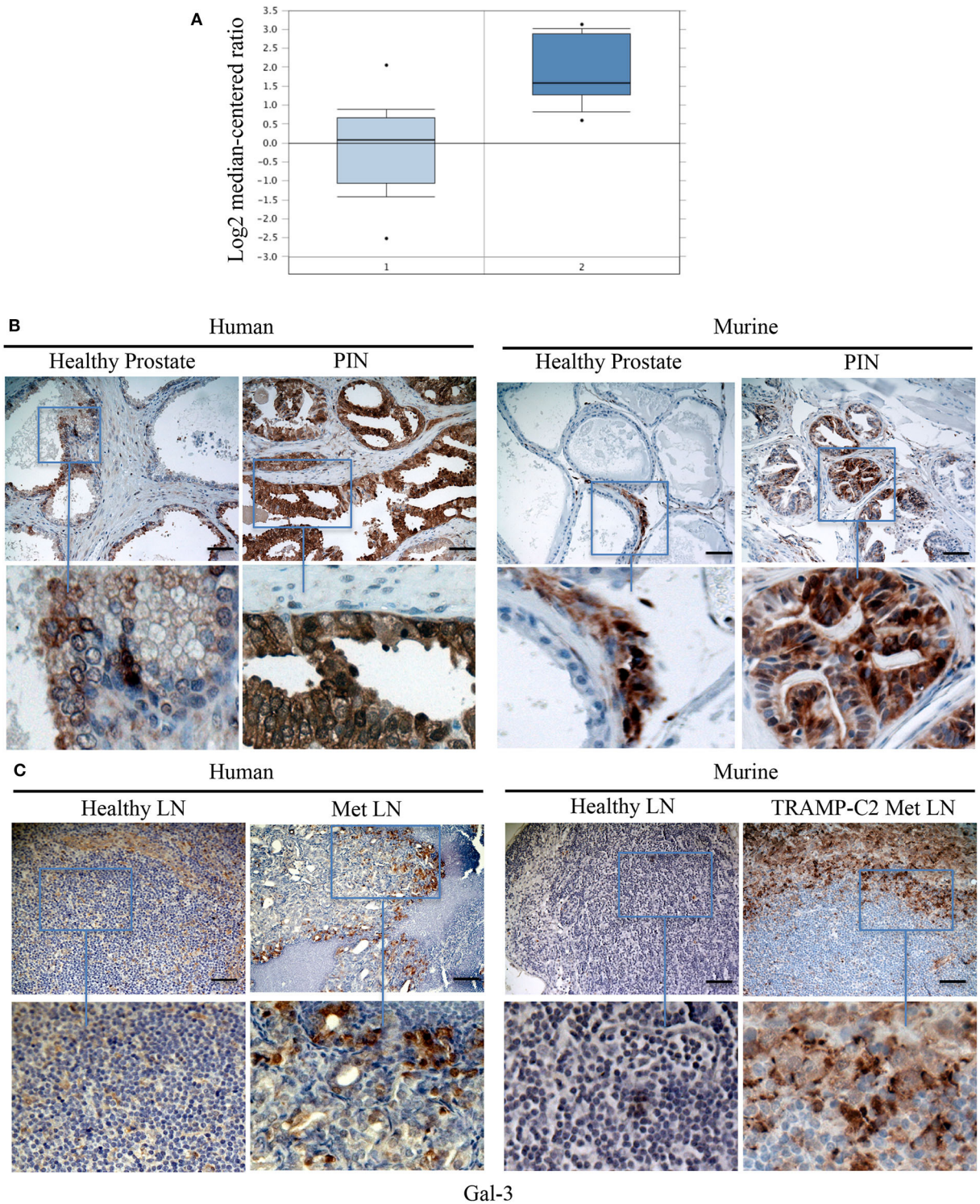


FIGURE 6 | Gal-3 is expressed in human and mouse PIN lesions and metastatic lymph nodes. **(A)** The levels of Gal-3 transcripts in normal and cancerous human prostate carcinoma tissues. Raw data were retrieved from the Oncomine (www.oncomine.org), Tomlins Prostate Dataset (50). Transcript level of Gal-3 in PIN samples (2), $n = 13$, was compared with samples prostate gland (1), $n = 23$. Box plot represents the median with 90th and 10th percentiles, and statistical significance was analyzed by Student T-test, $p = 0.00307$. Formalin fixed paraffin embedded sections from human and mouse: healthy prostate and PIN lesions **(B)** or healthy and metastatic (Met) lymph nodes (LN) **(C)** were stained by immunohistochemistry in order to evaluate Gal 3 expression. Scale Bar = 20 mm. In blue boxes zoom details. Slides are representative of at least three different cases. Images were optimized for brightness.

has a preferential cytoplasmic and nuclear localization in TPIN-SCs, irrespective of their differentiation stage.

Because Gal-3 may favor or protect from apoptosis depending on its intracellular localization (10), we investigated apoptosis in TPIN-SCs and the more differentiated TRAMP-C2 cells. TPIN-SCs were less prone to undergo apoptosis than TRAMP-C2 cells (**Figure 4E**, **Supplementary Figure 3**), thus suggesting that nucleus-cytoplasmic distribution of Gal-3 in CSCs protect them from apoptosis.

TPIN-SCs Use Gal-3 to Dampen T Cell Proliferation

Because Gal-3 is immunosuppressive (11), we asked if TPIN-SCs utilize Gal-3, together with Tenascin-C (30), to suppress T cell-mediated immune responses. To this aim, CD8 T cells from the spleen of naive or TCR transgenic RAG-OT1 mice were labeled with CFSE, and stimulated with anti-CD3/CD28 beads or the $OVA_{257-264}$ peptide, respectively, in the presence of prostate CSCs. Whereas, as expected (30), the addition of TPIN-SCshScram to the culture blocked T cell proliferation, Gal-3 silencing in TPIN-SCs substantially dampened their immunomodulatory effects (**Figures 5A,B**). The direct immunomodulatory role of Gal-3 was confirmed by adding the Gal-3 synthetic inhibitor LacNAc (49) to the co-culture with TPIN-SCs, and showing that CD8 T cell proliferation was rescued (**Figure 5C**).

We previously showed that CSCs precociously migrate to prostate-draining lymph nodes of TRAMP mice affected by PIN through the CXCR4/CXCL12 axis, and participate in generating a local immunosuppressive microenvironment (30, 46). Indeed, CD8 T cells obtained from prostate-draining lymph nodes of TRAMP mice proliferated less than T cells from prostate-draining lymph nodes of age-matched wild type littermates (**Figure 5D**). To investigate if Gal-3 was responsible for this phenomenon, CFSE-labeled naïve cells from prostate-draining lymph nodes of TRAMP mice were cultured with anti-CD3/CD28 beads and LacNAc (**Figure 5D**). Flow cytometry analysis of CD8 T cells showed that in the presence of LacNAc, T cell proliferation was restored to the levels of T cell proliferation in age-matched wild type mice, thus confirming that Gal-3 contributes to the immunosuppressive milieu in TRAMP lymph nodes. Altogether, these findings suggest that Gal-3 participates to the immunosuppressive activity of TPIN-SCs both in the primary tumor lesion and in precociously invaded lymph nodes.

Gal-3 Is Expressed in Human and Mouse PIN Lesions and Metastatic Lymph Nodes

Because TPIN-SCs originate from PIN lesions, we searched for a published gene signature of human normal prostate and PIN (50). As reported in **Figure 6A**, the Gal-3 transcript was found overexpressed in human PIN when compared to healthy prostate. We next validated Gal-3 expression at the protein level by immunohistochemistry. While in the healthy human and mouse prostate, Gal-3 showed a weak immunostaining primarily localized in the cytoplasm (**Figure 6B**), in human PIN lesions Gal-3 staining was rather heterogeneous and intense, and mainly cytoplasmic (**Figure 6B**), thus confirming previous findings (14). Interestingly, Gal-3 was also found in mouse PIN lesions, where it showed a patchy distribution (**Figure 6B**). Thus, both human and murine PIN lesions express Gal-3 preferentially in the cytoplasm of transformed cells, as we found in differentiated TRAMP-C2 cells (**Figure 4**).

Based on our findings in the TRAMP model (**Figure 3**), and the known role of Gal-3 in the metastatic process (51), we were interested in investigating the expression of Gal-3 in metastatic prostate cancer. To this aim, we stained with anti-Gal-3 antibodies human Du145 cells, which were derived from a central nervous system metastasis (35), and PC3 cells obtained from a metastatic lymph node (36). At flow cytometry, both cell populations clearly expressed Gal-3 (**Supplementary Figure 4**). By immunohistochemistry we found that Gal-3 was strongly expressed in metastatic bone from prostate cancer patients (**Figure 6C** and **Table 1**), thus confirming previous findings (52). We also originally observed that in some samples Gal-3 staining was stronger at the invading edge of lymph node metastasis (**Figure 6C** and **Table 1**).

We also investigated Gal-3 expression in mouse lymph nodes affected by measurable metastasis. The incidence of measurable lymph node metastasis in TRAMP mice has been reported to be very low (30, 53). A recent survey in our colony of 88 TRAMP mice found lymph node metastases by adenocarcinoma, which were confirmed by the pathologist, in two mice, accounting for ∼3% of the screened animals. To overcome the limitation of the autochthonous TRAMP model, we took advantage of the well-established model of lymph node metastasis upon subcutaneous challenge with TRAMP-C2 cells (39). Thus, C57BL/6 mice were challenged with TRAMP-C2 cells (**Supplementary Figure 5**). When the tumor area reached $\geq$80 mm^2, we surgically resected the primary tumor, and monitored mice for lymph node metastasis occurrence. Approximately 1 month after surgery, 86% of the mice (i.e., six out of seven mice) developed axillary and inguinal lymph node metastases. In 62% of the metastatic lymph nodes from TRAMP-C2-challenged mice we found Gal-3 expression in neoplastic cells invading the lymph node (**Table 1**). Similarly to the human counterpart, 25% of metastatic lymph nodes from TRAMP-C2-challenged mice had a more intense Gal-3 staining at the invading edge of the metastasis (**Figure 6C** and **Table 1**). Altogether, these findings demonstrate that Gal-3 is expressed in human and murine PIN lesions as well as in metastatic lymph nodes.

TABLE 1 | Gal-3 expression in tumor cells invading metastatic lymph nodes.

	Gal-3$^+$samples (%)	Gal-3 at leading edge (%)
Human	9/9 (100)	2/9 (22)
Mouse	8/13 (62)	2/8 (25)

Number of human and mouse metastatic lymph nodes stained for Gal-3 by immunohistochemistry. Human samples: pelvic lymph nodes from 9 patients. Murine samples: axillary/inguinal lymph nodes from 7 mice challenged with TRAMP-C2 cells. Leading edge refers to intense Gal-3 staining in cancer cells at the interface with lymphocytes in lymph nodes.

DISCUSSION

Prostate cancer is one of the most frequently diagnosed cancers, and it accounts for 19% of all estimated new cancer cases in men (54). Metastatic dissemination is a severe complication of prostate cancer, and the main cause of cancer mortality. The majority of prostate cancer patients harbor bone with lymph-node metastases, 6% develop exclusive lymph node disease recurrence and 20% have visceral metastases (55). When prostate cancer becomes castration-resistant it is essentially incurable. Indeed, prostate cancer is one of the major causes of death by cancer and accounts for 9% of estimated cancer deaths in men (54). Thus, a better understanding of the metastatic process in prostate cancer is essential to direct current and future therapeutic strategies.

The prostate cancer microenvironment is immunosuppressive (56, 57). We and others have previously reported that in TRAMP mice, especially in the early phases of cancer development and progression (33, 58), the tumor microenvironment is endowed with redundant immunosuppressive mechanisms. These are operated by several cell populations, including regulatory T cells (59, 60), myeloid derived suppressor cells (61, 62), and prostate CSCs (30). Our new findings suggest that Gal-3 is an additional mechanism of immune suppression that acts both in primary prostate lesions and in lymph nodes. Gal-3 also exerts pro-metastatic functions in CSCs. Several experimental evidences support our conclusions. Firstly, Gal-3 expressed in TPIN-SCs dampened T cell proliferation, and Gal-3 silencing in TPIN-SCs or the addition of LacNac to the co-culture recued T cell proliferation. Gal-3 silencing in TPIN-SCs also diminished *in vitro* cell proliferation, thus substantiating a direct function of Gal-3 in supporting proliferation not only of differentiated cancer cells (18), but also of prostate CSCs, as previously described for other CSCs (23, 26). More importantly, Gal-3 impacted TPIN-SC proliferative potential also *in vivo*, as Gal-3 silencing in TPIN-SC reduced tumor burden. Thus, our findings confirm data obtained *in vitro* and *in vivo* with PC3 cells (18), and extend the role of Gal-3 to prostate CSCs. Gal-3 also supported the metastatic potential of TPIN-SCs, and when expressed in TPIN-SCs, Gal-3 endowed prostate CSCs with tropism for draining lymph nodes. Hence, when expressed in prostate CSCs, Gal-3 supports tumor growth and metastatic dissemination through cell-intrinsic and cell-extrinsic mechanisms (**Figure 7**). It will be interesting to identify the molecular mechanism by which Gal-3 endows CSCs with metastatic potential.

Gal-3 has been already implicated in the biology of prostate cancer (12), and Gal-3 has been proposed as predictive biomarker of prostate cancer aggressiveness especially in the context of metastasis (20, 21). Our analyses on human and mouse tissues confirm and extend previous findings showing that Gal-3 is expressed in both human and mouse PIN lesions, as well as in metastases (13–17, 63). All together, these findings suggest that Gal-3 has a relevant role already at the stage of PIN. Whereas, in humans a direct link between PIN and prostate adenocarcinoma has not been demonstrated, in TRAMP mice, PIN invariably precedes adenocarcinoma (31). Because Gal-3 is expressed in mouse PIN lesions, prostate CSCs and lymph node metastasis, we hypothesize that Gal-3 links mouse PIN lesions to lymph node metastasis *via* CSCs. By acting directly on CSCs and indirectly on immune surveillance, Gal-3 might favor the precocious dissemination of the former.

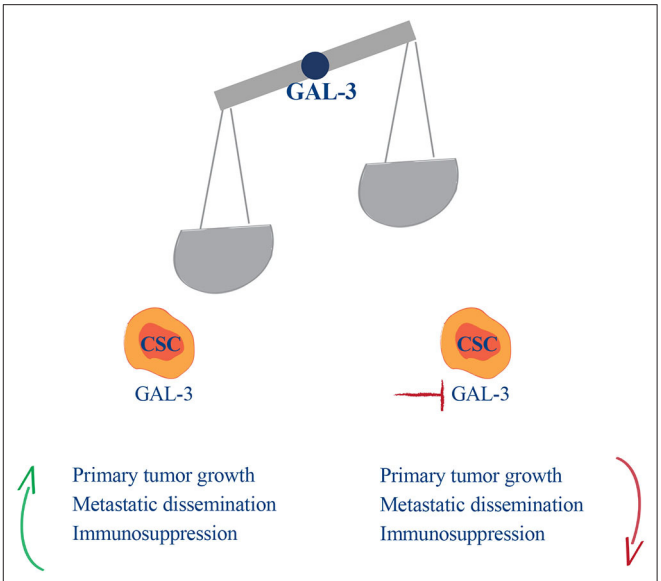


FIGURE 7 | Gal-3 balances cancer malignancy in the early phases of prostate cancer development. In the early phases of disease development and metastasis CSCs, through Gal-3, drive prostate cancer malignancy, and immunosuppression.

The finding that adenocarcinoma metastases are rare, but prostate CSCs can frequently be isolated from prostate draining lymph nodes of TRAMP mice is only apparently contradicting. In fact, autochthonous tumors in TRAMP mice lack genetic alterations that drive full prostate cancer metastatization (64). Nonetheless, our findings in the TRAMP model support a process of early lymph node seeding by prostate CSCs, and let us hypothesize that in some prostate cancer patients, metastasis occurs very early, and may account for recurrence after radical prostatectomy (65). Thus, measuring Gal-3 expression in prostate biopsies and/or Gal-3 levels in blood might be an early predictor of metastatic disease. This hypothesis requires investigation in a prospective clinical trial.

Importantly, a more intense Gal-3 staining was found at the leading edge of lymph node metastasis in some human and mouse samples. These findings further support a relevant role for Gal-3 in the invading process, and let us hypothesize that the leading edge is rich in CSCs. Because flow cytometry and immunohistochemistry analyses showed that Gal-3 expression is not restricted to CSCs, an alternative hypothesis is that Gal-3 might be upregulated in more invading tumor cells irrespective of their differentiation status. We are currently investigating between these two non-mutually exclusive possibilities.

Gal-3 overexpression at the leading edge of the lymph node metastasis, where cancer cells and lymphocytes get directly in contact, might also exert an important immunomodulatory activity. In support of this hypothesis, we have found that either silencing Gal-3 in TPIN-SC or the addition of LacNAc to the

cultures rescued T cell proliferation in the presence of TPIN-SCs. More importantly, incubation of T cells with LacNAc rescued T cell proliferation in prostate-draining lymph nodes of TRAMP mice affected by mouse PIN. Thus, CSCs that early migrated to prostate-draining lymph nodes contributed to the establishment of an immunosuppressive milieu (30, 46) also through Gal-3. LacNAc in prostate-draining lymph nodes might inhibit Gal-3 either drained through lymphatic vessels from the primary tumor lesion, or locally produced by migrated prostate CSCs. Against the former hypothesis are evidences that inhibition of CSCs migration to prostate-draining lymph nodes avoids local immune suppression (30), and that CSCs require a cell-to-cell contact to dampen T cell activation (30).

Silencing of either Tenascin-C or Gal-3 in TPIN-SCs only partially rescued T cell proliferation, suggesting that the two molecules are endowed with different and potentially independent immunosuppressive mechanisms. In fact, Tenascin-C binds to α5β1 integrin expressed on the cell surface of both human and mouse T cells (30, 46), and inhibits stress fiber formation, thus dampening T cell receptor-dependent activation, proliferation, and cytokine production. Gal-3 has been reported to impair T cell activation by destabilizing the immunological synapse (66), and inhibiting TCR clustering (67) and CD8-TCR interactions (49). Our findings, however, do not exclude interactions between Gal-3 and Tenascin-C in favoring prostate CSCs tumorigenic and metastatic potential. As an example, by interacting with N-linked oligosaccharides on the surface of mammary carcinoma cells, Gal-3 induces activation of α5β1 integrin, the target of Tenascin-C on T cells (30), favoring, in this case, fibronectin fibrillogenesis, and fibronectin-dependent spreading and motility of tumor cells (68). Additionally, Gal-3 is able to directly interact with Tenascin-C (69), and the two molecules have been involved in homotypic cancer cell adhesion in glioma (70). Analogously, we speculate that Gal-3 and Tenascin-C may directly interact, thus mediating important steps of the metastatic cascade in prostate cancer.

The consistent number of Gal-3$^+$ cells both in PIN lesions and in metastatic lymph nodes suggests that Gal-3 expression is not restricted to CSCs, and indeed, we have found that Gal-3 is also produced by more differentiated human and murine prostate cancer cells. Interestingly, intracellular distribution of Gal-3 appears to vary depending on the differentiation state of the cancer cells. While in less differentiated prostate CSCs, Gal-3 was equally distributed in nucleus and cytoplasm, in more differentiated cells Gal-3 was preferentially confined to the cytoplasm. In support of the latter finding, in most of the tumor cells in PIN and metastatic lesions, Gal-3 is cytoplasmic. The role of nuclear and cytoplasmic Gal-3 expression in prostate CSCs still need to be investigated. Within the cell, Gal-3 can shuttle between the nucleus and the cytoplasm, thus participating to cell cycle progression, cell growth, and apoptosis (18). We analyzed the apoptotic process in culture conditions, and found that TRAMP-C2 cells were more prone to apoptosis than CSCs, thus suggesting that nucleus-cytoplasm distribution of Gal-3 protects CSCs from apoptosis. This finding was unexpected, as cytoplasmic Gal-3 has been previously described to protect from apoptosis (71). Galectins can undergo post-translational proteolysis and phosphorylation (72), which might depend on the redox status of the microenvironment (73). Upon phosphorylation, Gal-3 acquires its anti-apoptotic and cell cycle arrest functions (74). Thus, we speculate that the culture conditions in which TRAMP-C2 cells were grown did not allow adequate post-translational modification of Gal-3. We also hypothesize that nuclear Gal-3 exclusion may associate with cell cycle progression in more differentiated prostate cancer cells. Thus, equal distribution of Gal-3 in the two cell compartments may favor CSC quiescence, and identify CSCs in prostate cancer. Further investigation is required to support our hypotheses.

Bresalier et al. (22) used surface Gal-3 expression as marker of CSCs in gastrointestinal tumors. Because we have found that both CSCs and more differentiated human and mouse prostate cancer cells express Gal-3 on the cell surface, surface Gal-3 does not appear to represent a stemness marker in prostate cancer.

Several Gal-3-specific therapeutic strategies are available (75). *In vitro*, PectaSol-C Modified Citrus Pectin and GCS-100 induced apoptosis, inhibition of cell proliferation and cell cycle arrest in cancer cells from the prostate and other tumors (76–78). Additionally, treatment with GCS-100 overcame the Gal-3-induced disfunction of tumor infiltrating T cells, and favored tumor rejection in mice (79). It has also been reported that the Thomsen-Friedenreich disaccharide TFD100 purified from cod blocked Gal-3-mediated angiogenesis and prostate cancer metastasis in mice, as well as apoptosis of activated T cells (80). Treatment with the Gal-3 inhibitor GR-MD-02 in combination with the stimulatory anti-OX40 monoclonal antibody promoted antigen specific T cell expansion and survival of mice bearing TRAMP-C1 tumors, reduced lung metastases in the 4T1 model, and showed anti-tumor activity in other mouse models (81). Gal-3 inhibitors, used either alone or in combination with immune checkpoint blockers or vaccination, have been and also are investigated in phase I-III clinical trials in melanoma, non-small cell lung cancer, squamous cell carcinoma of the head and neck, and chronic lymphocytic leukemia (NCT02575404, NCT02117362, NCT01723813, and NCT00514696). PectaSol-C Modified Citrus Pectin is proposed as dietary supplement in biochemical relapsed prostate cancer-affected patients [NCT01681823; refs. (75, 82)]. Our findings support the hypothesis that Gal-3 inhibitors also target CSCs, and could be tested in the early phases of prostate cancer.

DATA AVAILABILITY STATEMENT

The dataset has been uploaded to the GEO - GSE65502. Other raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Ospedale San Raffaele. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Ethical Committee, San Raffaele Hospital.

AUTHOR CONTRIBUTIONS

MB developed the concept of the study. MB, EJ, SC, ABre, AE, and CB designed and conceived the experiments. SC, MG, ABre, TB, AM, VP, AE, CB, and EJ performed the experiments. MG took care of the mouse colonies. AL supervised the experiments conducted to generate TPIN-SCkoGal-3 and TPIN-SCkoScram cell lines. IP analyzed microarray data. RG supervised western blot analyses. ABri provided patients samples. MF and CD supervised preparation of immunohistochemistry samples and analyzed them. SC and ABre prepared the figures and tables. MB and SC wrote and prepared the manuscript. All authors read and approved the manuscript.

FUNDING

This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC; Grant No. #IG16807 to MB). SC and ABre were supported by fellowships from the Fondazione Italiana per la Ricerca sul Cancro/AIRC (Grants #18314 to SC and #22316 to ABre).

ACKNOWLEDGMENTS

The authors acknowledge the Advanced Light and Electron Microscopy Bioimaging Center (ALEMBIC) (San Raffaele Scientific Institute, Milan, Italy) for excellent support in image acquisition; Dr. Vincenzo Russo and Dr. Raffaella C. Fontana (San Raffaele Scientific Institute, Milan, Italy) and Dr. Dario Sangiolo (Università degli Studi di Torino, Turin, Italy) for the help on lentiviral transduction with the vector carrying the GFP sequence under the control of the Oct4 promoter (San Raffaele Scientific Institute, Milan, Italy); Dr. Ilaria Malanchi (The Francis Crick Institute, London, England), and Dr. Giorgio Stassi (University Of Palermo, Palermo, Italy) for critical comments and helpful suggestions. SC conducted this study in partial fulfillment of her Ph.D. at San Raffaele University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01820/full#supplementary-material

REFERENCES

1. Maman S, Witz IP. A history of exploring cancer in context. *Nat Rev Cancer*. (2018) 18:359–76. doi: 10.1038/s41568-018-0006-7
2. Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. *Cell Stem Cell*. (2012) 10:717–28. doi: 10.1016/j.stem.2012.05.007
3. Chaffer CL, Weinberg RA. How does multistep tumorigenesis really proceed? *Cancer Discov*. (2015) 5:22–4. doi: 10.1158/2159-8290.CD-14-0788
4. Turley SJ, Cremasco V, Astarita JL. Immunological hallmarks of stromal cells in the tumour microenvironment. *Nat Rev Immunol*. (2015) 15:669–82. doi: 10.1038/nri3902
5. Hinshaw DC, Shevde LA. The tumor microenvironment innately modulates cancer progression. *Cancer Res*. (2019) 79:4557–66. doi: 10.1158/0008-5472.CAN-18-3962
6. De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer*. (2017) 17:457–74. doi: 10.1038/nrc.2017.51
7. Hamidi H, Ivaska J. Every step of the way: integrins in cancer progression and metastasis. *Nat Rev Cancer*. (2018) 18:533–48. doi: 10.1038/s41568-018-0038-z
8. Yang RY, Rabinovich GA, Liu FT. Galectins: structure, function and therapeutic potential. *Expert Rev Mol Med*. (2008) 10:e17. doi: 10.1017/S1462399408000719
9. Ruvolo PP. Galectin 3 as a guardian of the tumor microenvironment. *Biochim Biophys Acta*. (2016) 1863:427–37. doi: 10.1016/j.bbamcr.2015.08.008
10. Liu FT, Rabinovich GA. Galectins as modulators of tumour progression. *Nat Rev Cancer*. (2005) 5:29–41. doi: 10.1038/nrc1527
11. Hsu DK, Chen HY, Liu FT. Galectin-3 regulates T-cell functions. *Immunol Rev*. (2009) 230:114–27. doi: 10.1111/j.1600-065X.2009.00798.x
12. Martinez-Bosch N, Rodriguez-Vida A, Juanpere N, Lloreta J, Rovira A, Albanell J, et al. Galectins in prostate and bladder cancer: tumorigenic roles and clinical opportunities. *Nat Rev Urol*. (2019) 16:433–45. doi: 10.1038/s41585-019-0183-5
13. Pacis RA, Pilat MJ, Pienta KJ, Wojno K, Raz A, Hogan V, et al. Decreased galectin-3 expression in prostate cancer. *Prostate*. (2000) 44:118–23. doi: 10.1002/1097-0045(20000701)44:2<118::AID-PROS4>3.0.CO;2-U
14. Van Den Brule FA, Waltregny D, Liu FT, Castronovo V. Alteration of the cytoplasmic/nuclear expression pattern of galectin-3 correlates with prostate carcinoma progression. *Int J Cancer*. (2000) 89:361–7. doi: 10.1002/1097-0215(20000720)89:4<361::AID-IJC8>3.0.CO;2-U
15. Merseburger AS, Kramer MW, Hennenlotter J, Simon P, Knapp J, Hartmann JT, et al. Involvement of decreased galectin-3 expression in the pathogenesis and progression of prostate cancer. *Prostate*. (2008) 68:72–7. doi: 10.1002/pros.20688
16. Knapp JS, Lokeshwar SD, Vogel U, Hennenlotter J, Schwentner C, Kramer MW, et al. Galectin-3 expression in prostate cancer and benign prostate tissues: correlation with biochemical recurrence. *World J Urol*. (2013) 31:351–8. doi: 10.1007/s00345-012-0925-y
17. Ahmed H, Cappello F, Rodolico V, Vasta GR. Evidence of heavy methylation in the galectin 3 promoter in early stages of prostate adenocarcinoma: development and validation of a methylated marker for early diagnosis of prostate cancer. *Transl Oncol*. (2009) 2:146–56. doi: 10.1593/tlo.09118
18. Wang Y, Nangia-Makker P, Tait L, Balan V, Hogan V, Pienta KJ, et al. Regulation of prostate cancer progression by galectin-3. *Am J Pathol*. (2009) 174:1515–23. doi: 10.2353/ajpath.2009.080816
19. Pienta KJ, Naik H, Akhtar A, Yamazaki K, Replogle TS, Lehr J, et al. Inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin. *J Natl Cancer Inst*. (1995) 87:348–53. doi: 10.1093/jnci/87.5.348
20. Balan V, Wang Y, Nangia-Makker P, Kho D, Bajaj M, Smith D, et al. Galectin-3: a possible complementary marker to the PSA blood test. *Oncotarget*. (2013) 4:542–9. doi: 10.18632/oncotarget.923
21. Nakajima K, Heilbrun LK, Hogan V, Smith D, Heath E, Raz A. Positive associations between galectin-3 and PSA levels in prostate cancer patients: a prospective clinical study-I. *Oncotarget*. (2016) 7:82266–72. doi: 10.18632/oncotarget.12619
22. Ilmer M, Mazurek N, Byrd JC, Ramirez K, Hafley M, Alt E, et al. Cell surface galectin-3 defines a subset of chemoresistant gastrointestinal tumor-initiating cancer cells with heightened stem cell characteristics. *Cell Death Dis*. (2016) 7:e2337. doi: 10.1038/cddis.2016.239
23. Kang HG, Kim DH, Kim SJ, Cho Y, Jung J, Jang W, et al. Galectin-3 supports stemness in ovarian cancer stem cells by activation of the Notch1 intracellular domain. *Oncotarget*. (2016) 7:68229–41. doi: 10.18632/oncotarget.11920
24. Kuo HY, Hsu HT, Chen YC, Chang YW, Liu FT, Wu CW. Galectin-3 modulates the EGFR signalling-mediated regulation of Sox2 expression via c-Myc in lung cancer. *Glycobiology*. (2016) 26:155–65. doi: 10.1093/glycob/cwv088

25. Huang CS, Tang SJ, Lee MH, Chang Wang CC, Sun GH, Sun KH. Galectin-3 promotes CXCR2 to augment the stem-like property of renal cell carcinoma. *J Cell Mol Med*. (2018) 22:5909–18. doi: 10.1111/jcmm.13860
26. Nangia-Makker P, Hogan V, Raz A. Galectin-3 and cancer stemness. *Glycobiology*. (2018) 28:172–81. doi: 10.1093/glycob/cwy001
27. Oskarsson T, Batlle E, Massague J. Metastatic stem cells: sources, niches, and vital pathways. *Cell Stem Cell*. (2014) 14:306–21. doi: 10.1016/j.stem.2014.02.002
28. Klein CA. Parallel progression of primary tumours and metastases. *Nat Rev Cancer*. (2009) 9:302–12. doi: 10.1038/nrc2627
29. Mazzoleni S, Jachetti E, Morosini S, Grioni M, Piras IS, Pala M, et al. Gene signatures distinguish stage-specific prostate cancer stem cells isolated from transgenic adenocarcinoma of the mouse prostate lesions and predict the malignancy of human tumors. *Stem Cells Transl Med*. (2013) 2:678–89. doi: 10.5966/sctm.2013-0041
30. Jachetti E, Caputo S, Mazzoleni S, Brambillasca CS, Parigi SM, Grioni M, et al. Tenascin-C protects cancer stem-like cells from immune surveillance by arresting T-cell activation. *Cancer Res*. (2015) 75:2095–108. doi: 10.1158/0008-5472.CAN-14-2346
31. Greenberg NM, Demayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, et al. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci USA*. (1995) 92:3439–43. doi: 10.1073/pnas.92.8.3439
32. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. (1992) 68:869–77. doi: 10.1016/0092-8674(92)90030-G
33. Degl'innocenti E, Grioni M, Boni A, Camporeale A, Bertilaccio MT, Freschi M, et al. Peripheral T cell tolerance occurs early during spontaneous prostate cancer development and can be rescued by dendritic cell immunization. *Eur J Immunol*. (2005) 35:66–75. doi: 10.1002/eji.200425531
34. Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. *Cancer Res*. (1997) 57:3325–30.
35. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF. Isolation of a human prostate carcinoma cell line (DU 145). *Int J Cancer*. (1978) 21:274–81. doi: 10.1002/ijc.2910210305
36. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol*. (1979) 17:16–23.
37. Hess Michelini R, Freschi M, Manzo T, Jachetti E, Degl'innocenti E, Grioni M, et al. Concomitant tumor and minor histocompatibility antigen-specific immunity initiate rejection and maintain remission from established spontaneous solid tumors. *Cancer Res*. (2010) 70:3505–14. doi: 10.1158/0008-5472.CAN-09-4253
38. Iezzi G, Boni A, Degl'innocenti E, Grioni M, Bertilaccio MT, Bellone M. Type 2 cytotoxic T lymphocytes modulate the activity of dendritic cells toward type 2 immune responses. *J Immunol*. (2006) 177:2131–7. doi: 10.4049/jimmunol.177.4.2131
39. Kwon ED, Foster BA, Hurwitz AA, Madias C, Allison JP, Greenberg NM, et al. Elimination of residual metastatic prostate cancer after surgery and adjunctive cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade immunotherapy. *Proc Natl Acad Sci USA*. (1999) 96:15074–9. doi: 10.1073/pnas.96.26.15074
40. Elia AR, Grioni M, Basso V, Curnis F, Freschi M, Corti A, et al. Targeting tumor vasculature with TNF leads effector T cells to the tumor and enhances therapeutic efficacy of immune checkpoint blockers in combination with adoptive cell therapy. *Clin Cancer Res*. (2018) 24:2171–81. doi: 10.1158/1078-0432.CCR-17-2210
41. Lombardo A, Cesana D, Genovese P, Di Stefano B, Provasi E, Colombo DF, et al. Site-specific integration and tailoring of cassette design for sustainable gene transfer. *Nat Methods*. (2011) 8:861–9. doi: 10.1038/nmeth.1674
42. Cantore A, Ranzani M, Bartholomae CC, Volpin M, Valle PD, Sanvito F, et al. Liver-directed lentiviral gene therapy in a dog model of hemophilia B. *Sci Transl Med*. (2015) 7:277ra228. doi: 10.1126/scitranslmed.aaa1405
43. Sorrentino C, Ciummo SL, Cipollone G, Caputo S, Bellone M, Di Carlo E. Interleukin-30/IL27p28 shapes prostate cancer stem-like cell behavior and is critical for tumor onset and metastasization. *Cancer Res*. (2018) 78:2654–68. doi: 10.1158/0008-5472.CAN-17-3117
44. Jachetti E, Mazzoleni S, Grioni M, Ricupito A, Brambillasca C, Generoso L, et al. Prostate cancer stem cells are targets of both innate and adaptive immunity and elicit tumor-specific immune responses. *Oncoimmunology*. (2013) 2:e24520. doi: 10.4161/onci.24520
45. Bellone M, Caputo S. Crosstalk between prostate cancer stem cells and immune cells: implications for tumor progression and resistance to immunotherapy. In: Maccalli C, Todaro M, Ferrone S, editors. *Cancer Stem Cell Resistance to Targeted Therapy*. Cham: Springer International Publishing (2019). p. 173–221. doi: 10.1007/978-3-030-16624-3_8
46. Bellone M, Caputo S, Jachetti E. Immunosuppression via tenascin-C. *Oncoscience*. (2015) 2:667–8. doi: 10.18632/oncoscience.210
47. Pan GJ, Chang ZY, Scholer HR, Pei D. Stem cell pluripotency and transcription factor Oct4. *Cell Res*. (2002) 12:321–9. doi: 10.1038/sj.cr.7290134
48. Sangiolo D, Mesiano G, Gammaitoni L, Leuci V, Todorovic M, Giraudo L, et al. Cytokine-induced killer cells eradicate bone and soft-tissue sarcomas. *Cancer Res*. (2014) 74:119–29. doi: 10.1158/0008-5472.CAN-13-1559
49. Demotte N, Stroobant V, Courtoy PJ, Van Der Smissen P, Colau D, Luescher IF, et al. Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. *Immunity*. (2008) 28:414–24. doi: 10.1016/j.immuni.2008.01.011
50. Tomlins SA, Mehra R, Rhodes DR, Cao X, Wang L, Dhanasekaran SM, et al. Integrative molecular concept modeling of prostate cancer progression. *Nat Genet*. (2007) 39:41–51. doi: 10.1038/ng1935
51. Fortuna-Costa A, Gomes AM, Kozlowski EO, Stelling MP, Pavao MS. Extracellular galectin-3 in tumor progression and metastasis. *Front Oncol*. (2014) 4:138. doi: 10.3389/fonc.2014.00138
52. Nakajima K, Kho DH, Yanagawa T, Harazono Y, Hogan V, Chen W, et al. Galectin-3 cleavage alters bone remodeling: different outcomes in breast and prostate cancer skeletal metastasis. *Cancer Res*. (2016) 76:1391–402. doi: 10.1158/0008-5472.CAN-15-1793
53. Gelman IH. How the TRAMP model revolutionized the study of prostate cancer progression. *Cancer Res*. (2016) 76:6137–9. doi: 10.1158/0008-5472.CAN-16-2636
54. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin*. (2018) 68:7–30. doi: 10.3322/caac.21442
55. Halabi S, Kelly WK, Ma H, Zhou H, Solomon NC, Fizazi K, et al. Meta-analysis evaluating the impact of site of metastasis on overall survival in men with castration-resistant prostate cancer. *J Clin Oncol*. (2016) 34:1652–9. doi: 10.1200/JCO.2015.65.7270
56. Kwek SS, Cha E, Fong L. Unmasking the immune recognition of prostate cancer with CTLA4 blockade. *Nat Rev Cancer*. (2012) 12:289–97. doi: 10.1038/nrc3223
57. Rigamonti N, Bellone M. Prostate cancer, tumor immunity and a renewed sense of optimism in immunotherapy. *Cancer Immunol Immunother*. (2012) 61:453–68. doi: 10.1007/s00262-012-1216-6
58. Drake CG, Doody AD, Mihalyo MA, Huang CT, Kelleher E, Ravi S, et al. Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. *Cancer Cell*. (2005) 7:239–49. doi: 10.1016/j.ccr.2005.01.027
59. Degl'innocenti E, Grioni M, Capuano G, Jachetti E, Freschi M, Bertilaccio MT, et al. Peripheral T-cell tolerance associated with prostate cancer is independent from CD4+CD25+ regulatory T cells. *Cancer Res*. (2008) 68:292–300. doi: 10.1158/0008-5472.CAN-07-2429
60. Wada S, Yoshimura K, Hipkiss EL, Harris TJ, Yen HR, Goldberg MV, et al. Cyclophosphamide augments antitumor immunity: studies in an autochthonous prostate cancer model. *Cancer Res*. (2009) 69:4309–18. doi: 10.1158/0008-5472.CAN-08-4102
61. Bronte V, Kasic T, Gri G, Gallana K, Borsellino G, Marigo I, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. *J Exp Med*. (2005) 201:1257–68. doi: 10.1084/jem.20042028
62. Rigamonti N, Capuano G, Ricupito A, Jachetti E, Grioni M, Generoso L, et al. Modulators of arginine metabolism do not impact on peripheral T-cell tolerance and disease progression in a model of spontaneous prostate cancer. *Clin Cancer Res*. (2011) 17:1012–23. doi: 10.1158/1078-0432.CCR-10-2547
63. Ellerhorst J, Troncoso P, Xu XC, Lee J, Lotan R. Galectin-1 and galectin-3 expression in human prostate tissue and prostate cancer. *Urol Res*. (1999) 27:362–7. doi: 10.1007/s002400050164

64. Ding Z, Wu CJ, Chu GC, Xiao Y, Ho D, Zhang J, et al. SMAD4-dependent barrier constrains prostate cancer growth and metastatic progression. *Nature*. (2011) 470:269–73. doi: 10.1038/nature09677
65. Makarov DV, Humphreys EB, Mangold LA, Carducci MA, Partin AW, Eisenberger MA, et al. The natural history of men treated with deferred androgen deprivation therapy in whom metastatic prostate cancer developed following radical prostatectomy. *J Urol*. (2008) 179:156–61; discussion 161–52. doi: 10.1016/j.juro.2007.08.133
66. Chen HY, Fermin A, Vardhana S, Weng IC, Lo KF, Chang EY, et al. Galectin-3 negatively regulates TCR-mediated CD4+ T-cell activation at the immunological synapse. *Proc Natl Acad Sci USA*. (2009) 106:14496–501. doi: 10.1073/pnas.0903497106
67. Demetriou M, Granovsky M, Quaggin S, Dennis JW. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature*. (2001) 409:733–9. doi: 10.1038/35055582
68. Lagana A, Goetz JG, Cheung P, Raz A, Dennis JW, Nabi IR. Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells. *Mol Cell Biol*. (2006) 26:3181–93. doi: 10.1128/MCB.26.8.3181-3193.2006
69. Probstmeier R, Montag D, Schachner M. Galectin-3, a beta-galactoside-binding animal lectin, binds to neural recognition molecules. *J Neurochem*. (1995) 64:2465–72. doi: 10.1046/j.1471-4159.1995.64062465.x
70. Tews DS. Adhesive and invasive features in gliomas. *Pathol Res Pract*. (2000) 196:701–11. doi: 10.1016/S0344-0338(00)80122-3
71. Califice S, Castronovo V, Bracke M, Van Den Brule F. Dual activities of galectin-3 in human prostate cancer: tumor suppression of nuclear galectin-3 vs tumor promotion of cytoplasmic galectin-3. *Oncogene*. (2004) 23:7527–36. doi: 10.1038/sj.onc.1207997
72. Rabinovich GA, Croci DO. Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer. *Immunity*. (2012) 36:322–35. doi: 10.1016/j.immuni.2012.03.004
73. Stowell SR, Cho M, Feasley CL, Arthur CM, Song X, Colucci JK, et al. Ligand reduces galectin-1 sensitivity to oxidative inactivation by enhancing dimer formation. *J Biol Chem*. (2009) 284:4989–99. doi: 10.1074/jbc.M808925200
74. Yoshii T, Fukumori T, Honjo Y, Inohara H, Kim HR, Raz A. Galectin-3 phosphorylation is required for its anti-apoptotic function and cell cycle arrest. *J Biol Chem*. (2002) 277:6852–7. doi: 10.1074/jbc.M107668200
75. Wdowiak K, Francuz T, Gallego-Colon E, Ruiz-Agamez N, Kubeczko M, Grochola I, et al. Galectin targeted therapy in oncology: current knowledge and perspectives. *Int J Mol Sci*. (2018) 19:210. doi: 10.3390/ijms19010210
76. Streetly MJ, Maharaj L, Joel S, Schey SA, Gribben JG, Cotter FE. GCS-100, a novel galectin-3 antagonist, modulates MCL-1, NOXA, and cell cycle to induce myeloma cell death. *Blood*. (2010) 115:3939–48. doi: 10.1182/blood-2009-10-251660
77. Yan J, Katz A. PectaSol-C modified citrus pectin induces apoptosis and inhibition of proliferation in human and mouse androgen-dependent and-independent prostate cancer cells. *Integr Cancer Ther*. (2010) 9:197–203. doi: 10.1177/1534735410369672
78. Hossein G, Halvaei S, Heidarian Y, Dehghani-Ghobadi Z, Hassani M, Hosseini H, et al. Pectasol-C modified citrus pectin targets galectin-3-induced STAT3 activation and synergize paclitaxel cytotoxic effect on ovarian cancer spheroids. *Cancer Med*. (2019) 8:4315–29. doi: 10.1002/cam4.2334
79. Demotte N, Wieers G, Van Der Smissen P, Moser M, Schmidt C, Thielemans K, et al. A galectin-3 ligand corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. *Cancer Res*. (2010) 70:7476–88. doi: 10.1158/0008-5472.CAN-10-0761
80. Guha P, Kaptan E, Bandyopadhyaya G, Kaczanowska S, Davila E, Thompson K, et al. Cod glycopeptide with picomolar affinity to galectin-3 suppresses T-cell apoptosis and prostate cancer metastasis. *Proc Natl Acad Sci USA*. (2013) 110:5052–7. doi: 10.1073/pnas.1202653110
81. Farhad M, Rolig AS, Redmond WL. The role of galectin-3 in modulating tumor growth and immunosuppression within the tumor microenvironment. *Oncoimmunology*. (2018) 7:e1434467. doi: 10.1080/2162402X.2018.1434467
82. Elia AR, Caputo S, Bellone M. Immune checkpoint-mediated interactions between cancer and immune cells in prostate adenocarcinoma and melanoma. *Front Immunol*. (2018) 9:1786. doi: 10.3389/fimmu.2018.01786

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 02 October 2020
doi: 10.3389/fimmu.2020.02168

CXCR4 Inhibition Counteracts Immunosuppressive Properties of Metastatic NSCLC Stem Cells

Orazio Fortunato[1], Dimas Carolina Belisario[2], Mara Compagno[2], Francesca Giovinazzo[1], Cristiano Bracci[2,3], Ugo Pastorino[4], Alberto Horenstein[2,3], Fabio Malavasi[2,3], Riccardo Ferracini[5], Stefania Scala[6], Gabriella Sozzi[1], Luca Roz[1], Ilaria Roato[2]* and Giulia Bertolini[1]*

[1] Tumor Genomics Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Nazionale dei Tumori, Milan, Italy, [2] Center for Experimental Research and Medical Studies (CeRMS), Azienda Ospedaliera-Universitaria Città (AOU) della Salute e della Scienza di Torino, Turin, Italy, [3] Laboratory of Immunogenetics, Department of Medical Sciences, University of Turin, Turin, Italy, [4] Unit of Thoracic Surgery, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy, [5] Department of Surgical Sciences (DISC), Orthopaedic Clinic-IRCCS, A.O.U. San Martino, Genoa, Italy, [6] Functional Genomics, Istituto Nazionale Tumori "Fondazione G. Pascale", IRCCS, Naples, Italy

OPEN ACCESS

Edited by:
Cristina Maccalli, Sidra Medicine, Qatar

Reviewed by:
Claudia De Vitis, Sapienza University, Italy
Jörg Wischhusen, University Hospital Würzburg, Germany

***Correspondence:**
Luca Roz
luca.roz@istitutotumori.mi.it
Ilaria Roato
roato78@libero.it

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *21 March 2020*
Accepted: *10 August 2020*
Published: *02 October 2020*

Citation:
Fortunato O, Belisario DC, Compagno M, Giovinazzo F, Bracci C, Pastorino U, Horenstein A, Malavasi F, Ferracini R, Scala S, Sozzi G, Roz L, Roato I and Bertolini G (2020) CXCR4 Inhibition Counteracts Immunosuppressive Properties of Metastatic NSCLC Stem Cells. Front. Immunol. 11:02168. doi: 10.3389/fimmu.2020.02168

Cancer stem cells (CSCs) are functionally defined as the cell subset with greater potential to initiate and propagate tumors. Within the heterogeneous population of lung CSCs, we previously identified highly disseminating CD133+CXCR4+ cells able to initiate distant metastasis (metastasis initiating cells-MICs) and to resist conventional chemotherapy. The establishment of an immunosuppressive microenvironment by tumor cells is crucial to sustain and foster metastasis formation, and CSCs deeply interfere with immune responses against tumors. How lung MICs can elude and educate immune cells surveillance to efficiently complete the metastasis cascade is, however, currently unknown. We show here in primary tumors from non-small cell lung cancer (NSCLC) patients that MICs express higher levels of immunoregulatory molecules compared to tumor bulk, namely PD-L1 and CD73, an ectoenzyme that catalyzes the production of immunosuppressive adenosine, suggesting an enhanced ability of MICs to escape immune responses. To investigate *in vitro* the immunosuppressive ability of MICs, we derived lung spheroids from cultures of adherent lung cancer cell lines, showing enrichment in CD133+CXCR4+MICs, and increased expression of CD73 and CD38, an enzyme that also concurs in adenosine production. MICs-enriched spheroids release high levels of adenosine and express the immunosuppressive cytokine IL-10, undetectable in an adherent cell counterpart. To prevent dissemination of MICs, we tested peptide R, a novel CXCR4 inhibitor that effectively controls *in vitro* lung tumor cell migration/invasion. Notably, we observed a decreased expression of CD73, CD38, and IL-10 following CXCR4 inhibition. We also functionally proved that conditioned medium from MICs-enriched spheroids compared to adherent cells has an enhanced ability to suppress CD8+ T cell activity, increase Treg population, and induce the polarization of tumor-associated macrophages (TAMs), which participate in suppression of T cells. Treatment of spheroids with anti-CXCR4 rescued T cell cytotoxic activity and prevented TAM

polarization, likely by causing the decrease of adenosine and IL-10 production. Overall, we provide evidence that the subset of lung MICs shows high potential to escape immune control and that inhibition of CXCR4 can impair both MICs dissemination and their immunosuppressive activity, therefore potentially providing a novel therapeutic target in combination therapies to improve efficacy of NSCLC treatment.

Keywords: metastasis initiating cells, non-small cell lung cancer, CXCR4, immunosuppression, CD73, adenosine, tumor associated macrophage (TAM), PD-L1

INTRODUCTION

Lung cancer represents the first cause of cancer-related mortality worldwide (1). The predominant form of lung cancer is non-small cell lung cancer (NSCLC) for which available therapeutic options are largely ineffective because of its aggressiveness and diagnosis at metastatic phase (1). Treatment of NSCLC advanced stage disease used to rely on conventional platinum-based chemotherapy regimens that poorly impacted overall clinical outcome of patients, due to chemoresistance and frequent recurrence (2). Moreover, damage induced by chemotherapy in normal tissue has been proven to potentially cause the release of cytokines/chemokines that can sustain tumor cell survival and promote a receptive and immune-suppressive microenvironment able to chemoattract tumor cells at distant sites and foster metastasis initiation (3–5). Recently, immune checkpoint inhibitors (ICIs) have emerged as potentially revolutionary new drugs. First-line therapies combining cisplatin with ICIs may become the future mainstay of advanced NSCLC therapy (6–9). Unfortunately, a large number of patients still do not benefit from ICIs, and thus rationally designed combination strategies to extend ICIs effectiveness are mandatory (10).

We previously identified in NSCLC a subset of CD133+ lung cancer stem cells (CSCs), co-expressing CXCR4, endowed with stemness features and characterized by resistance to cisplatin and superior ability to seed distant site and initiate metastatic process (11–13).

CXCL12/CXCR4 axis has been described to play a pivotal role in CSCs maintenance, to guide tumor cell dissemination, and to foster chemoresistance (14–16). Cancer cells can up-regulate CXCR4 expression in response to extracellular adenosine, a potent immune suppressor molecule, thus acquiring increased ability to migrate and proliferate in response to CXCL12 (17, 18).

Due to its wide expression on several cell lineages, CXCR4 inhibition has been tested for different purposes and the CXCR4 inhibitor (Plerixafor) has been clinically approved for the mobilization of CD34+ hematopoietic stem cells for autologous transplantation in patients with lymphoma or multiple myeloma (19). Currently, several clinical studies are ongoing to test the efficacy of different CXCR4 inhibitors in metastatic patients with solid tumors (20–22). More recently, some studies have demonstrated that CXCR4 inhibition can reduce immunosuppression both by acting on Treg cells and myeloid derived suppressor cells (MDSC) that highly expressed CXCR4 receptor, overall resulting in the reactivation of T immune response against tumor cells (23–25). CXCL12/CXCR4 axis synergizes with CD38 to support migration as a central step in tumor disease progression (26). CD38 is a pleiotropic glycoprotein receptor with enzyme activity involved in the catabolism of extracellular nucleotides (27). Therefore, multifunctional protein CD38 can contribute to immune suppressor of T cell, activating the non-canonical adenosinergic pathway that provides AMP substrate to CD73 (28, 29).

CD73 can be expressed on cancer cells and different immune cell populations. This molecule dephosphorylates extracellular adenosine monophosphate (AMP) generating free adenosine, which contributes to the immune-suppressive and pro-angiogenic microenvironment at the tumor site (30, 31). It is known that adenosine is involved in tumor immune escape, and thus the block of CD73 enzymatic activity can reactivate an antitumor immune response (32) by synergizing with chemotherapeutic drugs known to promote immunogenic responses and enhance the therapeutic activity of ICIs (33–35). Anti-CD73 antibody has been demonstrated effective in reducing tumor growth and metastatization in mice (32, 35, 36). Remarkably, CD73 expression has been described as a poor prognostic factor for overall survival in NSCLC (37). A significant population of CD39+CD73+ myeloid derived suppressor cells, capable of inhibiting T and NK cell activity, has been shown in peripheral blood and tumor tissues of NSCLC patients (38).

Immunotherapy based on ICIs have achieved significant results in clinical practice, improving survival of patients with cancer (39). However, only a fraction of patients have shown long-term benefit, and the high rate of resistance still limits their efficacy (40). The mechanisms of resistance to ICIs are quite different, and among them the up-regulation of CD38 by tumor cells determines a functional impairment of CD8 T cells, with a consequent tumor immune escape (41). Chen et al. demonstrated that the co-inhibition of immune checkpoints and adenosine release improves anti-tumor immune response (41).

Interestingly, also CXCR4 inhibition results effective in reverting tolerogenic polarization of tumor microenvironment (42) and in restoring sensitivity to CTLA-4 and PD-1 checkpoints inhibitors (24, 43).

Here, we report that NSCLC CD133+CXCR4+ metastasis-initiating cells (MICs) are endowed with immunosuppressive properties allowing them to escape immune control, by the expression of high levels of PD-L1 and CD73/CD38 ectoenzymes, that mediate extracellular adenosine generation (28). We prove the ability of a new class of CXCR4 antagonists (44) to counteract the immune suppressive behavior of metastatic NSCLC stem cells,

pointing at CXCR4 as novel target to prevent metastatic dissemination and immune escape mechanisms exploited by MICs.

MATERIALS AND METHODS

Cell Cultures and Pharmacological Treatments

NSCLC cell lines (A549, H1299, H3122, SW900) were purchased from ATCC and cultured in adhesion in conventional medium, RPMI-1640 supplemented with 10% heat-inactivated bovine serum (RPMI 10%) (FBS, all from Lonza). Bronchial-epithelial cells (HBEC3KT), immortalized by hTERT and CDK4, were obtained from Prof J. Minna (UT Southwestern) and cultured in Keratinocyte SFM (ThermoFisher).

To obtain sphere cultures, cells were plated in Ultra-Low Attachment plates (Corning) at a density of 10^4 cells/ml in serum-free medium DMEM/F12 (Lonza), supplemented with commercial hormone mix, B27 (Gibco), EGF 20 ng/ml, bFGF10 ng/ml (PeproTech), and heparin 2 μg/ml, named Stem Cells Medium (SCM). Floating sphere cultures were expanded for 15 days in the above medium. Once a week, they were gently dissociated with Accumax (Sigma-Aldrich) and re-plated as single cells in fresh medium.

Adherent cells and dissociated spheroids were incubated with peptide R 1 μM for 2 h at 37°C at a density of $2.5x10^5$ cells/ml in respective complete medium. Next, the medium was removed and fresh medium was added and collected after 24 h to obtained cancer cell conditioned medium (CM).

PBMCs from healthy volunteers were plated at $1x10^6$ cells/well in well plates and incubated at 37°C for 4 h. After the incubation, non adherent cells (T cells) were removed and used for the experiments. At the same time, adherent cells (monocytes) were differentiated to macrophages for 7 days with 50 ng/mL of human M-CSF.

Stimulation of T cells was performed by Dynabeads Human T-Activator CD3/CD28 and cultured in 50% of CM from different cancer cell lines. According to cell lines, negative control of the experiment were T cells cultured in RPMI 10% or 50% Stem Cell Medium (SCM).

Spheroids were treated with different concentration of mAb anti-CD73 (10, 20, 50 ug/ml, clone CB73, generated and purified in house through a two-steps HPLC chromatography by FM) or Adenosine 5'-(α,β-methylene)diphosphate (APCP, at 25, 50, 100 uM, Sigma) every 48 h for 7 days.

Flow Cytometry Analysis

To analyze tumor cell surface markers, single cell solution was washed in staining buffer (PBS1×+ 0.5% BSA+ 2mM EDTA) and incubated for 30 min at 4C° with the following antibodies: anti-human PE-CD133/1 (clone AC133/1Miltenyi Biotech), APC anti-human CXCR4 (44717 clone-R&D system), BB515 Anti-human CD73 (clone AD2), BB700 Mouse Anti-human CD38 (clone HIT2), BV421 Mouse Anti-human CD274 (PD-L1 clone MIH1), AlexaFluor488 Anti-Human HLA-ABC (clone DX17), and BV510 CD39 (clone A1) (all from BD Biosciences).

Primary tumor cell suspensions were obtained by digesting primary tumors, from consenting patients, with human Tumor Dissociation Kit (Miltenyi), subsequent filtering of dissociated tumor tissue on 100 μm pore cell strainer (Falcon), and erythrocytes removal by Red Blood Cell Lysis Solution (Miltenyi Biotech). Tumor cells were then stained with CD133, CXCR4, CD73, or PD-L1 (as specified above). Stromal cells were identified by staining for PE-Cy7 anti-human CD45, CD31, CD34 (eBioscience) and excluded by a negative gating strategy to perform tumor cell analysis.

To analyze the different subtypes of macrophages, cultured cells were washed in staining buffer and incubated for 30 min at 4°C with the following antibodies: Alexa488 anti-human CD206 (clone 15-2) (Biolegend) and PE anti-human CD163 (clone GHI/61) (Biolegend), APC anti-human CD14 (clone M5E2) (BD Biosciences).

For staining of T cytotoxic cells, lymphocytes were incubated in staining buffer with BV510 Anti-Human CD3 (HIT3a) and BB515 Anti-Human CD8 (clone Leu3a) for 30 min at 4°C; then the cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Solution for 30 min at 4°C, washed in BD Perm/Wash buffer, and incubated with APC anti-human IFNγ (clone B27) (all from BD Bioscience), for 30 min at 4°C.

For analysis of Treg phenotype, T cells were first incubated with surface antibodies in staining buffer for 30 min at 4°C: BV510 Mouse Anti-Human CD3 (HIT3a), PE-Cy7 Anti-Human CD4 (clone Leu3a), APC Anti-Human CD25 (clone M-A251); then fixed and permeabilized with Transcription Factor Buffer Set, according to the datasheet instructions, and finally incubated with PE anti-Human FoxP3 (clone259D/C7) (all from BD Biosciences) for 30 min at 4°C. Tregs were identified within live cell gate as CD3+CD4+Foxp3+$CD25^{high}$.

For all analyses, dead cells were excluded by the use of Fixable Viability Stain 780 (BD Horizon). Data were acquired with a FACSCanto cytometer (BD) and analyzed by FlowJo software V10.

PBMCs Proliferation Assay

Two different tests were performed to assess T-cell proliferation: MTT and CSFE staining.

MTT assay: PBMCs derived from buffy coats were plated in a 96 well plate at $2x10^5$ cells/well in RPMI, 10% FBS. To induce proliferation PBMCs were stimulated with OKT-3 (7.5 μg/ml) and anti-CD28 (7.5 μg/ml) and cultured with 50% of CM from cancer cells for 72 h.

MTT assay was performed according to the manufacturer's instructions (Sigma-Aldrich).

CSFE staining: T cells were incubated with CFSE (BD Biosciences) to a final concentration of 1.5 uM, for 8 min at room temp. The reaction was blocked by incubating cells in FBS. Stained T cell were plated at $1x10^5$ cells/well in 24 well plates with RPMI+10% FBS and stimulated with antiCD3/CD8 microbeads and CM from tumor cells (ratio 1:1). Unstimulated T cells, plated in RPMI 10% or SCM +RPMI 10% (ratio 1:1) according to different tested CM, represent the negative control of the experiments. After 72 h T cells were analyzed by FACS to

assess the % of CSFE stained cells, which was inversely correlated to the proliferation rate.

Migration/Invasion Assay

For migration assays, 50.000 cells/well were incubated with peptide R inhibitor of CXCR4 (1μM) or AMD3100 (10 μM) and seeded in 200 μl of RPMI-1640 medium supplemented with 1% FBS onto 8 μm-pore Transwell® cell culture inserts (BD Falcon) in 24 well plate. The lower chamber was filled with 500 μl of RPMI supplemented with SDF-1 (50 ng/ml) as chemoattractant factor. For the invasion assay $1x10^5$ cells were plated onto 8 μm-pore Transwell® cell culture inserts covered with 20μl of Matrigel, which was allowed to solidify at 37°C.

After 48 h (migration assay) or 72 h (invasion assay), cells on the top of the insert membranes were removed by gentle scraping with a sterile cotton swab while migrated/invaded cells in the lower side of the insert were fixed in methanol and mounted on slides using the VECTASHIELD Mounting Medium, containing DAPI. For each insert, cells in 4 random fields were counted by fluorescence microscope visualization at 20X magnification, and the values were averaged. Each experiment was performed in triplicate.

Adenosine Quantification

Twenty-four hours before the adenosine assay, adherent cells were seeded on 24 well plates at a concentration of 5 x 10^5/500 μL, while lung spheroid cells were transferred into 24 well plates in new medium, after have being cultured for 15 days (as previously described).

Culture medium was removed from adherent cells simply by pipetting, while spheroids cells were collected in Eppendorf tubes, centrifuged at low speed to pellet them down, and medium was removed. The cells and derived lung cancer spheroids were incubated with 100 μL STOP solution (EHNA 100 μmol/L, DYP 10 μmol/L, and 10 μmol/LDEF) (Sigma-Aldrich) for 15 min at 37°C and then treated with 100 μL AMP 100 μmol/L for 10 min at 37°C on a basculant. After incubation, the cells were collected in a tube containing acetonitrile (ACN; 1:2; 4°C), centrifuged (13000 *g* for 5 min at 4°C). Tubes were transferred into a Speed Vac (Eppendorf), to remove the supernatant, reconstituted in HPLC-grade water, and assayed or stocked at -80°C.

Chromatography analyses of the supernatant were performed with an HPLC (Beckman Coulter) fitted with a reverse-phase column (Synergi 4U Polar-RP80A; 150 x 4.6 mm; Phenomenex). Nucleotides and nucleosides were separated using a mobile-phase buffer (0.025 mol/L K_2HPO_4, 0.01 mol/L sodium citrate, 0.01 mol/L citric acid, adjusted with phosphoric acid to a pH of 5.1 and 8% acetonitrile (ACN) for 13 min at a flow rate of 0.6 mL/min. Ultraviolet (UV) absorption was measured at 254 nm. Chromatography-grade standards used to calibrate the signals were dissolved in PBS 1X, pH 7.4 (Sigma-Aldrich), 0.2 μm-filtered, and injected in a volume of 15 μL. The retention times (R_t, in min) of standards were: AMP, 5.8; inosine (INO), 6.4; and adenosine (ADO), 10; using a R_t window of ± 5%. Peak area was calculated using Gold software (Beckman Coulter). Quantitative measurements were inferred by comparing percentage area of each nucleotide and nucleoside analyzed, as previously described (29).

Real-Time PCR

Automating RNA isolation was a performed by Maxwell RSC using simplyRNA Cells Kit (Promega). Expression levels of IL-10 and CD73 genes were determined by Real-Time PCR, using TaqMan® assays (Thermo Fisher) and normalized using the $2^{-\Delta\Delta Ct}$ method relative to B2M, and results are expressed as mean ± SD. For each PCR reaction, 5ng cDNA input was added.

Protein Extraction and Western Blot Analysis

Whole cell extracts were obtained from cell lines treated with 1 μM CXCR4 inhibitor using GST-FISH buffer (10 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, 2% Glycerol, 1 mM EDTA, 25 mM HEPES pH 7.5) supplemented with protease inhibitors (Roche), 1 mM phenylmethanesulfonylfluoride (PMSF), 10 mM NaF, and 1 mM Na_3VO_4. Extracts were cleared by centrifugation at 12,000 RPM for 15 min. The supernatants were collected and assayed for protein concentration using the Bio-Rad protein assay method. Twenty μg of proteins were loaded on 12% Mini-PROTEIN TGX gels (BIO-RAD), transferred on nitrocellulose membrane (GE Healthcare), and blocked with 5% skim milk (BIO-RAD). Primary antibodies for immunoblotting included monoclonal anti-rabbit NT5E/CD73 (D7F9A clone, Cell Signaling Technology, CAT NO #13160) and rabbit polyclonal anti-βactin (Sigma, CAT NO #A2066). Membranes were developed with ECL solution (GE Healthcare).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 6.0. Statistically significant difference between two groups was assessed by two-sided Student's t-test. Statistical analyses among more than two groups was performed by one-way Anova with Tukey's *post hoc* test. Data are expressed as means and standard deviation, unless otherwise indicated. Statistical significance was defined as a P value less than 0.05.

RESULTS

Lung Cancer Metastasis Initiating Cells Highly Express PD-L1 and CD73 Markers

We initially investigated by flow cytometry the expression of PD-L1 and CD73 on surgically resected primary NSCLC samples (n=22), within tumor bulk population and CD133+ CSC subsets.

PD-L1 was significantly more expressed in CD133+ CSC subset (median value= 20%; min 2.5%, max 98%) compared to total population (median= 9.5%, min 0.5%, max 96%) (**Figure 1A**). Among CSC subsets, we could detect the population of mesenchymal CD133+EpCAM-CXCR4+ metastasis initiating cells (MICs) in 17 cases of primary tumors. Notably, we verified that it was the highest expressor of PD-L1 (median value= 31.8%; min 6%, max 100%). Conversely, CD133+ CSCs

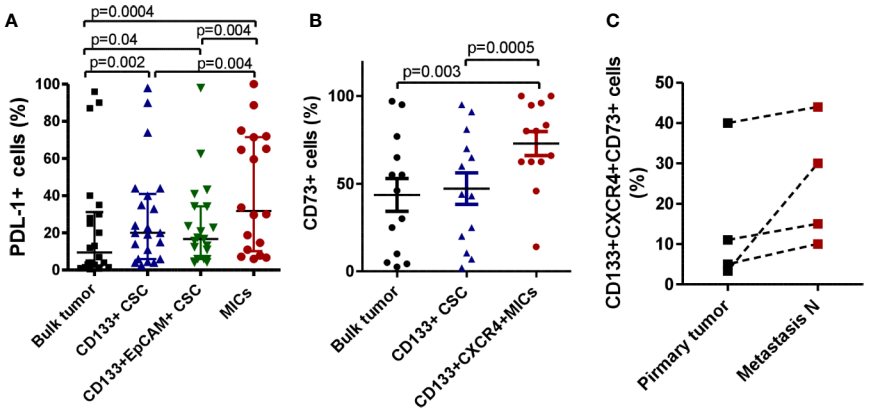


FIGURE 1 | MICs highly expressed immunoregulatory markers. **(A)** FACS analysis of N=22 NSCLC primary tumors. PD-L1 expression was assessed within bulk tumor population and different subsets of CD133+ Cancer Stem Cells, the epithelial one (EpCAM+) and the mesenchymal and metastatic one (CD133+CXCR4+ EpCAM- Metastasis initiating cells MICs). **(B)** FACS analysis of N=13 NSCLC primary tumors for the expression of CD73 within bulk tumor and different CSC subsets. **(C)** Comparison of CD73 expression by FACS analysis in n=4 primary tumors and synchronous lymph node metastases.

positive for the epithelial marker EpCAM showed lower expression of PD-L1 (median value = 16.6%; min 4%, max 98%) (**Figure 1A**).

We also observed a down-regulation of HLA class I, antigen presenting molecule, in CD133+ CSC compared to tumor bulk population, both in NSCLC primary tumors (n=6) and NSCLC cell lines (n=4) (0,7 fold decrease compared to bulk cells) (**Figures S1A, B**), confirming the ability of CSC to escape immune cells recognition. In 13 primary NSCLC samples, we also assessed CD73 expression within bulk population and CSC subsets. CD73 expression was significantly increased within the subset of CD133+CXCR4+ MICs (median value= 80%; min 14%, max 100%) compared to CD133+ CSCs (median= 44%; min 2%, max 95%) and bulk tumor (median= 46%; min 2.5%, max 65%) (**Figure 1B**).

Finally, in 4 cases, we were able to analyse primary tumors and corresponding synchronous lymph node metastases. The subset of metastatic and immunosuppressive CD133+CXCR4+ CD73+ MICs was 2.6 fold-enriched in metastasis compared to primary tumors (**Figure 1C**).

Overall, this immunophenotypic characterization of primary NSCLC indicates that CSCs and in particular the fraction of MICs displays high levels of molecules involved in immune suppression.

Lung Cancer Spheroids Are Enriched in MICs and Express Immunosuppressive Molecules

To study *in vitro* the immunosuppressive properties of MICs, we exploited a well-known method adopted to enrich for CSC population through the generation of cancer spheroids grown in selective medium, containing EGF and bFGF (45). We generated spheroids from 4 NSCLC cell lines: A549 and H3122 (adenocarcinoma), H1299 (large cell carcinoma), and SW900 (squamous cell carcinoma) (**Figure S2**). They were characterized for CD133+CXCR4+ phenotype, PD-L1, HLA class I, and for CD73, CD38, and CD39 expression, involved in the production of immunosuppressive adenosine (29).

Overall, compared to their parental adherent cell lines, spheroids were highly enriched in CD133+CXCR4+ MICs subset (30 fold-change), generally associated with an increase of either CD73 or CD38 markers (respectively 1.2 and 3 fold-change), both involved in immune regulation and generation of adenosine (**Figure 2A**). The expression of CD39, the ectoenzyme that functions in tandem with CD73 in the canonical adenosinergic pathway, was undetectable both in adherent cells and spheroids, suggesting that in our *in vitro* condition CD38/CD73 non-canonical pathway is uniquely responsible for adenosine production.

Finally, no significant modulation of PD-L1 or HLA class I was observed in any spheroids cell lines compared to parental adherent one (data not shown). To address whether the increase in CD73/CD38 observed by flow cytometry analysis in CSC-enriched spheroids could be functionally associated with an increased production of ADO, we added AMP to adherent cells and sphere cultures and quantified adenosine production by HPLC. Results showed an increase of adenosine levels in medium from spheroids compared to adherent cells (**Figure 2B**). These data suggested a direct connection between high membrane expression of CD73/CD38 and production of adenosine.

We also investigated the modulation of IL-10, a cytokine known to trigger immunosuppressive effects by inducing T reg and pro-tumorigenic macrophages. Gene expression Real-Time analysis showed that spheroids expressed different levels of IL-10, whereas in all tested adherent cell lines IL-10 expression was undetectable (**Figure 2C**).

Overall, our results show that spheroids generated *in vitro* can be exploited to investigate the immunosuppressive phenotype of MICs.

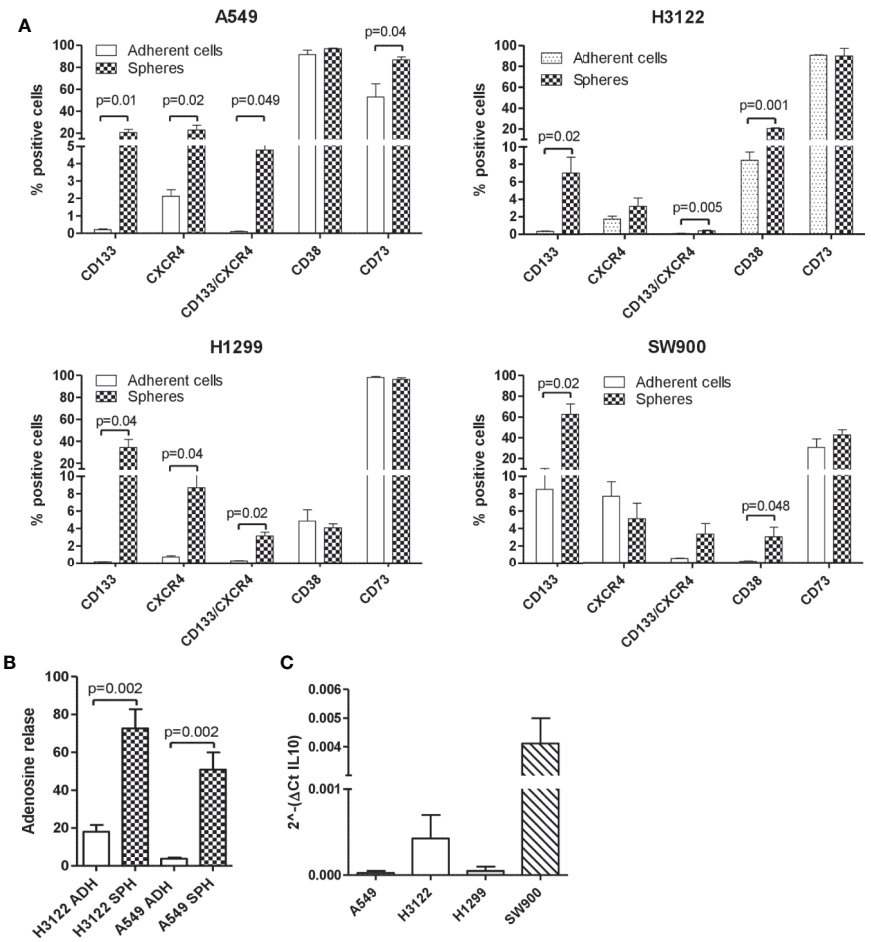


FIGURE 2 | Lung spheroids are enriched in MICs and expressed high level of immunosuppressive markers then adherent cells. **(A)** FACS analysis of adherent NSCLC cell lines (A549, H3122, H1299, SW900) and corresponding spheroids for expression of CD133, CXCR4, CD38, and CD73 markers. Data are the mean value ± SD of n=4 analyses for each cell line. **(B)** AMP substrate was added to culture medium and generation of adenosine was quantified by HPLC in the medium of spheroids and adherent cells (A549 and H3122 cell lines). Data are the mean value ± SD of n=2 analyses for each cell line. **(C)** IL-10 gene expression evaluated by Real-Time PCR in lung spheroids cultures. Bar are the mean value ± SD of $2^{-(CT\ IL\text{-}10\text{-}CT\ B2m)}$.

Inhibition of CXCR4 Pathway Prevents Tumor Dissemination and Reduces Expression of Immunosuppressive Molecules

To block migration of CD133+CXCR4+ MICs, we tested a novel peptide inhibitor of CXCR4, peptide R, an analogue of SDF-1 (44).

Firstly, we assessed the ability of peptide R (1μM) to prevent both migration/invasion induced by SDF-1, similarly to AMD3100, a CXCR4 antagonist that has been clinically approved (**Figure 3A**). The experiments were performed in our panel of lung cancer cell lines.

We analyzed the phenotype of adherent cell lines after treatment with peptide R. Notably, we observed a reduced expression of markers, such as CD38 and CD73 (**Figure 3B**). We verified that the modulation of CD38 and CD73 expression induced by CXCR4 blockade was a rapid event, with the greatest effect observed 2 h post treatment and that rapidly reverted to basal expression (**Figure S1A**). We also confirmed the down-regulation of CD73 after CXCR4 inhibition by WB and Real-Time analyses (**Figures S3B, C**).

However, since in adherent cell lines only a small percentage of cells expressed CXCR4 (median value 1.2%; min 0.7%, max 4.8%), we speculated that in lung cancer spheroids, highly enriched for CXCR4+ cells (median value 7.5%; min 2%, max 31.6%), treatment with peptide R might result in a more marked effect. Indeed, we proved that short-term treatment of spheroids with CXCR4 inhibitor was able to significantly reduce the expression of CD38 and/or CD73 in all cell lines (except for A549), likely indicating an adenosine decrease, and average 50% decrease of immunosuppressive IL-10 cytokine expression in all cell lines (**Figures 3C, D**). These results suggest a link between CXCR4 pathway and induction of immunosuppressive phenotype in MICs.

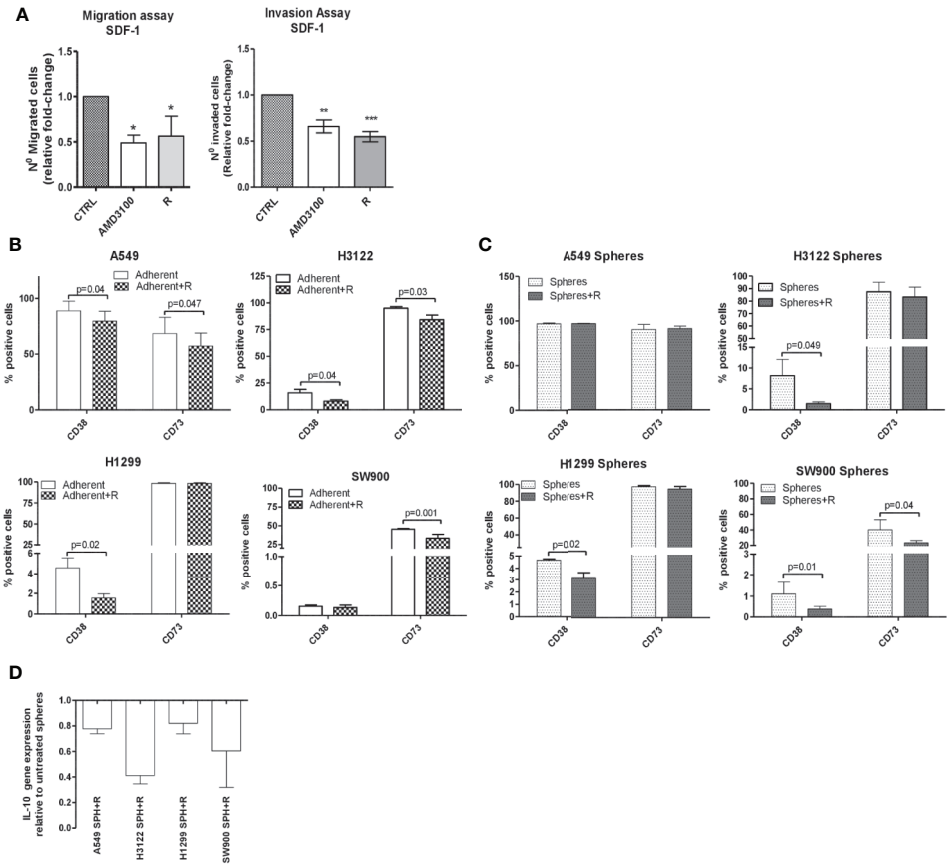


FIGURE 3 | Inhibition of CXCR4 axis prevents MICs migration and decreases immunomodulatory marker expression. **(A)** Migration and invasion assay performed *in vitro* on A549, H3122, H1299 cell lines. Cells were treated with CXCR4 inhibitors: peptide R 1μM or AMD3100 10 μM and chemoattracted by SDF-1 50 ng/ml. Data represent the median fold change of number of migrated/invaded cells after treatment relative to untreated control. Duplicate experiments were performed for each cell line *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$ **(B)** FACS analysis of adherent cells and **(C)** lung spheroids for the expression of CD38 and CD73 markers 2 h after treatment with peptide. Data are the mean value ± SD of n=3 analyses for each cell line. **(D)** Real-Time PCR quantification of IL-10 gene expression in spheroids after treatment with CXCR4 inhibitor compared to untreated cells.

CXCR4 Axis Inhibition Partially Rescues T Cells Suppression Caused by MICs

To functionally prove the relevance of effects on immunosuppressive molecules modulation induced by CXCR4 inhibition, we tested the ability of CM collected from treated cell lines versus untreated controls, in both adherent and spheroids condition, to induce T cell suppression.

Firstly, we assessed the effects of CM from adherent cells and spheroids in modulating T cells having regulatory function (T reg: CD4+Foxp3+CD25^{high}). T cells from healthy volunteers were stimulated with anti CD3/CD28 micro beads and cultured in presence of CM from cancer cells lines. We showed that spheroids CM were able to increase the percentage of T reg compared to control, at higher extent than adherent cells (respectively 1.6 and 1.3 fold-increase). Notably, blockade of CXCR4 in both adherent and spheroid cells was sufficient to prevent the increase of T reg population induced by untreated counterpart (**Figure 4A**).

Next, since MIC-enriched spheroids were able to induce T reg phenotype, we assessed their potential to suppress T-cell activity. We demonstrated that lung spheroid CM were able to significantly suppress the proliferation of T cells, isolated from healthy donors PBMCs, after stimulation with anti CD3/CD28 antibodies (**Figure 4B**). When we compared the effect of spheroids and adherent cells, we observed that T cells from healthy donors proliferated significantly less in the presence of spheroids CM than adherent cells CM, and importantly, CM from spheroids treated with peptide R partially counteracted the suppressive effect on T cells (**Figure 4C**).

Finally, we verified that CM from spheroids were able to partially suppress (0.8 fold-change) the release of IFN-γ from CD8+ T cytotoxic cells (**Figure 4D**), derived from PBMCs of healthy volunteers, whereas CM from adherent cancer cell lines did not. CM from adherent and spheroid cancer cell lines treated with peptide R were able to relieve suppression of T cells and increase the subset of CD8+ T cells expressing IFN-γ compared to untreated cells (**Figure 4D**).

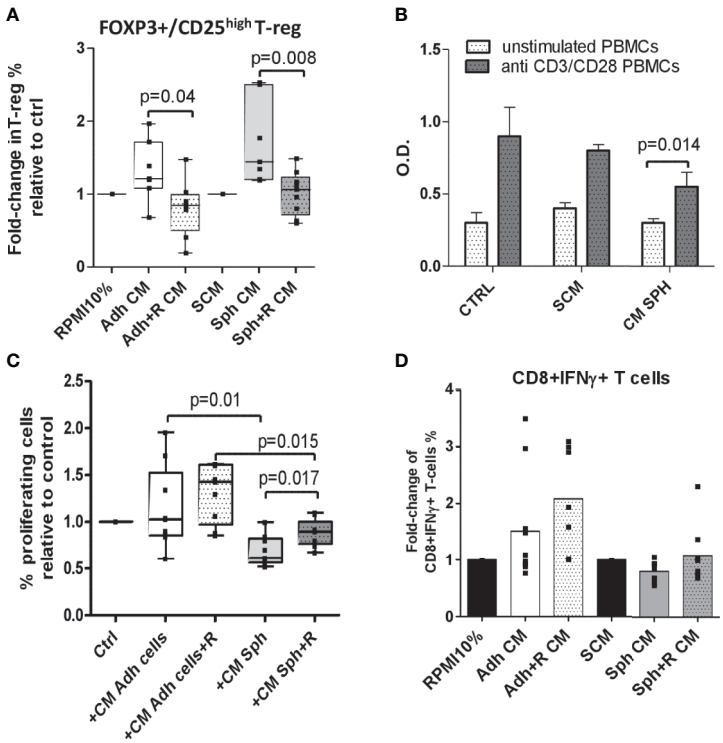


FIGURE 4 | CM from spheroids induces T cells suppression that can be prevented by CXCR4 inhibition. **(A)** FACS analysis for Treg population within T lymphocytes, from N=8 healthy volunteers. T lymphocytes were stimulated with anti CD3/CD28 beads and incubated for 72 h with CM from adherent or spheroids cell lines, untreated or treated with peptide R. Data are the fold-change in % T reg population compared to proper control medium (RPMI 10% for adherent cells and Stem cells medium- SCM- for spheroids). Data are the mean value ± SD. N=2 independent experiments were performed for each tested NSCLC cell lines (A549/H3122/H1299/Sw900). **(B)** MTT assay measuring the proliferation of healthy volunteer T cells, unstimulated or stimulated with anti-CD3 and anti-CD28 antibodies, after exposure for 72 h to CM from A549 and H3122 spheroids or control RPMI or SCM medium for 72 h. Data are the mean value ± SD of N=4 independent experiment for each cell line. **(C)** CSFE assay measuring proliferation of healthy volunteers T cells, stimulated with anti-CD3 and anti-CD28 microbeads, after exposure for 72 h to CM from adherent or spheroids, treated or not with peptide R. Data are the fold-change in % of proliferating cells compared to proper control medium (RPMI 10% for adherent cells and SCM for spheroid). Data are the mean value ± SE of each NSCLC cell line (A549/H3122/H1299/SW900) tested in triplicate experiment. **(D)** FACS analysis for CD8+ T cytotoxic cells expressing IFNγ in N=8 healthy volunteers incubated for 72 h with CM from adherent or spheroids cell lines, untreated or treated with peptide R. Data are the fold-change in % CD8 T cytotoxic population compared to proper control medium (RPMI 10% for adherent cells and Stem cells medium- SCM- for spheroids). Data are the mean value ± SE of N=2 independent experiments were performed for each tested NSCLC cell lines.

Overall, our data functionally prove that spheroids enriched in MICs possess an enhanced ability to suppress T-cell activity, concomitantly with the above reported increase in adenosine and IL-10 production. CXCR4 blockade is able to impair MIC immune suppression activity, preventing T reg generation and rescuing T cell activity.

CXCR4 Inhibition Impairs CSC Ability to Promote TAM Polarization

Finally, we tested the ability of CM from lung cancer cell lines treated or not with peptide R to induce M0 macrophages polarization toward tumor-associated macrophages (TAM), known to possess immunosuppressive properties (46).

Macrophage cultures were derived from healthy volunteers. We evaluated by FACS the increased percentage of CD206+, CD163+, and CD14-CD206+ cell subsets and by Real-Time PCR an increased expression of IL-10, VEGF, and, conversely, a decreased expression of pro-inflammatory cytokines IL-12 and IL-6 as a read out of the induction of TAM phenotype after exposure to cancer cells CM, as reported by Benner et al. (47).

Despite the variability across macrophage cultures from different volunteers, we found that CM from spheroid cell lines enriched in MICs were more prone to induced TAM polarization compared to adherent cell lines, confirming the immunosuppressive behavior of MICs (**Figures 5A, B**). Indeed, CM from spheroids proficiently expanded the subset of CD206+/CD163+ and CD14-CD206+ macrophages **Figure 5A**) and induced the up-regulation of IL-10 and VEGF with a concomitant decrease of IL-12 and IL-6 (**Figure 5B**), a phenotype typically associated with TAM.

To exclude that different medium composition (RPMI 10% or SCM) could drive modulation of immune regulation induced by cancer cells, we treated macrophage cultures with both RPMI

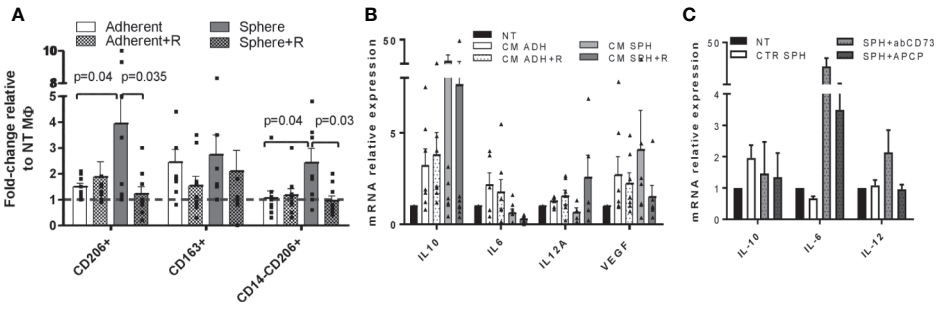


FIGURE 5 | CM from spheroids induces TAM polarization that can be prevented by CXCR4 inhibition. **(A)** FACS analysis for CD206, CD163, and CD14 expression in macrophages derived from PBMCs of healthy volunteers treated with CM from adherent or spheroids, treated or not with peptide R. Data are the fold-change in % of positive cells compared to control macrophages cultured in proper control medium (RPMI 10% for adherent cells and SCM for spheroids). N=2 independent experiments were performed for each tested NSCLC cell lines. **(B)** Real-Time PCR quantification of IL-10, IL-6, IL-12, VEGF gene expression in macrophages derived from PBMCs of heavy smoker volunteers treated with CM from adherent or spheroids, treated or not with peptide R. Control macrophages cultured in proper control medium (RPMI 10% for adherent cells and SCM for spheroids) were used as calibrator. N=2 independent experiments were performed for each tested NSCLC cell lines. **(C)** Real-Time PCR quantification of IL-10, IL-6, IL-12 genes expression in macrophages derived from PBMCs of heavy smoker volunteers treated with CM from spheroids, untreated or treated with anti CD73 antibody or APCP. Control macrophages cultured in SCM were used as calibrator.

10% or SCM media conditioned by adherent NSCLC cell lines. We verified that the effects of the two CMs in the induction of TAM phenotype were very similar, indicating that different medium composition does not modify the intrinsic ability of cancer cells to induce TAM polarization (**Figure S4A**).

Finally, we assessed whether the observed increased immunosuppressive effects of spheroids reflect specific properties of selected cancer cells or can be related to the different *in vitro* culture conditions (adherent vs suspension). We exploited the immortalized but not tumorigenic human bronchial epithelial cell line (HBEC3-KT) that is not expected to induce an immunosuppressive effect on PBMCs. HBEC cells were grown in adhesion and in suspension as spheroids and SCM conditioned medium was collected by both cultures. Macrophages treated with SCM-CMs from both adherent and spheroids HBEC failed to show TAM polarization, as assessed by FACS and Real-Time PCR analyses (**Figure S4B**).

Overall, these data confirm that differences observed between CM from adherent and spheroids NSCLC cell lines are not due to medium composition or different culture conditions, but instead related to the intrinsic properties of spheres enriched in MIC population, with higher potential to induce immunosuppressive effects.

The treatment of lung spheroid cultures with peptide R was able to partially prevent TAM polarization, significantly reducing CD206/CD163 surface expression and IL-10, VEGF gene expression while increasing IL-12 production compared to untreated control (**Figures 5A, B**).

To prove the role of adenosine as a key mediator of immunosuppressive properties of MICs, we treated spheroids with the Adenosine 5'-(α,β-methylene)diphosphate (APCP) and with a neutralizing antibody against CD73, both able to impair adenosine production (29, 32). We observed that collected media from Ab-treated cells were able to revert TAM phenotype induced by lung cancer spheroids as indicated by a decrease of IL-10 and an increase of IL-6 and IL-12 (the latter was observed only after moAb treatment) (**Figure 5C**).

Overall, our data suggest that MICs-enriched spheroids not only directly act on T cell regulation but also induce polarization of TAM, which can exacerbate an immune suppressive environment.

DISCUSSION

Cancer stem cells are composed of heterogeneous populations, each with a specific function (48, 49). The subset of CSCs deputed to metastasis initiation possesses features allowing primary tumor escape, survival in circulation, and distant organs seeding (50, 51). Immune escape mechanisms adopted by MICs are supposed to be essential to complete all the steps leading to metastasis generation (52, 53).

Some evidence has reported that CSCs are characterized by specific immunological properties, which protect them against chemotherapeutic drugs but also increase their resistance toward apoptosis-inducing immune effectors, like T or NK cells (54). Several mechanisms can be exploited by CSCs to escape immune surveillance, such as down-regulation of MHC class I and II molecules, inefficient antigen presentation, and release of immunosuppressive factors (52). These strategies would help CSCs to survive, sustain tumor progression, and metastasize (53).

Moreover, it has been reported that there is a correlation between immunosuppressive environment and activation of epithelial to mesenchymal transition (EMT) program, endowing primary tumor cells with disseminating and stemness properties (52, 55). Dongre et al. showed that mesenchymal traits of tumor cells are associated with high levels of PD-L1 expression, content of T reg cells, and M2-like macrophages, proving that EMT activation in tumor cells promotes the recruitment of immunosuppressive cells and immune surveillance escape (56). In NSCLC the activation of EMT by the up-regulation of ZEB1 transcriptional factors causes the up-regulation of PD-L1 by tumor cells, leading to CD8+ T cells immune suppression and increased metastasis (57).

All together, these evidences suggest that a deeper understating of the immune profile of CSCs, and in particular of the mesenchymal subset deputed to metastasis initiation, can pave the way for specific anti-CSC immunotherapy, necessary to achieve a complete eradication of tumors and control of metastatic diseases.

In NSCLC, we previously showed that the population of CD133+/CXCR4+ MICs is endowed with stemness and EMT features, enhanced resistance to cisplatin, and superior ability to seed distant organs and initiate metastasis (11, 13). However, the immunological characterization of this subset has never been reported.

Here, we show that NSCLC MICs express the highest levels of both PD-L1 and CD73, compared to bulk tumor cells and epithelial CSC subset, suggestive for increased potential to suppress T cell activity.

An increased expression of PD-L1 has also been reported in CSCs of other tumor types. In head and neck carcinomas the subset of CD44+ CSCs expressing high level of PD-L1 can selectively evade host immune responses. The use of an immune check point inhibitor against PD-1 partially restored the immunogenicity of CD44+CSCs, providing the rationale for an anti CSC-immunotherapy (58).

In triple negative breast cancer, ALDH/CD44+ CSCs exhibited increased levels of PD-L1 versus non-CSC tumor cells. ALDH/CD44+/PD-L1+ CSCs were found in close contact with PD-1+ T cells both in murine and human tumor samples, suggesting a direct effect of CSCs in immune control (59).

In our study, we report that NSCLC CSCs co-expressing CXCR4 and CD73 are enriched in lymph node metastasis compared to primary tumors, indicating that the cells able to initiate metastasis may have an enhanced immunosuppressive activity.

This result confirms previously published literature reporting increased CD73 levels in metastatic tumors (31, 60). Moreover, studies deriving from different solid tumors reported CD73 expression as a poor prognostic factor (37, 61), suggesting that CD73-adenosine pathway plays a fundamental role in tumor dissemination, likely promoting immune suppression.

To investigate *in vitro* the immunosuppressive phenotype of CD133+/CXCR4+ CSCs overcoming the limitation of the paucity of CSCs in established adherent NSCLC cell lines, we adopted the sphere forming assay, a method commonly recognized to enrich for CSC subset (45). Spheroid cultures generated from NSCLC cell lines recapitulate the immunosuppressive phenotype of CD133+ CXCR4+ MICs subset, also expressing higher levels of CD73 and CD38 as compared to adherent cells.

Adenosinergic signaling is a physiopathological regulator of tissue homeostasis, particularly upon injury and stress. Indeed, adenosine rapidly increases in response to stress, hypoxia, or tissue injury inducing repair processes (62). High levels of extracellular adenosine, generated by canonical CD39/CD73 or non-canonical CD38/CD73 adenosinergic pathways in tumor microenvironment (28, 29), can promote tumor progression by directly stimulating tumor proliferation, migration, invasion, and metastatic dissemination and by favoring immune escape of tumor cells (33). From a functional point of view, CD133+CXCR4+ MICs subset showed an increased release of immunosuppressive adenosine, due to the activation of CD38/CD73 pathway, and indeed CD38 and CD73 resulted highly expressed, while CD39 expression was undetectable Further, we also detected the increase release of IL-10, known to trigger immunosuppressive effects by inducing T reg cells and pro-tumorigenic immunosuppressive polarization of macrophages (63).

When we functionally tested *in vitro* immune regulatory properties of lung spheroids and corresponding adherent cells, we demonstrated that MICs-enriched spheroids possess an increased ability to induce T reg cells and consequently to suppress T cell proliferation as well as to reduce cytotoxic ability of CD8+ T cells.

Similarly, it was demonstrated that CSCs from glioblastoma inhibited T cell proliferation of healthy donors and showed lower immunogenicity and higher suppressive activity compared to corresponding adherent cell lines (64).

We also assessed the effect of spheroids to induce polarization of macrophages toward TAM phenotype that are very well known to promote immune suppression, tumor cell invasion, and metastasis (46, 65).

Conditioned media from cancer cells can be exploited to induce TAM polarization (47). In particular, TAM phenotype is associated with a high expression of immunosuppressive IL-10 and pro-angiogenic VEGF and low levels of inflammatory cytokines (IL-6 and 12). Besides, there is generally an increase of CD206/CD163 markers and reduced CD14 surface expression (47). All of these features were detected in cultures of macrophages derived from PBMCs of volunteers exposed to spheroids CM, thus *bona fede* providing support to the ability of MICs to induce TAM polarization that can exacerbate immunosuppressive environments.

It has been previously reported that one of the pathways stimulated by adenosine is the up-regulation of CXCR4 in cancer cells, increasing their ability to migrate and proliferate in response to CXCL12 (17). CXCR4 expression is an important factor for maintenance of stemness and endowment of metastatic potential of NSCLC CSCs (66). Thus, targeting CXCR4 could be useful both to block CSCs and to decrease tumor microenvironment immune suppression.

Moreover, CXCR4 is highly expressed also by the subset of immunosuppressive Treg cells. CXCR4 and its inhibition have been demonstrated in different tumor types to efficiently revert Treg suppression of T effectors proliferation, improving anticancer immune responses (23, 67).

CXCR4/CXCL12 axis inhibition has been demonstrated to revert tolerogenic polarization of tumor microenvironment (42) and to restore sensitivity to CTLA-4 and PD-1 ICIs (23, 43), overall representing a novel and effective way to counteract ICIs resistance. In the present study, we tested a novel peptide inhibitor of CXCR4, peptide R, analogue of CXCL12 (44), to target CD133+CXCR4+ MICs. We show that the treatment of NSCLC spheroids with Peptide R, besides preventing tumor cell dissemination, decreases expression of immunosuppressive molecules, such as CD73, CD38, and IL-10.

Furthermore, the functional blockade of CXCR4 in tumor cells is sufficient to prevent the immunosuppressive ability of MICs by restoring T cell proliferation and IFN_{γ} expression, as well as partially preventing TAM polarization.

Our study has some limitations, mainly related to the small effects observed among treatment groups and the lack of *in vivo* validation of the findings. Indeed, treatment of PBMC with CM in some experiments resulted in biological effects that did *not* reach *statistical significance* mainly due to the great variability among PBMC from different healthy volunteers and to the use of several NSCLC cell lines. Despite the expected variability, we decided to test different NSCLC cell lines to take into consideration the heterogeneity of NSCLC histological subtypes and to avoid the potential bias of single cell line-dependent effects.

In vivo validation of our observation could definitely strengthen our conclusions. However, the *in vivo* investigation of the immunosuppressive ability of human tumor cells is hampered by the necessity to use immunocompromised mice, lacking adaptive immunity, to grow xenograft tumors. The establishment of a more sophisticated humanized murine model reconstituted with human immune cells might provide in the near future further validation of our *in vitro* evidence.

Finally, the validation of the potential of CXCR4 blockade to counteract MICs immune escape may be challenging *in vivo*. Since CXCR4 is wildly expressed both by tumor and stroma/immune cells, the systemic delivery of CXCR4 inhibitors *in vivo* could affect these different cell subsets, impairing the possibility to finely dissect the players involved in the generation of the immunosuppressive microenvironment and the impact of CXCR4 inhibition on this tumor-stromal crosstalk.

Despite these limitations, taken together our data suggest the high ability of MICs to escape immune control and corroborate the link between CXCR4 pathway and the induction of immunosuppressive phenotype in CSCs. Consequently, they point at CXCR4 inhibitors as potential innovative agents to implement efficacy of immunotherapy, by concurring in reverting immune suppression and preventing metastatic dissemination.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB of Fondazione IRCCS Istituto Nazionale dei Tumori. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GB and IR conceived the study. OF, DB, FG, MC, CB, AH, IR, and GB performed the experiments and analyzed the data. LR, IR, and GB supervised data acquisition and analysis. UP provided clinical samples. SS provided the CXCR4 inhibitor. GB, IR, and OF wrote the manuscript. MC, AH, FM, RF, SS, GS, and LR reviewed and critically assessed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work is supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) (grant number IG21431 to LR and IG18812 to GS); Roche Italia (Roche per la Ricerca 2017 GB); CRT Foundation (CRT ordinary grant 2018 to IR).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02168/full#supplementary-material

REFERENCES

1. Hirsch FR, Scagliotti GV, Mulshine JL, Kwon R, Curran WJJr., Wu YL, et al. Lung cancer: current therapies and new targeted treatments. *Lancet* (2016) 389:299–311. doi: 10.1016/S0140-6736(16)30958-8
2. Chang A. Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. *Lung Cancer* (2011) 71:3–10. doi: 10.1016/j.lungcan.2010.08.022
3. D'Alterio C, Scala S, Sozzi G, Roz L, Bertolini G. Paradoxical effects of chemotherapy on tumor relapse and metastasis promotion. *Semin Cancer Biol* (2019) 60:351–61. doi: 10.1016/j.semcancer.2019.08.019
4. Karagiannis GS, Condeelis JS, Oktay MH. Chemotherapy-induced metastasis: mechanisms and translational opportunities. *Clin Exp Metastasis* (2018) 35:269–84. doi: 10.1007/s10585-017-9870-x
5. Vyas D, Laput G, Vyas AK. Chemotherapy-enhanced inflammation may lead to the failure of therapy and metastasis. *Onco Targets Ther* (2014) 7:1015–23. doi: 10.2147/OTT.S60114
6. Reck M. Pembrolizumab as first-line therapy for metastatic non-small-cell lung cancer. *Immunotherapy* (2018) 10:93–105. doi: 10.2217/imt-2017-0121
7. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. *Nature* (2018) 553:446–54. doi: 10.1038/nature25183
8. Chatwal MS, Tanvetyanon T. Combination chemotherapy and immunotherapy in metastatic non-small cell lung cancer: a setback for personalized medicine? *Transl Lung Cancer Res* (2018) 7:S208–10. doi: 10.21037/tlcr.2018.07.15
9. Giroux LE, Dumenil C, Julie C, Giraud V, Dumoulin J, Labrune S, et al. Immunotherapy revolutionises non-small-cell lung cancer therapy: Results, perspectives and new challenges. *Eur J Cancer* (2017) 78:16–23. doi: 10.1016/j.ejca.2016.12.041
10. Pitt JM, Vetizou M, Daillere R, Roberti MP, Yamazaki T, Routy B, et al. Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors. *Immunity* (2016) 44:1255–69. doi: 10.1016/j.immuni.2016.06.001
11. Bertolini G, Roz L, Perego P, Tortoreto M, Fontanella E, Gatti L, et al. Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci U S A* (2009) 106:16281–6. doi: 10.1073/pnas.0905653106
12. Bertolini G, Gatti L, Roz L. The "stem" of chemoresistance. *Cell Cycle* (2010) 9:628–9. doi: 10.4161/cc.9.4.10821
13. Bertolini G, D'Amico L, Moro M, Landoni E, Perego P, Miceli R, et al. Microenvironment-Modulated Metastatic CD133+/CXCR4+/EpCAM- Lung Cancer-Initiating Cells Sustain Tumor Dissemination and Correlate with

Poor Prognosis. *Cancer Res* (2015) 75:3636–49. doi: 10.1158/0008-5472.CAN-14-3781
14. Scala S. Molecular Pathways: Targeting the CXCR4-CXCL12 Axis–Untapped Potential in the Tumor Microenvironment. *Clin Cancer Res* (2015) 21:4278–85. doi: 10.1158/1078-0432.CCR-14-0914
15. Wang Z, Sun J, Feng Y, Tian X, Wang B, Zhou Y. Oncogenic roles and drug target of CXCR4/CXCL12 axis in lung cancer and cancer stem cell. *Tumour Biol* (2016) 37:8515–28. doi: 10.1007/s13277-016-5016-z
16. Cojoc M, Peitzsch C, Trautmann F, Polishchuk L, Telegeev GD, Dubrovska A. Emerging targets in cancer management: role of the CXCL12/CXCR4 axis. *Onco Targets Ther* (2013) 6:1347–61. doi: 10.2147/OTT.S36109
17. Richard CL, Tan EY, Blay J. Adenosine upregulates CXCR4 and enhances the proliferative and migratory responses of human carcinoma cells to CXCL12/SDF-1alpha. *Int J Cancer* (2006) 119:2044–53. doi: 10.1002/ijc.22084
18. Allard B, Turcotte M, Stagg J. CD73-generated adenosine: orchestrating the tumor-stroma interplay to promote cancer growth. *J BioMed Biotechnol* (2012) 2012:485156. doi: 10.1155/2012/485156
19. Jantunen E. Novel strategies for blood stem cell mobilization: special focus on plerixafor. *Expert Opin Biol Ther* (2011) 11:1241–8. doi: 10.1517/14712598.2011.601737
20. Porvasnik S, Sakamoto N, Kusmartsev S, Eruslanov E, Kim WJ, Cao W, et al. Effects of CXCR4 antagonist CTCE-9908 on prostate tumor growth. *Prostate* (2009) 69:1460–9. doi: 10.1002/pros.21008
21. Huang EH, Singh B, Cristofanilli M, Gelovani J, Wei C, Vincent L, et al. A CXCR4 antagonist CTCE-9908 inhibits primary tumor growth and metastasis of breast cancer. *J Surg Res* (2009) 155:231–6. doi: 10.1016/j.jss.2008.06.044
22. Richert MM, Vaidya KS, Mills CN, Wong D, Korz W, Hurst DR, et al. Inhibition of CXCR4 by CTCE-9908 inhibits breast cancer metastasis to lung and bone. *Oncol Rep* (2009) 21:761–7. doi: 10.3892/or_00000282
23. Chen IX, Chauhan VP, Posada J, Ng MR, Wu MW, Adstamongkonkul P, et al. Blocking CXCR4 alleviates desmoplasia, increases T-lymphocyte infiltration, and improves immunotherapy in metastatic breast cancer. *Proc Natl Acad Sci U S A* (2019) 116:4558–66. doi: 10.1158/1538-7445.SABCS18-2744
24. Zeng Y, Li B, Liang Y, Reeves PM, Qu X, Ran C, et al. Dual blockade of CXCL12-CXCR4 and PD-1-PD-L1 pathways prolongs survival of ovarian tumor-bearing mice by prevention of immunosuppression in the tumor microenvironment. *FASEB J* (2019) 33:6596–608. doi: 10.1096/fj.201802067RR
25. Jiang K, Li J, Zhang J, Wang L, Zhang Q, Ge J, et al. SDF-1/CXCR4 axis facilitates myeloid-derived suppressor cells accumulation in osteosarcoma microenvironment and blunts the response to anti-PD-1 therapy. *Int Immunopharmacol* (2019) 75:105818. doi: 10.1016/j.intimp.2019.105818
26. Vaisitti T, Aydin S, Rossi D, Cottino F, Bergui L, D'Arena G, et al. CD38 increases CXCL12-mediated signals and homing of chronic lymphocytic leukemia cells. *Leukemia* (2010) 24:958–69. doi: 10.1038/leu.2010.36
27. Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, et al. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev* (2008) 88:841–86. doi: 10.1152/physrev.00035.2007
28. Horenstein AL, Bracci C, Morandi F, Malavasi F. CD38 in Adenosinergic Pathways and Metabolic Re-programming in Human Multiple Myeloma Cells: In-tandem Insights From Basic Science to Therapy. *Front Immunol* (2019) 10:760. doi: 10.3389/fimmu.2019.00760
29. Horenstein AL, Chillemi A, Zaccarello G, Bruzzone S, Quarona V, Zito A, et al. A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes. *Oncoimmunology* (2013) 2:e26246. doi: 10.4161/onci.26246
30. Allard D, Chrobak P, Allard B, Messaoudi N, Stagg J. Targeting the CD73-adenosine axis in immuno-oncology. *Immunol Lett* (2019) 205:31–9. doi: 10.1016/j.imlet.2018.05.001
31. Jiang T, Xu X, Qiao M, Li X, Zhao C, Zhou F, et al. Comprehensive evaluation of NT5E/CD73 expression and its prognostic significance in distinct types of cancers. *BMC Cancer* (2018) 18:267. doi: 10.1186/s12885-018-4073-7
32. Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, et al. Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci U S A* (2010) 107:1547–52. doi: 10.1073/pnas.0908801107
33. Allard B, Pommey S, Smyth MJ, Stagg J. Targeting CD73 enhances the antitumor activity of anti-PD-1 and anti-CTLA-4 mAbs. *Clin Cancer Res* (2013) 19:5626–35. doi: 10.1158/1078-0432.CCR-13-0545
34. Allard B, Longhi MS, Robson SC, Stagg J. The ectonucleotidases CD39 and CD73: Novel checkpoint inhibitor targets. *Immunol Rev* (2017) 276:121–44. doi: 10.1111/imr.12528
35. Young A, Ngiow SF, Barkauskas DS, Sult E, Hay C, Blake SJ, et al. Co-inhibition of CD73 and A2AR Adenosine Signaling Improves Anti-tumor Immune Responses. *Cancer Cell* (2016) 30:391–403. doi: 10.1016/j.ccell.2016.06.025
36. Terp MG, Olesen KA, Arnspang EC, Lund RR, Lagerholm BC, Ditzel HJ, et al. Anti-human CD73 monoclonal antibody inhibits metastasis formation in human breast cancer by inducing clustering and internalization of CD73 expressed on the surface of cancer cells. *J Immunol* (2013) 191:4165–73. doi: 10.4049/jimmunol.1301274
37. Inoue Y, Yoshimura K, Kurabe N, Kahyo T, Kawase A, Tanahashi M, et al. Prognostic impact of CD73 and A2A adenosine receptor expression in non-small-cell lung cancer. *Oncotarget* (2017) 8:8738–51. doi: 10.18632/oncotarget.14434
38. Li J, Wang L, Chen X, Li L, Li Y, Ping Y, et al. CD39/CD73 upregulation on myeloid-derived suppressor cells via TGF-beta-mTOR-HIF-1 signaling in patients with non-small cell lung cancer. *Oncoimmunology* (2017) 6:e1320011. doi: 10.1080/2162402X.2017.1320011
39. Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J Clin Invest* (2015) 125:3384–91. doi: 10.1172/JCI80011
40. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat Commun* (2016) 7:10501. doi: 10.1038/ncomms10501
41. Chen L, Diao L, Yang Y, Yi X, Rodriguez BL, Li Y, et al. CD38-Mediated Immunosuppression as a Mechanism of Tumor Cell Escape from PD-1/PD-L1 Blockade. *Cancer Discov* (2018) 8:1156–75. doi: 10.1158/2159-8290
42. Righi E, Kashiwagi S, Yuan J, Santosuosso M, Leblanc P, Ingraham R, et al. CXCL12/CXCR4 blockade induces multimodal antitumor effects that prolong survival in an immunocompetent mouse model of ovarian cancer. *Cancer Res* (2011) 71:5522–34. doi: 10.1158/0008-5472.CAN-10-3143
43. Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, et al. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. *Proc Natl Acad Sci U S A* (2013) 110:20212–7. doi: 10.1073/pnas.1320318110
44. Portella L, Vitale R, De LS, D'Alterio C, Ierano C, Napolitano M, et al. Preclinical development of a novel class of CXCR4 antagonist impairing solid tumors growth and metastases. *PLoS One* (2013) 8:e74548. doi: 10.1371/journal.pone.0074548
45. Ishiguro T, Ohata H, Sato A, Yamawaki K, Enomoto T, Okamoto K. Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications. *Cancer Sci* (2017) 108:283–9. doi: 10.1111/cas.13155
46. Quatromoni JG, Eruslanov E. Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *Am J Transl Res* (2012) 4:376–89.
47. Benner B, Scarberry L, Suarez-Kelly LP, Duggan MC, Campbell AR, Smith E, et al. Generation of monocyte-derived tumor-associated macrophages using tumor-conditioned media provides a novel method to study tumor-associated macrophages in vitro. *J Immunother Cancer* (2019) 7:140. doi: 10.1186/s40425-019-0622-0
48. Kreso A, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell* (2014) 14:275–91. doi: 10.1016/j.stem.2014.02.006
49. Magee JA, Piskounova E, Morrison SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell* (2012) 21:283–96. doi: 10.1016/j.ccr.2012.03.003
50. Sowa T, Menju T, Sonobe M, Nakanishi T, Shikuma K, Imamura N, et al. Association between epithelial-mesenchymal transition and cancer stemness and their effect on the prognosis of lung adenocarcinoma. *Cancer Med* (2015) 4:1853–62. doi: 10.1002/cam4.556
51. Adorno-Cruz V, Kibria G, Liu X, Doherty M, Junk DJ, Guan D, et al. Cancer stem cells: targeting the roots of cancer, seeds of metastasis, and sources of therapy resistance. *Cancer Res* (2015) 75:924–9. doi: 10.1158/0008-5472.CAN-14-3225
52. Lorenzo-Sanz L, Munoz P. Tumor-Infiltrating Immunosuppressive Cells in Cancer-Cell Plasticity, Tumor Progression and Therapy Response. *Cancer Microenviron* (2019) 12:119–32. doi: 10.1007/s12307-019-00232-2

53. Maccalli C, Volonte A, Cimminiello C, Parmiani G. Immunology of cancer stem cells in solid tumours. A review. *Eur J Cancer* (2014) 50:649–55. doi: 10.1016/j.ejca.2013.11.014
54. Abdullah LN, Chow EK. Mechanisms of chemoresistance in cancer stem cells. *Clin Transl Med* (2013) 2:3. doi: 10.1186/2001-1326-2-3
55. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. *Nat Rev Mol Cell Biol* (2019) 20:69–84. doi: 10.1038/s41580-018-0080-4
56. Dongre A, Rashidian M, Reinhardt F, Bagnato A, Keckesova Z, Ploegh HL, et al. Epithelial-to-Mesenchymal Transition Contributes to Immunosuppression in Breast Carcinomas. *Cancer Res* (2017) 77:3982–9. doi: 10.1158/0008-5472.CAN-16-3292
57. Chen L, Gibbons DL, Goswami S, Cortez MA, Ahn YH, Byers LA, et al. Metastasis is regulated via microRNA-200/ZEB1 axis control of tumour cell PD-L1 expression and intratumoral immunosuppression. *Nat Commun* (2014) 5:5241. doi: 10.1038/ncomms6241
58. Lee Y, Shin JH, Longmire M, Wang H, Kohrt HE, Chang HY, et al. CD44+ Cells in Head and Neck Squamous Cell Carcinoma Suppress T-Cell-Mediated Immunity by Selective Constitutive and Inducible Expression of PD-L1. *Clin Cancer Res* (2016) 22:3571–81. doi: 10.1158/1078-0432.CCR-15-2665
59. Castagnoli L, Cancila V, Cordoba-Romero SL, Faraci S, Talarico G, Belmonte B, et al. WNT signaling modulates PD-L1 expression in the stem cell compartment of triple-negative breast cancer. *Oncogene* (2019) 38:4047–60. doi: 10.1038/s41388-019-0700-2
60. Monteiro I, Vigano S, Faouzi M, Treilleux I, Michielin O, Menetrier-Caux C, et al. CD73 expression and clinical significance in human metastatic melanoma. *Oncotarget* (2018) 9:26659–69. doi: 10.18632/oncotarget.25426
61. Buisseret L, Pommey S, Allard B, Garaud S, Bergeron M, Cousineau I, et al. Clinical significance of CD73 in triple-negative breast cancer: multiplex analysis of a phase III clinical trial. *Ann Oncol* (2018) 29:1056–62. doi: 10.1093/annonc/mdx730
62. Ferrari D, Gambari R, Idzko M, Muller T, Albanesi C, Pastore S, et al. Purinergic signaling in scarring. *FASEB J* (2016) 30:3–12. doi: 10.1096/fj.15-274563
63. Karin N. Chemokines and cancer: new immune checkpoints for cancer therapy. *Curr Opin Immunol* (2018) 51:140–5. doi: 10.1016/j.coi.2018.03.004
64. Di TT, Mazzoleni S, Wang E, Sovena G, Clavenna D, Franzin A, et al. Immunobiological characterization of cancer stem cells isolated from glioblastoma patients. *Clin Cancer Res* (2010) 16:800–13. doi: 10.1158/1078-0432.CCR-09-2730
65. Jinushi M, Baghdadi M, Chiba S, Yoshiyama H. Regulation of cancer stem cell activities by tumor-associated macrophages. *Am J Cancer Res* (2012) 2:529–39.
66. Jung MJ, Rho JK, Kim YM, Jung JE, Jin YB, Ko YG, et al. Upregulation of CXCR4 is functionally crucial for maintenance of stemness in drug-resistant non-small cell lung cancer cells. *Oncogene* (2013) 32:209–21. doi: 10.1038/onc.2012.37
67. Santagata S, Napolitano M, D'Alterio C, Desicato S, Maro SD, Marinelli L, et al. Targeting CXCR4 reverts the suppressive activity of T regulatory cells in renal cancer. *Oncotarget* (2017) 8:77110–20. doi: 10.18632/oncotarget.20363

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 21 October 2020
doi: 10.3389/fimmu.2020.02166

Tuning Cancer Fate: Tumor Microenvironment's Role in Cancer Stem Cell Quiescence and Reawakening

Antonella Sistigu[1,2†], *Martina Musella*[1†], *Claudia Galassi*[1], *Ilio Vitale*[3,4] *and Ruggero De Maria*[1,5*]

[1] *Istituto di Patologia Generale, Università Cattolica del Sacro Cuore, Rome, Italy,* [2] *Tumor Immunology and Immunotherapy Unit, IRCCS Regina Elena National Cancer Institute, Rome, Italy,* [3] *IIGM - Italian Institute for Genomic Medicine, c/o IRCSS Candiolo (TO), Candiolo, Italy,* [4] *Candiolo Cancer Institute, FPO - IRCCS, Candiolo, Italy,* [5] *Fondazione Policlinico Universitario "A. Gemelli" - IRCCS, Rome, Italy*

Cancer cell dormancy is a common feature of human tumors and represents a major clinical barrier to the long-term efficacy of anticancer therapies. Dormant cancer cells, either in primary tumors or disseminated in secondary organs, may reawaken and relapse into a more aggressive disease. The mechanisms underpinning dormancy entry and exit strongly resemble those governing cancer cell stemness and include intrinsic and contextual cues. Cellular and molecular components of the tumor microenvironment persistently interact with cancer cells. This dialog is highly dynamic, as it evolves over time and space, strongly cooperates with intrinsic cell nets, and governs cancer cell features (like quiescence and stemness) and fate (survival and outgrowth). Therefore, there is a need for deeper insight into the biology of dormant cancer (stem) cells and the mechanisms regulating the equilibrium quiescence-*versus*-proliferation are vital in our pursuit of new therapeutic opportunities to prevent cancer from recurring. Here, we review and discuss microenvironmental regulations of cancer dormancy and its parallels with cancer stemness, and offer insights into the therapeutic strategies adopted to prevent a lethal recurrence, by either eradicating resident dormant cancer (stem) cells or maintaining them in a dormant state.

Keywords: tumor microenvironment, cancer stem cells (CSC), disseminated cancer cells (DCC), reawakening, dormancy, immunoediting of cancer, immune escape, tumor evolution

OPEN ACCESS

Edited by:
Cristina Maccalli, Sidra Medicine, Qatar

Reviewed by:
Soldano Ferrone, Massachusetts General Hospital and Harvard Medical School, United States
Pier Paolo Claudio, University of Mississippi, United States

***Correspondence:**
Ruggero De Maria
ruggero.demaria@unicatt.it

†These authors share first authorship

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *15 April 2020*
Accepted: *10 August 2020*
Published: *21 October 2020*

Citation:
Sistigu A, Musella M, Galassi C, Vitale I and De Maria R (2020) Tuning Cancer Fate: Tumor Microenvironment's Role in Cancer Stem Cell Quiescence and Reawakening. Front. Immunol. 11:2166. doi: 10.3389/fimmu.2020.02166

INTRODUCTION

Despite the many noteworthy improvements in early diagnosis and treatment of primary tumors in recent years, in many cases, cancer patients develop distant metastases that, almost invariably, portend a poor prognosis. The current view is that metastatic relapse is caused by the reawakening of disseminated cancer cells (DCCs) from a dormant and asymptomatic state, after a time-lag lasting from a few months to several years.

Cancer dormancy is broadly defined as a stalled phase of cancer progression during which single cancer cells or microscopic tumor bulks remain clinically undetectable, yet retain the ability to progress into overt disease (1). Pristine mentions of cancer dormancy date back to the 1950s, when clinicians hypothesized that dormancy could explain cases of relapse observed several years

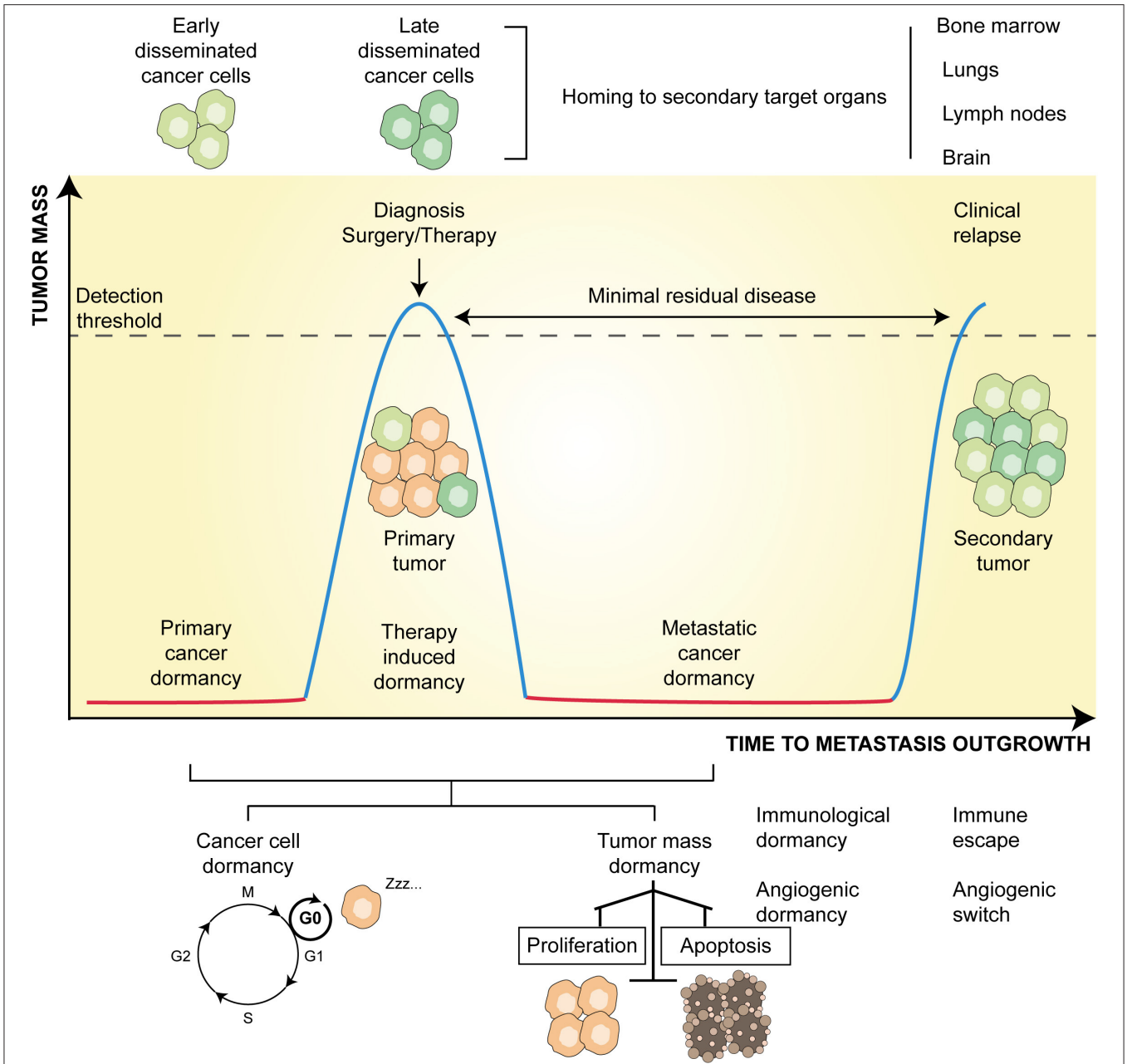


FIGURE 1 | Principles and temporal course of cancer dormancy. Along with primary tumor development, a state of dormancy (red line) allows the survival of microscopic bulk cancer cells. The progressive evolution of the tumor, accumulating genetic and epigenetic changes, and its microenvironment, molding immune, and angiogenic contextures, eventually lead to tumor outgrowth (blue line). At this time, early disseminated cancer cells may develop and home to metastatic sites (mainly bone marrow, lungs, lymph nodes, and brain). Following treatment (either surgery or therapy or both) leading to tumor regression, resistant cells may persist latent (red line) and constitute an undetectable minimal residual disease. At this time, late disseminated cancer cells may develop and join early counterparts at secondary organs. After a time lag, which can last from a few months to many years, these disseminated cells may overgrow and give rise to metastatic clinical relapse (blue line). Dormancy might be due to solitary cells entered into a G_0 phase of cell cycle arrest (cancer cell dormancy) or to the equilibrium between the rate of proliferation and apoptosis (tumor mass dormancy) mainly influenced by angiogenic and immunological cues.

after post-surgical and post-therapy remission (2). Nowadays, it is well-proven that dormancy is an adaptive and protective mechanism that malignant cells adopt to survive stress conditions of the tumor microenvironment (TME) (3). Moreover, cancer dormancy is considered a crucial part of the natural history of cancer evolution, irrespective of whether it occurs during primary tumor development (primary dormancy) or metastatic colonization (metastatic dormancy) (4) (**Figure 1**). In this setting, if the TME is growth permissive, cancer cells proliferate and give rise to overt diseases. If instead, the TME is

not-permissive, cancer cells either are eradicated via the activation of regulated cell death or an irreversible proliferative arrest known as cellular senescence or survive by entering reversible dormancy. Dormant cells could then contribute to disease evolution by increasing their fitness via enforcement of genetic and epigenetic editing (5), and/or by promoting the remodeling of the TME, which then becomes "fertile soil" for outgrowth (3).

Three additional layers of complexity are emerging in the field of cancer dormancy, all of which have therapeutic relevance. First, at the molecular level, both the entry to and exit from dormancy are finely regulated by the cooperative action of cellular and molecular components of the TME (3). Of note, these contextual cues trigger a multitude of dormancy inducing signaling, and almost all overlap with those that induce cancer stemness (6, 7). This is supported by the evidence that cancer stem cells (CSCs)—the subset of cancer cells endowed with self-renewal ability, therapy-resistance, and immune evasion (8–10)—may switch between dormant and proliferative states (6, 7), resulting in an increased metastatic potential (11). Second, at the mechanistic level, tumor dormancy encompasses cellular dormancy (i.e., the condition in which solitary cancer cells temporarily arrest their cell cycle), and tumor mass dormancy, which refers to the condition in which clusters of indolent malignant cells enter a state of balanced proliferation/apoptosis rate (1). This balance, which prevents a tumor from increasing in size, seems to rely on (i) the absence of new vessel sprouting (so-called angiogenic dormancy) (3, 12), and (ii) immunosurveillance (so-called immunologic dormancy) (3, 13, 14). Finally, even though cellular senescence is widely considered as an irreversible and persistent cell cycle arrest (15), instances of reversible senescence and a causal link of the latter to disease recurrence have also been reported (7, 16).

In this study, we first describe the process of metastasization and the experimental models developed to study cancer dormancy and then discuss the role of the TME factors in regulating cancer dormancy and reawakening at metastatic sites. In particular, we focus on the intimate cooperation between different TME signals as we cover the complex relationship between immune-mediated dormancy and dormancy-mediated immune escape. During these discussions, we highlight the striking parallels between cancer dormancy and cancer stemness and summarize the current use of and ensuing therapeutic opportunities to prevent the occurrence of life-threatening metastatic relapse.

METASTASIS: MODELS, EVOLUTION AND DORMANCY

It is estimated that metastatic relapse is responsible for as much as 90% of cancer-related deaths (17, 18). This is ascribed to the fact that progressing metastases rapidly become incurable, spread to additional sites, and compromise the function of vital organs (17). The clinical importance of cancer metastasis has been undeniable since the recognition of cancer as a disease, which has fostered massive experimental efforts to understand its origins and nature (19).

Taking stock of the increasingly large body of research to date, metastasis can be depicted as a sequential, multi-step process collectively conceptualized as the invasion-metastasis cascade (19–22). This sequence of events includes: (i) single cancer cell detachment from the primary tumor and infiltration of the surrounding tissues (invasion); (ii) stimulation of neo vessel sprouting (neoangiogenesis); (iii) entering of cancer cells into blood vessels (intravasation), where these cells acquire the status of circulating tumor cells (CTCs); (iv) survival of CTCs to the hematogenous environment; (v) the leakage of CTCs from the bloodstream (extravasation) followed by their homing to distant organs, where they acquire the status of DCCs; and (*vi*) formation of micro metastatic bulks by DCCs and their adaptation to the new microenvironment (colonization) (23, 24). The metastatic cascade is full of rate-limiting steps, which explains why only a small percentage (0.02%) of DCCs successfully take root and rise into overt metastases (25). Indeed, after homing to a distant site, DCCs face a new microenvironment almost always devoid of growth permissive factors, resulting in DCC demise/senescence or entry into dormancy (1, 23, 26). As anticipated above, the acquisition of a dormant state is a strategy that enables cancer cells to perpetuate the disease while remaining under the radar for a protracted time, until both their fitness and the environmental conditions become permissive for growth (5). In this evolutionary process, the more DCC variants acquire genetic and epigenetic alterations, the higher is their probability of outgrowing in target organs.

Based on genetic comparative analysis studies, different evolutionary models have been proposed to explain the process of metastasization. In the linear progression model, metastases are late, even final events of primary tumor development (27) arising from the progressive accumulation of somatic alterations in cancer cells of the primary tumor (28, 29) that are under the selective pressure of heterotypic signals from the TME (30). Such a unidirectional timeline of events is initiated by the emergence of a cancer cell clone with metastatic capability followed by its dissemination to distinct organs. As a result, primary and metastatic sites are genetically related, although major differences can derive from the development of metastases from rare subclones (27, 31, 32) or the acquisition of specific genetic/epigenetic variation at the primary and/or colonization site. On the contrary, the parallel progression model assumes that DCCs develop early during tumor onset, perhaps even before the formation of overt primary lesions (33–38). This model implies that primary and metastatic tumors evolve independently from each other, resulting in them having a completely different genetic makeup (39, 40). Hence, cancer cells may constantly disseminate during primary tumor progression and evolve, giving rise to different cell variants, outside of the primary lesion. Finally, the tumor self-seeding model postulates a bidirectional exchange of cancer cells between parallel primary and metastatic lesions, denying the hypothesis of independent tumor evolution at primary and colonization sites (41).

Irrespective of the precise metastatic model, DCCs surviving this process are generally incompetent at growing in colonization

sites and enter dormancy. This is clinically relevant, as beyond enhancing cancer cell fitness and aggressiveness, metastatic dormancy also induces resistance to therapy (5). Indeed, as conventional anticancer therapies target rapidly proliferating cancer cells, quiescence appears as the most consistent defense strategy of tumors to resist therapy. In particular, therapy-related dormancy preserves the survival of such cell subpopulations, which are the precursors of tumor relapse constituting the so-called minimal residual disease (MRD) (42) (**Figure 1**).

Cancer dormancy stands out as more than simple quiescence and clinical undetectability, instead, it is a multifaceted and plastic phenomenon with a tremendous impact on therapy outcome and patient survival. This is the reason why dormancy represents a major clinical conundrum and a hot research topic in oncology. We need to gain further insights into the mechanisms governing cancer dormancy and reawakening, as this would open new avenues for preventing or treating metastatic disease. To accomplish this need, a number of experimental preclinical and computational models have been developed.

MODELS OF CANCER DORMANCY: PRINCIPLES AND APPLICATIONS

Over the past two decades, an intensive wave of investigation in the field of tumor dormancy has led to the development of various experimental models that investigate the molecular mechanisms and circuitries regulating dormancy as well as the intricate cross-talk between dormant cancer cells and host immune cells (3, 43). Experimental strategies conceived to study cancer dormancy encompass: (i) *in vitro* and *ex vivo* models; (ii) *in vivo* models; (iii) mathematical and computational models. **Table 1** summarizes these current methods, which are also briefly described here.

In vitro and *ex vivo* Models of Cancer Dormancy

Despite constituting a highly simplified depiction of the TME, *in vitro* models of cancer dormancy provide major advantages including the unique possibility (i) to study, at a single cell resolution, the crosstalk between cancer cells and the other cellular and non-cellular components of the TME; and (ii) to functionally suppress or completely remove specific cell populations that are essential for animal survival and as such, difficult to be studied in *in vivo* models. The regulatory mechanisms identified through *in vitro* models, however, always need validation in more complex and realistic *in vivo* models.

Two-dimensional (2D) and three-dimensional (3D) cell cultures are the standard *in vitro* tools for investigating the mechanisms of cellular dormancy as well as the interactions with selected players of the microenvironment regulating major steps of dormancy such as cell cycle arrest, immunogenicity, differentiation, and therapeutic resistance. In the simplest 2D cell culture setting, cancer cells from either immortalized or primary cell lines are seeded on selected stromal components [e.g., fibronectin 1 (FN1), collagen I, collagen IV, among others] at clonogenic densities to favor cell interaction with the substratum and in the presence of microenvironmental soluble factors [e.g., epidermal growth factor (EGF) and basic fibroblast growth factor]. The effect of such extracellular matrix (ECM) factors on cancer cell dormancy, survival, and metastatic potential can then be evaluated by analyzing (as examples) cell clonogenic potential upon staining with crystal violet or cancer cell morphology, phenotype, cell cycle arrest, proteome and transcriptome employing standard methods of cellular and molecular biology (e.g., by microscopy, flow cytometry, western blot, qRT-PCR, and other techniques) (44, 45). In this setting, the 2D system can be easily perturbed by the addition of blocking antibodies, inhibitors, or peptides, partially mimicking the tumor microenvironmental conditions (44, 45).

TABLE 1 | Models for studying cancer dormancy.

***In vitro and ex vivo* models**	**References**
2D cultures:	
Cancer cells are cultivated on extracellular matrix (ECM) component-coated plates.	(44)
Breast cancer + fibronectin + fibroblast growth factor-2	(45)
3D cultures:	
Dormant cancer cells remain quiescent in 3D bioengineered models.	
Biomaterial based model	(46)
Breast Cancer + Basement Membrane Matrix	(47)
Breast Cancer + Bone Marrow and Lung Niche Cells + laminin-rich ECM	(48)
Breast Cancer + Bone Marrow Niche Cells + Collagen biomatrix	(49) (50)
Breast, Colon and Pancreatic Cancer + Stiff Col-Tgel	(51)
Bladder, Prostate Cancer + Prostate Niche Cells + Amikagel	(52)
Breast and Ovarian Cancer + Collagen gel	(53)
Melanoma + Fibrin gel	(54)
Brain Metastatic Breast Cancer + Hyaluronic Acid Hydrogel	
Microfluidic based models/Organ-on-a-Chip	
Breast Cancer + Hepatic Niche Cells + PEG hydrogel LiverChip and Breast Cancer Lung Cancer-on-a-Chip	(55–58)
Bioreactor based model	
Breast Cancer + Bone Niche Cells	(59, 60)
***In vivo* models**	
Mouse vaccination and tumor challenge	
BCL1 mouse lymphoma model	(61)
DA1-3b of acute myeloid leukemia	(62)
Experimental metastasis assays:	
Cancer cells are injected directly into the circulation (e.g., tail vein, left cardiac ventricle, iliac artery)	(63) (64–66)
Spontaneous metastasis assays:	
Cancer cells are injected orthotopically or subcutaneously.	(67)
Spontaneous tumor models:	(68, 69)
Genetically engineered mouse models of oncogene ablation/induction (e.g., *Myc*, *Kras*)	(70–72)
Transgenic mouse models (e.g., MMTV-PyMT, MMTV-HER2, RET)	(33, 73)
Resection mouse models	(74, 75)
PDX models	(76–78)
Mathematical and Computational models	
Ordinary differential equations	(79–81)
Mechanistic modeling	(82, 83)
Gene regulatory networks	(84, 85)
Systems biology models	(86)

In this context, the recent development of microfluidic devices, bioreactors, and biomaterials, has driven researchers into a 3D cell culture-based multidisciplinary approach to detect, profile and even treat dormant cancer cells, spanning from fundamental biology to high-throughput screening (87–91). Indeed, cells cultured in a 3D model system more closely mimick the *in vivo* conditions and address most of the factors that can impact cancer dormancy, such as cell-to-cell and cell-to-ECM interactions, tissue architecture, proteomic and metabolomics profiles, and oxygen levels (92). 3D cell cultures can be generated by using either natural (Cultrex, laminin-rich ECM, collagen) (46–49) or synthetic biomaterials (collagen-based and fibrin-based hydrogels, amikagels, and hyaluronic acid hydrogels) (50–54). Moreover, organ-on-chip 3D models provide a way to study cancer dormancy at growing steps of complexity from a cell, to tissue till organ levels, and offer the possibility to perform a real-time, high-resolution analysis taking into consideration the inter-tissue interfaces, the fluid flows, and mechanical strengths, which are all features known to affect tumor dormancy (55–59). Similarly, bioreactors allow researchers to monitor and alter the chemical composition of the culture and thus to identify key chemical contributors to cancer dormancy and reawakening under controlled conditions (60).

Although highly informative and relatively simple, *in vitro* models are not devoid of caveats. The most significant hurdles of the *in vitro* systems are: (i) the need, in multicellular cultures, to optimize culturing protocols allowing the growth and survival of different cell types, (ii) the needs of organ-specific stromal cells, which are usually difficult to obtain, (iii) the difficulty of mimicking the dynamic evolution of the TME composition, and (iv) the challenge of replicating the complexity of the TME, and most notably the role of the immune system. Indeed, *in vivo* models represent a logical extension of *in vitro* findings providing a more comprehensive approach and enabling data validation.

In vivo Models of Cancer Dormancy

Five broad approaches are currently employed to investigate cancer dormancy *in vivo*: (i) vaccination assays, (ii) metastasis assays, either in induced or spontaneous settings, (iii) spontaneous tumor models, (iv) resection mouse models, and (v) patient-derived xenograft (PDX) models.

In the vaccination assay, irradiated or otherwise killed malignant cells are inoculated into immunocompetent syngeneic mice. One-to-2 weeks later, the immunized animals are challenged with living cancer cells and monitored for the presence of persistent dormant cancer cells over long term follow up (from a few months to 1 year) (61, 62). As it stands, the gold-standard approach to evaluate the multi-organ dormancy of tagged cancer cells relies on metastasis assays. Metastases can be experimentally induced by injecting cancer cells into the tail vein (63–65) or the iliac artery (66). Otherwise, cancer cells can be injected subcutaneously or orthotopically and spontaneous metastatic potential can be monitored over time (67–69), or into genetically engineered mice that develop metastatic cancers (33, 70–73), or even humanized PDX models (76–78) can be used. All these assays allow *in vivo* live animal imaging and real-time monitoring of metastasis formation and growth, they provide countless insights into the mechanisms of metastatic dormancy and tumor persistence. Of note, as surgery could trigger metastatic relapse in patients with breast cancer (93), are so-called resection mouse models, which offer the possibility to link primary cancer surgery to the appearance of secondary disease at distant anatomical sites (74, 75) potentially helping unveil mechanisms of cancer cell dissemination and reawakening.

These multiplicities of *in vivo* models offer a holistic view of cancer dormancy and represent pre-clinical tools for clinical validation and intervention. However, *in vivo* studies also have some limitations. Indeed, cancer dormancy takes place over a long time frame and asynchronous heterogeneous dormant cancer cell populations are difficult to track. In this sense, the integration and merging of experimental data with mathematical models and computational simulations may provide insights and a better understanding of the regulatory circuits and the biological behaviors underlying dormancy, with invaluable benefits to translational research.

Mathematical and Computational Models of Cancer Dormancy

The last 15 years have witnessed significant advances in mathematical modeling and computational simulations of complex biological processes such as cancer evolution, response to therapy, and even dissemination and dormancy. The use of mathematics in cancer research, known as mathematical oncology, encompasses knowledge-based differential equation models that simulate and predict tumor dynamics and response to therapy. Mathematical oncology offers insights into the complexity and multiscale nature of cancer cell dormancy and dissemination, (i) by integrating experimental and clinical information (79–81), (ii) by mechanistically modeling tumor evolution and progression as a functional consequence of the complex interaction between cancer cells and the surrounding TME (82, 83), and (iii) by predicting and simulating the molecular pathways involved (84, 85). More recently, systems biology, a multidisciplinary approach that integrates cancer research and medicine, genetics and epigenetics, mathematics, physics, and bioinformatics has gained momentum in the study of cancer dormancy and reawakening, as provides a more comprehensive view of the dynamics of these complex processes (86).

The optimization, application, and integration of all these models will help our understanding of the complexity of cancer dormancy and the multiscale nature of cancer progression. Undoubtedly, this is a promising path forward to validate and translate experimental findings in clinical settings and overcome therapeutic resistance in cancer.

CANCER DORMANCY AND CANCER STEMNESS: PARALLELS AND DIFFERENCES BETWEEN CULPRITS OF RELAPSE

CSCs are the subpopulation of stem-like cells within the tumor mass that possess unique stem-like features such as long-term self-renewal capability, multi-lineage differentiation, and high resistance to stress and apoptosis (9, 94). Based on these

properties, CSCs are considered the seeds of tumor initiation, progression, and metastatic relapse and mainly responsible for therapy failure and poor clinical outcomes (9, 94). Historically presumed to be a very small and quiescent subpopulation, it is now clear that CSCs may not always adhere to this model. Indeed, recent evidence shows that CSCs can be relatively abundant (at least in some tumors), able to alternate between dormant and proliferating states, characterized by a high degree of heterogeneity and plasticity over space (i.e., in distinct tumor regions) and time (i.e., at distinct tumor progression stages) (9). Moreover, subsets of CSCs were reported to differentiate into heterogeneous lineages of cancer cells including non-stem cells, and *vice versa* differentiated cells to undergo cell dedifferentiation and even adopt CSC features (95, 96). CSCs reside in niches, which are specialized regions within the TME, preserving CSC survival and metastatic potential and regulating dormancy-reawakening switches (97). However, to date, a univocal definition of CSCs is still missing, and a unified model of genetic and phenotypic biomarkers is very difficult to achieve. In light of this evidence, resting CSC functional markers on the most threatening properties of CSCs may likely be the key.

One such property is the ability of CSCs to enter and exit from dormancy that, in the majority of cancer types, is the *sine qua non* condition for surviving therapy and initiating metastases, which are the two lethal features of CSCs. Based on this striking analogy, some investigations have proposed that CSCs and dormant cells are two sides of the same coin (6, 98). Indeed, ever-increasing data show the parallels at the molecular level, between dormant DCCs and CSCs. To give some examples, the activation of the p38 mitogen-activated protein kinase 1 (MAPK1) can induce dormancy in differentiated cancer cells (99) as well as in CSCs (100). Similarly, the induction of the mammalian target of the rapamycin (mTOR) signaling pathway could preserve both the survival of dormant DCCs (101) and the quiescence of CSCs (102).

Strengthening these findings, more recently, the activation of mTOR was able to enrich the pool of CSCs within DCCs in bone marrow (BM) metastatic niches in prostate cancer models, through a mechanism involving the release of growth arrest specific 6 (GAS6) by osteoblasts (103). Along with this, the Notch and Wingless (Wnt) pathways, which are essential for the maintenance of cancer stemness (104–106), were proven to promote cancer cell reawakening in different solid tumors (107, 108). Notably, these pathways could promote cell cycle progression in a fashion dependent on the protooncogene c-Myc, while their inactivation was associated with CSC senescence and tumor dormancy (109–112). Furthermore, c-Myc could trigger the polycomb repressor complex 1 component (PRC1) Bmi-1 expression, which in turn seems to correlate with breast cancer patient relapse years after treatment (113) and to influence the self-renewal capability of breast CSCs (114). Other examples proving the molecular similarity between dormant DCCs and CSCs include the interleukin 6 (IL-6) cytokine leukemia inhibitory factor (LIF)-LIF receptor (LIFR) axis, which appears to have a role in preserving both dormancy and cancer stemness, at least in the breast cancer setting (115). Autophagy, an evolutionarily conserved process through which cells survive metabolic stress conditions (116), can regulate the survival of dormant cancer cells and CSCs (117–120).

Finally, mechanical cues of the ECM and the epithelial-to-mesenchymal transition (EMT) process, may be functionally important for inducing stem traits in cancer cells and for promoting their metastatic outgrowth (121–123). For example, the Zinc Finger E-Box Binding Homeobox 1 (ZEB1), a key regulator of EMT, was shown to contribute to the cellular response to microenvironmental *stimuli*, such as local inflammation and the tumor promoter transforming growth factor-β (TGF-β), by activating a transcriptional program that pushes DCCs out of dormancy, committing them with stem-like features (124, 125). Similarly, the hypoxia-induced lysyl oxidase like-2 protein (LOXL2) can promote EMT and endow breast cancer cells with the ability to switch from dormant non-CSCs into proliferating metastatic CSCs (123). In this context, analyses in colorectal cancer models have recently revealed that the EMT-related factor ZEB2 coordinates a program of therapy resistance of quiescent cancer cells (126). Of note, these cells, which pre-exist in therapy-naïve tumors, show recognizable stem-like traits and behaviors (126). On the whole, these findings suggest again that the binomial dormant DCCs and CSCs could be interchangeable.

However, not all CSCs are dormant (9); and not all dormant cells are CSCs (127). Dormant cancer cells likely comprise both CSC and non-CSC subpopulations (7). Moreover, CSCs do not necessarily retain dormant-like features owing to their capacity to switch from dormant to proliferative states (128). Based on their tendency to enter dormancy, cancer (stem) cells can be broadly grouped into (i) dormancy-competent CSCs, (ii) dormancy-incompetent CSCs, (iii) cancer repopulating cells, and (iv) DCCs (7, 129). Dormancy-competent CSCs are endowed with the ability to switch between dormancy and reawaking states, a plasticity that fosters their metastatic potential and resistance to therapy (7, 129). Conversely, dormancy-incompetent CSCs are usually enriched in advanced diseases and are characterized by a loss in the ability to enter dormancy, possibly due to the progressive accumulation of somatic mutations in the mechanisms governing dormancy entry (7, 129).

Indeed, as the tumor progresses and the microenvironment evolves, CSCs accumulate epigenetic and genetic alterations despite their robust DNA damage response (130), and dormancy-competent CSCs may turn into dormancy-incompetent CSCs (129). Cancer-repopulating cells are the subset of CSCs able to self-renew post-therapy and thus responsible for relapse and metastatic onset (7, 129). Finally, DCCs, either with stem-like or differentiated features, lie in secondary distant organs and the bloodstream (in this latter case, acting as CTCs) and preserve the ability to reawaken and fuel metastatic outgrowth (7, 129).

As above described, striking parallels exist between dormant DCCs and dormant CSCs. These analogies also apply to the microenvironmental cues, encompassing biological, biochemical, and biophysical factors, that coordinate both DCC outgrowth and CSC self-renewal. Further research in this area could uncover new similarities that ultimately may offer therapeutic solutions for unmet medical needs.

MICROENVIRONMENTAL CUES COOPERATE TO TIP THE BALANCE BETWEEN CANCER DORMANCY AND REAWAKENING

The TME is a complex and dynamic ecosystem made up of a heterogeneous population of cancer cells and resident or infiltrating non-cancer cells [mainly leukocytes, including lymphocytes and tumor-associated macrophages (TAMs), cancer-associated fibroblasts, endothelial cells, and pericytes]. These are surrounded by the ECM and a mixture of secreted molecules encompassing lymphokines, cytokines, growth factors, and metabolites, among others. Cancer cell behavior and fate are profoundly influenced by the constant and evolving interplay with microenvironmental players, which often corrupt cancer cells to survive and eventually give rise to overt disease. The TME thus represents the background where physical and chemical perturbations tip the balance quiescence vs. proliferation. Quiescence and proliferation, in turn, come into sharp focus as by-products of the co-evolution of cancer cells and their microenvironments. Indeed, it is emerging that, in response to mitogenic and stress-signaling pathways, cancer cells trigger a set of complex intracellular molecular programs, thus underscoring a situation in which intrinsic mechanisms perfectly meet the cooperative action of extrinsic factors (3). Such intrinsic molecular pathways are beyond the scope of this review and have been extensively reviewed elsewhere (131–134). In this review, we will only cover the different microenvironmental cues governing dormancy regulation, with particular emphasis on CSCs and metastatic outgrowth.

Cancer Niches: More Than Just Fertile Soils

Niches are specialized areas of the TME that regulate cancer (stem) cell fate and properties by the joint action of cell-cell and cell-ECM crosstalks and the messages delivered by paracrine factors.

Metastatic niches are the fertile environments of secondary organs (i.e., BM, lymph nodes, lungs, liver, and brain) that provide favorable conditions for the seeding of DCCs with stem-like and non-stem-like features (135). Indeed, metastatic niches guarantee the nutrient and oxygen supply required for cell proliferation, thus setting the point for cancer (stem) cell proliferation or quiescence (135).

A body of evidence indicates that the BM frequently hosts DCCs derived from different primary organs, including breast, colon, prostate, head, and neck (136), although these DCCs rarely develop bone metastases (137). This observation suggests that BM metastatic niches could delay or even prevent tumor mass sprouting by inducing a state of dormancy (138), a situation observed in expanded hematopoietic stem cells (HSCs) undergoing differentiation (139). In line with this hypothesis, metastatic niches reportedly provide unique signals promoting quiescence and long-term survival. For example, Notch2, which is known to induce cancer cell proliferation in primary breast carcinomas (108), was recently shown to have an opposite effect in metastatic BM niches, favoring the quiescence and long-term survival of disseminated breast CSCs (140).

The Wnt pathway, which in its canonical form acts as a regulator of processes like cell proliferation and cell stemness (141), is also inversely associated with cancer cell dormancy (107, 142), was reported to induce dormancy of prostate cancer cells populating the BM niches, via a mechanism involving the non-canonical receptor tyrosine kinase-like orphan receptor 2 (ROR2)/Siah E3 Ubiquitin Protein Ligase 2 (SIAH2) signal, resulting in the inhibition of the canonical Wnt/β-catenin pathway (143). In this study, a negative correlation between ROR2 expression and metastasis-free survival in patients with prostate cancer was observed, potentially offering new potential therapeutic opportunities. These data are in line with previous observations of a role for non-canonical Wnt signaling in maintaining HSCs in a quiescent G_0 state (144). At odds with this is the fact that canonical Wnt signaling, out of the BM, is generally inversely associated with cancer cell dormancy in different tumor types (107, 142). On the whole, these observations show opposite effects led by the same factors in different metastatic niches, where they likely face different microenvironmental factors. This further supports the hypothesis that HSC niches may host dormant cancer cells.

Other microenvironmental signals involved in dormancy at the metastatic site include TGF-β, bone morphogenetic proteins (BMPs), and LIFR. Firstly described as a potent inhibitor of HSC proliferation (145, 146), TGF-β is now recognized as another major factor that, once released by osteoblasts (one main BM stromal cell type), keeps DCCs and CSCs in a state of protracted dormancy (147, 148). This effect mainly relies on the triggering of the Gas6 receptor Axl (148) and the downstream activation of the p38 MAPK signaling (147). Similarly, the production of BMPs by BM stromal cells was associated with DCC hibernation. Specifically, the presence of BMP7 induced dormancy of prostate CSCs by activating the MAPK p38, and by fostering the expression of the cell cycle inhibitor p21 and the metastasis suppressor gene N-myc downstream-regulated gene 1 (NDRG1) (100). Accordingly, a variant of BMP7 (BMP7v) reportedly halted the metastatic spreading of colorectal CSCs by inhibiting the EMT program and by forcing cancer cell differentiation (149). In line with these observations, blocking BMP ligands via the TGF-β inhibitor Coco reawakened dormant breast CSCs and favored disease outgrowth in lung niches, which are known permissive soils (150). Notably, in a large cohort of patients, Coco-related metagenes predicted metastatic relapse in the lung, but not in the BM nor the brain, suggesting that Coco could be an organ-specific regulator (150). Finally, in breast cancer patients, low LIFR levels were shown to correlate with poor prognosis and with the appearance of overt metastasis along with the loss of CSC-associated genes (115). This is in line with previous observations which indicate that IL-6 plays a role in reawakening breast CSCs from therapy-induced dormancy (151).

Beyond reacting to soluble factors, DCCs also engage with other cell types of the metastatic niche, as well as with the ECM. Experimental studies show that breast cancer cells prime mesenchymal stem cells (MSCs) residing in BM niches to transfer microRNAs (miRNAs) via exosomes, which in turn

promote cancer cell quiescence and drug resistance (152, 153). Apparently at odds with these observations, using a 3D co-culture model, Bartosh et al. demonstrated that DCCs from breast tumors cannibalize surrounding MSCs, resulting in an increased survival and tumor mass dormancy (154). Osteoblasts and osteoclasts, which are BM stromal cells with opposite physiological functions (155), also play opposite roles in the regulation of DCC dormancy. This was shown in myeloma DCCs, which entered dormancy while engaging with osteoblasts in the endosteum, while they started proliferating (i.e., reawakened) upon interaction with osteoclasts (77). Accordingly, as reported above, in a prostate cancer model, osteoblasts induce mTOR signaling by releasing GAS6, and this preserves CSC dormancy (103). Moreover, in breast cancer models, osteoclasts were recruited in the proximity of DCCs, supporting DCC growth into overt metastases (156). At the molecular level, cancer cell reawakening appears dependent on soluble receptor activator of nuclear factor- kappa B Ligand (sRANKL) signaling (77).

Accordingly, in breast cancer models, osteoclasts were found to be recruited in the proximity of DCCs and to support their growth into overt metastases (156). Along with this, a recent study in lung metastatic niches demonstrated that sustained inflammation and the interaction of DCCs with immune cells promote the formation of neutrophil extracellular traps (NETs, networks of neutrophil-derived extracellular fibers) in turn driving the switch from dormancy to reawakening (157). This effect was associated with the activity of neutrophil elastase and matrix metalloproteinase 9 (MMP9), two NET-associated proteases which sequentially remodel the ECM and activate the integrin α3β1 on cancer cells, eliciting downstream mitogenic signaling culminating in cellular dormancy.

The ***perivascular niche***, a tumor promoting milieu made up of a multitude of microvessels, regulates dormancy of cancer cells disseminated into BM, the lungs, and brain from various primary tumors (48, 158–160). Perivascular niches are characterized by the high availability of oxygen, nutrients, and paracrine factors, which renders them a permissive environment for the proliferation of DCCs and CSCs (161, 162). Accordingly, distinct types of CSCs and DCCs localize in the perivascular niches, growing in the proximity of capillaries (97, 163). It recently emerged that bidirectional interactions between these cells and components of the perivascular niche, including endothelial cells, are relevant for tumor evolution. The pool of glioblastoma CSCs residing at perivascular niches were shown to engage integrin α7-laminin interactions that foster invasiveness as well as self-renewal and growth potential (164), all features correlating with a dismal prognosis (165). Moreover, breast cancer cells that infiltrate lung metastatic niches induced the expression of the matricellular protein periostin (POSTN) in endothelial cells. In turn, POSTN contributed to CSC survival, nurturing micro to full macrometastases via a mechanism dependent on the activation of the Wnt signaling (166) and the activity of TGF-β1 (48). Other ECM components of the perivascular niche that influence metastasis include osteopontin and tenascin C (167–169). Emerging evidence indicates that these proteins act as primary regulators of CSC survival, self-renewal, and reawakening via the activation of transcriptional programs centered on Wnt, Nanog, and POU domain, class 5, transcription factor 1 (POU5F1, best known as Oct-4) (167–169).

Of note, is the fact that there is a certain degree of heterogeneity in endothelial cells of the perivascular niches. Thus, while endothelial cells of the sprouting neovasculature were shown to foster metastatic outgrowth, those of stable microvasculature mostly preserved and promoted cancer cell dormancy through the tumor suppressor thrombospondin-1, acting as a rate-limiting step for disease re-occurrence (48). Moreover, dormant and proliferating breast cancer cells displayed a distinct localization in perivascular areas (160). More precisely, dormant cells were shown to reside predominantly close to perisinusoidal venules expressing high levels of the inflammatory vascular cell adhesion molecule E-selectin, which favors the entry of cancer cells into the BM, and of the stromal cell-derived factor 1 (SDF-1), which anchors cells to the niche through its interaction with the C-X-C chemokine receptor type 4 (CXCR4), respectively (160).

The ECM: A Biochemical and Biophysical Niche for Cancer Cells

The ECM, commonly defined as the non-cellular component of a tissue, is a highly dynamic and physiologically active structure, that provides biochemical and biophysical support for surrounding cellular components (170). Characterized by a continuous remodeling over space and time, the ECM also represents a biological barrier, an anchorage site, and a movement track, playing major roles in regulating cellular interactions and communications (170). The ECM is tightly organized during embryogenesis and tissue homeostasis, but becomes extremely deregulated and deranged in cancer (171).

Emerging evidence suggests that the ECM may serve as a niche for DCCs and CSCs, influencing cell survival and proliferation, and thus dormancy (171, 172). Thus, downregulation of the urokinase plasminogen activator receptor (uPAR), which is involved in cell/ECM interactions, affected the capability of head and neck squamous cell carcinoma cells to interact with integrins, in turn causing deactivation of mitogenic pathways and induction of dormancy (173). Along with this, tissue stiffness (a mechanical property of the TME) and its underlying mechanotransduction pathways are also involved in tumor progression and metastasis (122, 174). Thus, in breast cancer models, the crosslink between fibrosis-associated deposition of type I collagen and integrin β1 or lysyl oxidase (LOX), was described to create a growth-permissive microenvironment capable of reawakening DCCs, thus supporting proliferative metastatic growth (46, 175). This occurred through the activation, downstream of integrin β1, of players including focal adhesion kinase (FAK), non-receptor tyrosine kinase (Src), ERK, and myosin light chain kinase (MLCK) (46). In this context, there is interesting evidence that pharmacological co-inhibition of Src and MEK1/2 prevented disease recurrence by killing dormant breast and ovarian DCCs (176, 177). Similarly, interstitial collagen I was described to favor the interaction between the tetraspanin Transmembrane 4 L Six Family Member 1 (TM4SF1) and the collagen receptor tyrosine kinase Discoidin domain

receptor family, member 1 (DDR1). This led to the expression of the stem related factors SRY (sex determining region Y)-box 2 (SOX2) and NANOG, driving multiorgan metastatic reactivation in the lung, bone, and brain (178).

The dormant-to-proliferative metastatic switch is also favored by a global reconfiguration of the cytoskeletal architecture of DCCs often mediated by the integrin β1 signaling. Thus, using a model of lung disseminated breast cancer cells, Green et al., demonstrated that, cancer cells respond to integrin β1-mediated fibronectin production and signaling by activating MLCK, resulting in the generation of actin stress fibers and entry into a proliferative state (179). The ability of integrin β1 signaling to promote cell-cycle progression seems also to rely on FAK activation (180). In particular, Weinberg's team showed that soon after extravasation into the lungs, breast cancer cells arrest their proliferation due to their inability to engage stable adhesions with ECM components. Later on, some cancer cells acquiring an elongated morphology developed abundant cell-matrix adhesion plaques, which in turn triggered the integrin β1-FAK signaling and promoted exit from dormancy (180). Src family kinases (SFKs) also act downstream the integrin-triggered dormant-to-proliferative switch (176). Moreover, using an *in vitro* model of stiff-soft tunable matrix it was revealed that fibrosis related integrin β1 and FAK signaling increased mitogenic *stimuli* by inducing protein kinase B (PKB/Akt) and signal transducer and activator of transcription 3 (STAT3). In this setting, only cells grown in soft matrix supports expressed CSC markers (181), suggesting that a pliable microenvironment might support cancer cell stemness, a hypothesis that is intriguing but which still requires *in vivo* validation. Finally, the association between matrix stiffness and cancer cell proliferation appears to be influenced by endothelial cells (182). More precisely, in a stiff environment, endothelial cells express the matricellular protein cysteine-rich angiogenic inducer 61 (CYR61), which in turn induces a β-catenin-dependent upregulation of N-cadherin levels. This lets cancer cells stably interact with the endothelium and thus enter the bloodstream and metastasize (182).

To add further layers of complexity, a recent study demonstrated that a stiff matrix could also induce dormancy (183). In this study, cancer-repopulating cells, when coping with a harsh environment, activate an epigenetic program that leads to the transcription of ten-eleven translocation 2 (Tet2) hydroxymethylating enzyme. Tet2, in turn, activates the cell cycle suppressors p21–p27 and induces integrin β3 downregulation, respectively, promoting and preserving dormancy (183). Moreover, a recent deep single cell analysis revealed a high phenotypic heterogeneity in dormant cancer cells, encompassing pools of quiescent, senescent, and actively proliferating cells (184). The characterization of cells entering long-term dormancy demonstrated that these cells adhere stably to a stiff matrix through integrin α5β1 and rho-associated kinase (ROCK)–mediated cell tension. Moreover, the capability to exit from dormancy appears strictly connected to the ability to trigger MMP-mediated FN1 degradation (184).

In conclusion, disseminated cancer (stem) cells and their environment engage in an intricate molecular cross-talk, regulating the entry into and the exit from dormancy and thus determining cancer cell fate (**Figure 2**).

ANGIOGENIC SWITCH AND ANGIOGENIC DORMANCY

A hallmark of progressive cancer growth, in both primary and secondary tumors, is the induction of tumor vasculature, a process termed the "angiogenic switch" (185, 186). Indeed, like healthy tissues, tumors need both an appropriate supply of oxygen/nutrients and a way to remove waste products (187). However, unlike physiological angiogenesis, in which new vessel sprouting is a highly regulated and self-limited process, tumor angiogenesis lacks growth controls resulting in continuous and deregulated vessel production (185). This leads to a structurally and functionally abnormal tumor vascular network characterized by new vessels with dead ends, which results in low oxygen tension (hypoxia), the paucity of metabolites, and imbalanced expression of angiogenic factors. This latter eventually stimulates further abnormal angiogenesis (185). As neovascular supply is crucial for tumor growth, cancer cells, including those integrated into the vessel walls (188), undergo adaptive dormancy, also known as angiogenic dormancy (186, 189). During angiogenic dormancy, cancer cell proliferation rate is balanced by enhanced apoptosis induction. This equilibrium maintains tumors that are microscopic and undetectable, for extended times (12).

Currently, there are three subtypes of hypoxia and related cancer cell adaptive mechanisms (190). First, acute hypoxia is characterized by transient perturbation in perfusion lasting from a few minutes up to a few days. Reportedly, cancer cells facing acute hypoxia decrease oxidative metabolism and activate autophagy, yet retaining high proliferative potential (191–193). Second, chronic hypoxia is mainly related to the presence of abnormal neo-vessels, leading to limited perfusion and oxygen supply. This long-lasting phenomenon is linked to a state in which cancer cells remain persistently dormant (192). Finally, cycling hypoxia is characterized by oxygen fluctuations in parallel with intermittent phases of cancer cell dormancy and reawakening that have been associated with increased tumor aggression (190, 194).

The balance between the angiogenic switch and angiogenic dormancy is a finely-tuned process regulated by integrated microenvironmental factors, including the pro-angiogenic vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), anti-angiogenic thrombospondin-1, angiostatin, and endostatin (189). Prosaposin has been described as another regulator of metastatic growth arrest (195). Once produced by cancer cells, prosaposin acts in a paracrine and endocrine fashion inducing the expression of thrombospondin-1 in stromal cells at primary and distant tumor sites, which blocks neoangiogenesis and delays tumor growth (195). Many niche components also play a role in regulating angiogenesis. Indeed, CSCs, seem able to transdifferentiate and directly contribute to the formation of abnormal vessels, thus supplying for the absence of true angiogenesis (196, 197). Moreover, CSCs often promote a considerable enhancement of VEGF levels, both by a

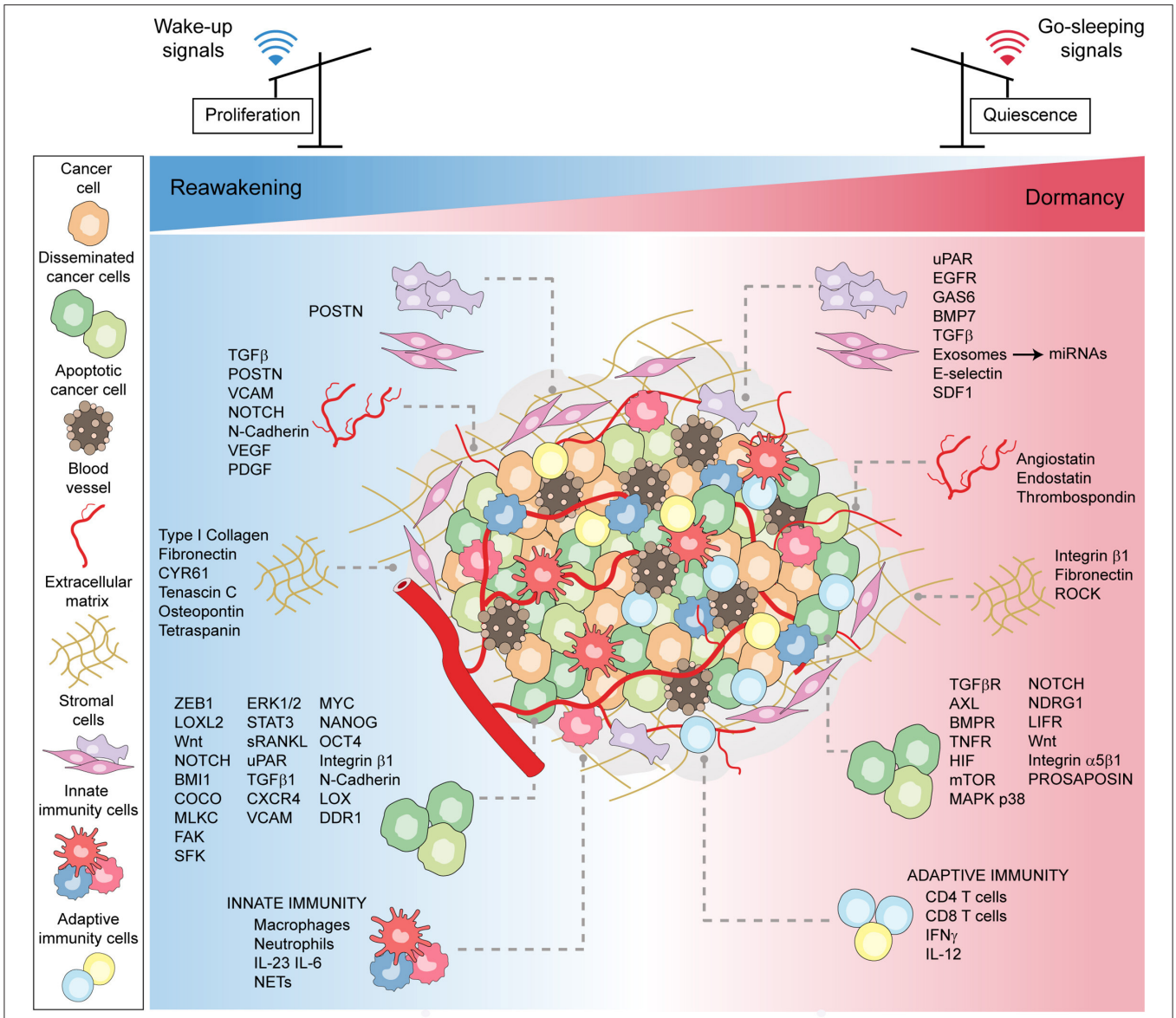


FIGURE 2 | Microenvironmental patterns tuning cancer dormancy, reawakening, and stemness. A schematic model showing the plethora of microenvironmental cues, encompassing the cellular, molecular, and physical factors, that converge to induce either stress-related or mitogenic signals to cancer cells. The bulk of cancer cells, encompassing disseminated and stem cells, in the face of contextual signals, either enter or exit dormancy.

direct production and by stimulating a pro-angiogenic activity in stromal cells localized in the proximity of the niche (198–200). Along with this, some stem-related factors such as Notch also act as angiogenesis promoters (201, 202), while anti-angiogenic factors (i.e., thrombospondin-1) are associated with inactivation of the stem-related transcriptional factors (i.e., MYC) (203), which in turn promote dormancy (109). Of note is the fact that CSCs adopt further adaptive mechanisms to cope with hypoxia, among which the expression of the hypoxia-inducible factors (HIFs) and HIF-regulated genes (204) that induce cellular dormancy by activating p21 signaling (205). In a seminal work, Almog et al. characterized a transcriptional rewiring of cancer cells undergoing an angiogenic switch (206). This switch was associated with downregulation of the angiogenesis inhibitor thrombospondin and upregulation of genes not hitherto linked to tumor dormancy, such as endothelial cell-specific molecule 1 (ESM1), 5'-ectonucleotidase, tissue inhibitor of metalloproteinase 3 (TIMP3), epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGF1R), phosphatidylinositol 3-kinase (PI3K) signaling, Eph receptor A5 (EphA5), and histone H2BK (206).

In summary, these myriad microenvironmental components and their reciprocal interactions, represent the major culprits governing cancer (stem) cell dormancy and outgrowth, and

are a clear index of the complexity of this regulation, offering additional potential targets for therapeutic intervention.

CANCER (STEM) CELL DORMANCY AND IMMUNITY: PRODUCTIVE DIALOGS AND RECIPROCAL REGULATIONS

Immune-Induced Dormancy: the Equilibrium Phase of Cancer Immunoediting

Over the past two decades, understanding of tumor biology has increased and revealed that the host immune system plays a dual role in cancer: it may both constrain and paradoxically aid tumor outgrowth. This phenomenon, which has been referred to as cancer immunoediting, passes through three phases, namely elimination, equilibrium, and escape (207).

During the elimination phase or immunosurveillance, cancer cells that escaped intrinsic control are destroyed by extrinsic, immune-mediated tumor suppressor mechanisms (208). The successful completion of this phase ensures cancer cell clearance and prevents the onset of the clinically apparent disease. However, rare cancer subclones may survive and progress into a phase of equilibrium, during which the immune system, by inducing a functional state of dormancy, might contain but not fully extinguish cancer cell growth. Of note, is the strong and relentless pressure exerted by the immune system during this phase, which may either control the outgrowth of occult tumors throughout the life of the host or sculpt less immunogenic variants that ultimately evade immune attack (208). Such immunoedited cancer cells, that are no longer susceptible to immune control, progress in the escape phase, emerging into clinically visible tumors (208). Of the three phases of cancer immunoediting, equilibrium is probably the longest and the most difficult to characterize. Clinical evidence on the existence of an equilibrium or tumor-dormancy phase came from the unintentional transmission of cancer from transplant organ donors to immunosuppressed recipients. In these cases, donors either were in durable clinical remission (209–211) or had no known history of malignancy (212, 213). Notably, cases of the rapid outgrowth of occult metastases were even reported when donors had glioblastoma, which usually does not metastasize (214). Similarly, metastatic recurrence of primary renal cell carcinoma soon after the post-transplant immunosuppressive medication was reported (215). These observations suggest a mechanism of immune-mediated control for occult malignancies and a progressive outgrowth of cancer cells under pharmacologically-induced immunosuppression, a condition required to prevent the recipient's rejection of the organ. The median time frame between transplantation and metastasis detection is relatively short, ranging between 3 and 36 months, with no differences between cancer types and the organ transplanted (211). As metastases generally take 6 to 12 years to emerge (35), it is plausible that under immune suppression, adaptive immunity cannot hold dormant cells in check, which thus exit from the equilibrium/dormant/persistent state (13). In line with these observations, in a variety of human tumors, it was reported that a 20 to 50 year interval from initial carcinogen exposure to the clinical detection of disease. Moreover, epidemiologic studies in autopsies revealed that microscopic foci of disease frequencies considerably exceed clinical incidence rates in various cancer types (e.g., thyroid cancer, prostate and breast carcinoma) (216–218). This gets stronger during the theoretical existence of periods of subclinical dormancy during tumor progression (219). However, none of these reports visualized tumor dormancy *de facto*, and they did not describe the immune effectors involved. Admittedly, clinical cancer dormancy is still poorly characterized and the role of innate and adaptive immunity in initiating and then stabilizing the dormant state is a matter of debate (220). However, we have strong evidence supporting the existence of an equilibrium phase governing clinical cancer dormancy. Indeed, tumors may chronically persist without symptoms for years and even decades before recurring either locally or at distant metastatic sites (4, 25, 221). Moreover, late relapses are relatively frequent in breast and prostate carcinoma patients after radical surgery (222, 223), in melanoma, thyroid and renal cell carcinoma (224, 225), non-Hodgkin's lymphoma (226), and acute myeloid leukemia (227).

In parallel, clinical and experimental studies have provided evidence that cancer cells can disseminate during premalignant stages of the disease, thus entering a protracted period of metastatic dormancy into target organs (35). Early preclinical suggestions of the capability of the immune system to hold cancer cells in a dormant/equilibrium phase were provided by transplant experiments in which immunodeficient mice adoptively transferred with T cells and then challenged with the murine B lymphoma BCL1 cells, were endowed with the capability to induce and maintain a state of tumor latency (228). Similarly, BCL1 dormant cancer cells resident in the spleen of immunized mice showed no evidence of disease 250 days after tumor rechallenge (229). In line with these findings, the adoptive cell transfer of tumor specific lymphocytes provided long-term protection from tumor development, retaining minor foci of dormant cancer cells on mouse models of prostate cancer (230) and lymphomas (231). Additional studies with mouse models of skin malignancies confirmed that the immune system may induce long-term latency of occult primary and metastatic carcinomas (232, 233). These findings are consistent with a role for anti-tumor immunity, and in particular T cells, in the maintenance of an equilibrium dormant state preventing tumor-cell growth. Pivotal studies from Schreiber's lab have further provided evidence and unveiled mechanisms of immune mediated dormancy. It was observed that the treatment of mice with low-dose methylcholanthrene (MCA) was followed by the development of aggressive tumors in only a few animals, with a sizeable percentage of the surviving mice free of disease. Deceptively, however, these mice bear dormant tumors that were held in check by the immune system. Indeed, when animals were treated with antibodies blocking T lymphocytes or neutralizing the cytokines IL-12 or interferon-γ (IFN-γ), tumors were released from immune control and outgrew (13). These findings validate previous observations of dormancy induced by CD8 T cell derived factors (228, 234). Moreover, MCA-induced sarcomas from immunodeficient mice were more immunogenic

than those arising in immunocompetent hosts (235). Follow-up studies showed opposing, complementary roles for ILs, during the equilibrium phase. Specifically, IL-23 seemed to promote the survival and outgrowth of occult cancer cells, while IL-12 seemed to favor dormancy and thus prevent immune escape (236). At odds with previous reports, innate immune signaling is associated with the awakening of dormant cancer cells. Local inflammation in the lungs was shown to ignite the exit of DCCs from latency, and thus the growth into overt metastases through the activation of a previously silent EMT transition program (125). This provided a newfound knowledge of the dual role of the immune system in protecting the host against tumor outgrowth and in sculpting the immunogenic profile of evolving tumors, finally rendering them more fit to survive and progress in an immunocompetent environment (235).

On the whole, these observations suggest that immunity can maintain cancer cells in a transient dormant state, which as a matter of course, end in either tumor elimination or tumor escape. CSCs may play pivotal roles in preserving the cancer dormant state. Indeed, they cope with robust anticancer immune responses by subverting immune effector functions and by drastically reducing their visibility (237). At the same time, however, such an immunopriviledge may foster immune escape and cancer outgrowth (238). It remains to be elucidated whether immune-mediated dormancy is either always a matter of a bulk tumor or may also resemble cellular dormancy. Mining the mechanisms regulating immune-mediated tumor equilibrium will help solve this question, and will open the possibility of uncovering predictive signatures with invaluable prognostic and therapeutic implications.

Dormancy as a Mechanism of Immune Escape: Sleeping in the Name of Survival

Immune escape is central to tumor persistence and relapse. Dormant cancer (stem) cells constitute the most critical, yet heterogeneous fraction of malignant cells able to evade host antitumor immunity (6, 7). Effective mechanisms of escaping immune control are (i) prevention of immune detection, (ii) prevention of immune activation, and (iii) activation of immune suppression (239, 240).

The immunogenicity of a tumor relies on a combination of antigenicity, i.e., the expression and presentation of tumor-associated antigens, and adjuvanticity, i.e., the release of alarmins and damage signaling (241). Cancer cells defective in either antigen presentation or production of adjuvant-like signals (or both) remain relatively invisible to the immune system and escape immune detection. The capability of dormant cancer cells to evade immune surveillance by reducing antigenicity has been reported (242, 243) and more recently confirmed through clinical immunogenomics (244, 245). Downregulation of the major histocompatibility complex class I (MHC-I) was ostensibly observed in quiescent cancer cells and CSCs isolated from different cancer types (238, 246). In a model of liver disseminated pancreatic cancer, dormancy-related loss of MHC-I was attributable to unresolved endoplasmic reticulum stress, and was responsible for hiding and protecting DCCs from T cell-mediated surveillance (247). Interestingly, the observation that, in hair follicles, Lgr5-GFP stem cells survive the adoptive transfer with antiGFP T cells by persisting in a dormant state, and reducing the expression of MHC-I molecules (248), further confirms that loss of antigen presentation is a common mechanism in quiescent cells, which CSCs adopt to escape immune attack. If the tumor does not manage to escape detection, then it can evolve to prevent the activation of a robust anticancer immune response. The immunosuppressive effects of cancer cells are mediated by (i) the secretion of soluble factors, (ii) the expression of inhibitory molecules, and (iii) the turning of infiltrating leukocytes into tolerogenic cells that, in turn, can suppress other tumor-specific immune cells. In a model of acute myeloid leukemia, the expression by cancer cells of the immune checkpoints CD274 (best known as PD-L1) and CD80 (also known as B7.1) prevented T cell activity and preserved cancer dormancy (62). Furthermore, the microenvironment itself can help quiescent cells elude immune control. Indeed, within the perivascular niche, the activity of effector T cells can be inhibited through the release of immune suppressive cytokines (such as IL-6) and the activation of the programmed cell death 1 (PDCD1, best known as PD1)-PD-L1 axis (249–251). In addition, tumor evolution seems to select for cancer cell clones resistant to the death effector mechanisms of the immune system. We recently discussed the genetic inactivation of the oncosuppressor caspase 8 (CASP8) and the death receptor FAS as strategic mechanisms cancer cells may adopt to evade apoptosis-mediated eradication by immune cells, mainly T and natural killer (NK) cells (5). These reports are in line with previous observations of dormant cancer cell-mediated escape from T cell induced apoptosis through deregulation of the suppressor of cytokine signaling 1 (SOCS1) cascade and overexpression of the pro-tumorigenic cytokine IL-3 (252).

Cancer cells defective for MHC-I molecules are optimal targets for NK cells, in which activation is MHC-unrestricted (253, 254). Evidence of evasion from NK mediated immunosurveillance by quiescent disseminated CSCs firstly came from Massague's lab. This team showed that by overexpressing the WNT inhibitor Dickkopf-related protein 1 (DKK1), CSCs enter a self-imposed quiescent state and downregulate the expression of UL16 binding protein (ULBP) ligands for NK cells, thus evading innate immunity and remaining latent in the long-term (255).

Additionally, dormant cancer (stem) cells may enter immune protected niches (also called immune-privileged niches), where they lie quiescent for extended periods (256). The capability of dormant niches to protect (cancer) stem cells from immune control is mainly due to the recruitment of regulatory immune cells, encompassing regulatory T (T_{REG}) cells, myeloid-derived suppressor cells (MDSCs), and immunosuppressive TAMs and neutrophils (TANs) (257, 258). In particular, TAMs are recruited by diverse chemotactic factors—including tumor-derived colony-stimulating factor 1 (CSF1), vascular endothelial growth factor A (VEGFA), semaphorin 3A, CC-chemokine ligand 2 (CCL2), and CXC-chemokine ligand 12 (CXCL12)—and nullify the cytotoxic activity of $CD8^+$ T cells by expressing the immune checkpoints PDL1 and B7-H4 (259, 260).

In addition, TAMs and regulatory dendritic cells can recruit T_{REG} cells and MDSCs, and foster their expansion and immunosuppressive functions (261–263). In brain metastatic loci, reactive astrocytes prevent CD8 T cell activation and recruit TAMs through the signal transducer and activator of transcription 3 (STAT3) activation program (264). Similarly, once expanded and polarized under gamma delta (γδ) T cell control (265), TANs act as pro-tumorigenic players in particular in metastatic niches, conferring highly immunosuppressive properties to the TME through the release of leukotrienes (266).

Finally, some tumors seem to evolve and acquire the capability to corrupt and turn immune effectors against themselves, thereby causing immune cell death through mechanisms that physiologically limit the antitumor immune response (240, 267, 268). These immune escape mechanisms can act in combination and make the tumor a formidable foe for the immune system, ultimately fostering the neoplastic outgrowth. Current integrated and single-cell based approaches that have been adopted to mine the immunome of primary and metastatic tumors seem extremely powerful, and may offer data that will soon implement the list of factors, cells, and mechanisms involved in immune escape.

Clinical Detection of Dormant DCCS and CTCS

The identification and possible targeting of dormant DCCs which persist during MRD is of utmost importance to prevent disease recurrence. However, the clinical detection (and monitoring) of cancer dormancy is a challenge, making it difficult to validate the cancer dormancy model in patients. Indeed, per definition, cancer dormancy is a controlled chronic disease that persists without any symptom or sign until its underlying equilibrium is disturbed and local or systemic relapse occurs. Two major obstacles need to be overcome for the clinical detection of cancer dormancy. First, micrometastatic, dormant DCCs are almost undetectable using conventional high resolution, whole-body imaging tools. Second, the entire process involves a long time frame of disease latency. In the last two decades, a flurry of research efforts have focused on the identification and standardization of highly sensitive and specific assays to identify and characterize occult micrometastatic cancer cells, in particular, DCCs in BM aspirates, and CTCs in peripheral blood.

The Current State of DCC and CTC Detection

Three methods are commonly used to detect and quantify DCCs and CTCs in liquid biopsies: (i) immunocytochemistry (IHC)/immunofluorescence (IF) staining followed by bright field/fluorescence microscopy; (ii) multicolor flow cytometry (MFC); and (iii) real time-polymerase chain reaction (RT-PCR). In this context, IHC and IF are the most widely used approaches as they provide the major advantage of evaluating and characterizing morphological criteria at a single-cell level (269, 270). On the contrary, MFC analyses are largely used to analyze biopsies from advanced stage metastatic cancer patients as they allow the rapid screening of tens of thousands of cells per second coupled with the possibility of isolating pure, viable cell subsets for further experimentation. As examples, isolated cells can be expanded either *in vitro*, by establishing primary cell cultures, or *in vivo*, by using xenograft models, and then used for functional analyses (271). A major drawback of these antibody-based technologies is the possibility of false positives, due to an "illicit" expression of markers in non-malignant cells—which can be the result of inflammation or injury (272), or even of the formation of chimeras by the fusion of cancer cells with immune cells (273)—and false negatives, due to the loss of marker expression (270). Finally, RT-PCR-based transcriptome analyses allow for the simultaneous and high sensitive detection of multiple factors, although the probability of false positive results due to contamination and amplification of transcripts from non-cancer cells is high. Besides, the presence of degrading enzymes could also give rise to false negative results (274). In these experimental settings, as DCCs and CTCs are a few tens dispersed in millions-to-billions of hematopoietic cells per milliliter of BM aspirate or blood, prior enrichment approaches through density gradient centrifugation and/or immunomagnetic bead separation are mandatory (269).

Markers of DCC and CTC Detection, Isolation, and Characterization

As hematopoietic cells circulating in the peripheral blood and residing in the BM are mainly of mesenchymal origin, epithelial cancer cells from different solid tumors can be identified through epithelium-specific antigens such as (i) cytoskeletal-associated cytokeratins (CKs, in particular CK 8, 18, 19, and 20) (275, 276), (ii) surface adhesion molecules, such as the epithelial cell-adhesion molecule (Ep-CAM) (269), and (iii) growth factor receptors, such as the erb-b2 receptor tyrosine kinase 2 (ERBB2, best known as HER2) for breast cancer and the epidermal growth factor receptor (EGFR) for lung cancer. Moreover, to disseminate in distant anatomical sites, cancer cells lose cell-to-cell adhesion molecules and enter the EMT process. Therefore, markers of EMT, such as vimentin, FN1, twist family bHLH transcription factor 1 (TWIST1), snail family transcriptional repressor 1 (SNAI1) and 2 (SNAI2, best known as SLUG) can be used to detect cancer dormancy (277, 278). As described above, DCCs can show stem cell features (7), such as the expression of cell surface adhesion receptor CD44, the cell surface CD24, prominin (best known as CD133), and CD49 antigens, and the functional marker aldehyde dehydrogenase 1 family member A1 (ALDH1) (279). Notably, co-staining with specific markers helps discriminate between quiescent and actively proliferating DCCs and CTCs. The most common dormancy-specific markers are the lack of the nuclear antigen Ki67, and the expression of the nuclear receptor subfamily 2 group F member 1 (NR2F1), the basic helix-loop-helix family member e41 (BHLHE41, also known as DEC2), and the cyclin dependent kinase inhibitor 1 B (CDKN1B, best known as p27) (280). Because dormant cells activate cytoprotective programs (i.e., the UPR) to cope with environmental stresses, including hypoxia and glucose starvation, the expression of UPR proteins, such as the heat shock protein family A

(Hsp70) member (HSPA5, best known as Grp78), can be analyzed (281).

Major Limitations of DCC and CTC Detection and Future Directions

Despite the successful detection and enumeration of DCCs and CTCs, and the unceasing development of automated and high sensitive analytical methodologies (e.g., CellSearch, ImageStream, FAST, Epic, CytoTrack, and EPISPOT platforms) (270, 282, 283), achieving high yield and high purity remains a major challenge. Moreover, the high variability of the results is due to multiple reasons, including the heterogeneity of marker expression, the difficulties to recover intact and live cells, the bias of false positive and false negative data, and the lack of standardized protocols. This has prevented the implementation of DCC and CTC usage into the routine clinical practice (284–288).

Currently, next-generation sequencing (NGS) multi-"omics" technologies are providing large-scale data and more comprehensive characterization of the intricate molecular mechanisms underlying the hallmarks of cancer (289). The in-depth knowledge of disease development, treatment resistance, and recurrence risk facilitated by this will be fundamental in guiding treatment decisions. Very recent advances in single-cell analyses have enabled researchers to characterize intra-tumor heterogeneity (i.e., the heterogeneity among the cancer cells of a single patient, at the spatial or temporal level), identify rare cell subsets, and measure the mutational landscapes of different cancer cell populations, and thus guide diagnosis and treatment. However, mainly due to the prohibitive costs (single-cell), multi-omics analyses have not yet been implemented in the clinical setting, preventing the advancement of precision medicine. As there is a widely recognized need to detect and characterize dormant DCCs and CTCs in more detail, there will undoubtedly be a rapid development of new, standardized, and exploitable technologies in the near future, that will expedite DCC and CTC implementation in clinical settings to prevent relapse and thus improve outcome.

IMPLICATIONS FOR THERAPY

After years of bench studies on cancer dormancy, discoveries of the mechanisms regulating dormancy and reawakening could provide an opportunity for bedside translation. There are essentially two clinical options to target dormant cancer (stem) cells: (i) forcing them out of quiescence, so-called "lock-out" approaches, or (ii) sustaining their perpetual dormancy, so-called "lock-in" strategies (138, 290). Clinical trials were launched to study the safety and efficacy of both strategies (**Table 2**). Nonetheless, these strategies require detailed knowledge of the mechanisms underlying dormancy and tumor evolution, a clear view of which mechanisms are tissue-specific or instead common and thus universally exploitable, and the possibility/ability to stratify patients and distinguish those who could benefit from therapies targeting dormancy and those who could not.

As dormancy represents a mechanism by which cancer cells evade current conventional antiproliferative therapies, ***lock-out*** strategies aim at reawakening and forcing dormant cells into proliferation before treatment. According to this principle, exit from dormancy wakes up cancer cell sensitivity to conventional chemo and radiation therapy as well as some types of target therapy. Inhibitors of Polo-like kinase1 (Plk1), for instance, appear highly effective against proliferating colorectal CSCs (128). Notably, dormant CSCs survive the treatment with Plk1 inhibitors but retain sensitivity once out from quiescence (128). In patients with chronic myeloid leukemia and non-small cell lung cancer, ablation of F-box/WD repeat-containing protein 7 (FBXW7), a ubiquitin ligase that regulates dormancy by degrading cMyc and Notch (291), pushes CSCs out of dormancy and thus significantly enhances the benefit of imatinib and gefitinib, respectively (292, 293). Likewise, human leukemia stem cells efficiently exit the quiescent state and enter an active cell cycle following the administration of granulocyte colony-stimulating factor (GCSF) and IFN-α (294, 295). Proliferating stem cells are then vulnerable to cytarabine- and 5-fluoro-uracil-based chemotherapy (294, 295).

In a more recent study, inhibition of macroautophagy could force quiescent ovarian CSCs out of G_0 and prevent further entry into quiescence (296). The dependence on specific niches (see above) represents a therapeutic opportunity for preventing or reducing metastasis outgrowth. This is exemplified by the targeting of E-selectin- and SDF-1 in the bone perivascular metastatic niche, which disrupts the anchorage of dormant breast cancer cells (160). This forces the mobilization of dormant cells into the bloodstream, where they are more vulnerable to chemotherapy, thus preventing metastatic colonization. Along similar lines, breaking the foothold of dormancy by targeting blood vessels, the ECM, or effector immune cells may prove effective in inhibiting dormant cancer cell survival and eventually relapse (297–299). Indeed, the blockade of the CCL2-C-C Motif Chemokine Receptor 2 (CCR2) axis, involved in breast cancer cell metastatic seeding in the lungs and recruitment of metastasis-associated macrophages (300), has provided therapeutic benefit in fibrosarcoma models (301). Similarly, inhibition of neutrophil infiltration by targeting the Notch1 signaling prevented lung metastatic spread of breast, ovary, and colorectal carcinoma, as well as melanoma (302). Overall, these pieces of evidence may offer new opportunities to specifically target DCCs and strategically eliminate MRD.

Data from clinical trials are emerging, and the results are promising (**Table 2**). As an example, in breast cancer patients docetaxel treatment following adjuvant fluorouracil, epirubicin, and cyclophosphamide (FEC) therapy successfully erased dormant DCCs (as detected in BM aspirates) while increasing the rates of metastasis-free survival [(303) NCT00248703]. Moreover, multiple on-going trials are exploiting immunotherapeutic protocols to target dormant cells. To reach a successful outcome, a few parameters have to be properly addressed. First, as dormant cancer (stem) cells develop early during tumor progression, their antigenic

TABLE 2 | Clinical trials targeting the dormancy window in cancer patients.

Description	ClinicalTrials.gov identifier	Drug(s)	Number of patients	Recruitment status	Phase	Results
Pilot study to evaluate the impact of Denosumab on DTCs in patients with early stage breast cancer	NCT01545648	Denosumab	4	Terminated (low accrual)	2	N/A
Pilot study of mobilization and treatment of DTCs in men with metastatic prostate cancer	NCT02478125	Burixafor hydrobromide, G-CSF, Docetaxel, or in combination	3	Terminated (low accrual)	1	N/A
Effect of Trastuzumab on DFS in early stage HER2-negative breast cancer patients with ERBB2 expressing DTCs	NCT01779050	Trastuzumab	7	Active, not recruiting	2	All patients experienced eradication of HER2/neu-positive ITCs from bone marrow; reduction in the number of ITC-positive patients
Zoledronic acid in the treatment of breast cancer with minimal residual disease in the bone marrow (MRD-1)	NCT00172068	Zoledronic acid in combination with calcium/vitamin D	96	Terminated	2	All patients treated became DTC negative; untreated patients 12 months after diagnosis had significantly shorter OS
Secondary adjuvant treatment for patients with ITCs in bone marrow	NCT00248703	Docetaxel	1,028	Active, not recruiting	2	79% of patients became DTC negative; enhanced metastasis-free survival in patients with DTC elimination
Gedatolisib, Hydroxychloroquine or the combination for prevention of recurrent breast cancer (GLACIER)	NCT03400254	Hydroxychloroquine, Gedatolisib, or combination	0	Withdrawn	3	N/A
Phase II pilot trial of Hydroxychloroquine, EVErolimus or the combination for prevention of recurrent breast cancer (CLEVER)	NCT03032406	Hydroxycholorquine, everolimus, or combination	60	Recruiting	2	N/A
Prolonged Tamoxifen compared with shorter Tamoxifen in treating patients who have breast cancer	NCT00003016	Tamoxifen citrate	20,000	Terminated	N/A	N/A
Pilot study of 5-Azacitidine and All-trans retinoic acid for prostate cancer with PSA-only recurrence after local treatment	NCT03572387	Combination of 5-Azacitidine and All-trans retinoic acid, or no treatment	20	Recruiting	2	N/A
Phase II study comparing chemotherapy in combination with OGX-427 or placebo in patients with bladder cancer	NCT01454089	Gemcitabine and Cisplatin in combination with OGX-427	183	Completed	2	N/A
OGX-427 in castration resistant prostate cancer patients	NCT01120470	OGX-427 and prednisone in combination	74	Completed	2	N/A
Safety and efficacy of ABT-510 in subjects with advanced renal cell carcinoma	NCT00073125	ABT-510/Thrombospondin-1 mimetic	103	Completed	2	N/A
PROvenge treatment and early cancer treatment	NCT00779402	Sipuleucel-T	176	Completed	3	N/A
Sunitinib malate or Sorafenib tosylate in treating patients with kidney cancer that was removed by surgery	NCT00326898	Sunitinib malate or sorafenib tosylate	1,943	Completed	3	None of patients treated showed survival benefit relative to placebo

DC, dendritic cell; DFS, disease free survival; DTC, disseminating tumor cell; ITC, isolating tumor cell; N/A, not applicable; OS, overall survival.

cargo is relatively poor. This, coupled with a reduced capability to present antigen on MHC-I, renders dormant cells poorly immunogenic. Alternative strategies based on chimeric antigen receptor (CAR) T (304) and NK cells (305) can be developed to overcome these limitations. Second, the high intra- and inter-patient heterogeneity of most tumors represents an additional challenge, that could be only addressed with cost-prohibitive personalized protocols. However, preclinical studies have shown

the possibility of rapidly and easily reprogram circulating T cells *in situ* (306). Third, not all patients presenting DCCs in BM aspirates *de facto* develop metastases (137), and so it is of utmost importance to identify the additional parameters that characterize high-risk patients, thus avoiding the over-treatment of low-risk patients.

Lock-in strategies aim at artificially keeping cancer cells in a dormant state, thus preventing their outgrowth (138). To date, the Adjuvant Tamoxifen: Longer Against Shorter (ATLAS NCT00003016) is the most significant trial that has used a strategy specifically based on forcing dormancy maintenance (**Table 2**). In this clinical trial, ER positive breast cancer patients showed a significant reduction of disease recurrence and metastasis outgrowth, when the standard 5 year adjuvant tamoxifen administration was extended to 10 years (307, 308).

A plethora of signaling pathways previously identified as regulators of cancer cell quiescence in preclinical studies can be exploited as potential therapeutic targets. Specifically, two strategies can be conceived. The first strategy is based on the activation of dormancy-maintaining factors. Thus, the activation of the stress-activated protein kinase p38 was shown to preserve a state of protracted dormancy in different cancer types (99, 309). Similarly, in breast cancer models, induction of the morphoregulatory gene Homeobox (Hox)D10 reverted tumorigenic cells into a growth-arrested phenotype (310, 311). The same effects are ascribed to the multiple microenvironmental factors described above, which drive quiescence by triggering low-mitogenic and high-stress signaling. For instance, metastasis-incompetent primary tumors promoted the conversion of recruited myeloid cells from pro- to anti-metastatic by forcing them to produce the antitumorigenic factor thrombospondin-1 (312). Moreover, stromal BMP7 triggered dormancy of prostate CSCs by activating p38, inducing the cell cycle inhibitor p21, and the metastasis suppressor NDRG1 (100), which is in line with the evidence that the inhibition of BMP4 reawakened dormant breast CSCs and favored lung colonization (150). Finally, the TGF-β2 signaling was also involved in the maintenance and/or induction of a quiescent state for BM DCCs in a head and neck squamous cell carcinoma model (147).

A second strategy is based on the chronic silencing of reawakening pathways. Specifically, a blockade of uPAR affected the FN1-dependent mitogenic signaling, resulting in a lack of ERK1/2 activity and induction of dormancy in head and neck squamous cell carcinoma cells (173, 309). Similarly, suppression of MAPK/ERK axis and SFK signaling, favored quiescence in breast cancer models (176, 313). Also, the inhibition of the lysophosphatidic acid receptor 1 (LPAR1) induced dormancy of breast metastatic lesions by activating p38 signaling (74). The DNA methylation inhibitor 5-azacytidine interrupted the $G_0 \rightarrow G_1$ switch in leukemia and breast cancer cells (314). In a subsequent study, the same authors showed that a combination of 5-azacytidine with bortezomib induces long-term dormancy multiple myeloma cells (315).

The advent of omics-based approaches disclosed single cell snapshots of molecular signatures associated with cancer dormancy (106, 316–318), some of which represent every promising target. Although theoretically highly attractive and clinically highly beneficial, the idea of keeping cancer (stem) cells asleep, may be difficult to translate into clinical settings. Some patients with a good prognosis and no more evidence of disease may be reluctant to continue therapy indefinitely. Moreover, long-term follow-ups and accumulating costs are additional challenges that need to be carefully considered. Interestingly, screening of the Prestwick Library, made up of Food and Drug Administration (FDA) approved drugs, led to the identification of the stimulant laxative drug bisacodyl as the sole agent specifically inhibiting quiescent, but not proliferating, glioblastoma stem-like cells (319). This opens the avenue to a third therapeutic strategy: the targeting of cancer (stem) cells while they are dormant. Intense basic and clinical research is developing, for example, target therapy with ABT-737, an inhibitor of anti-apoptotic BCL2 family members exerted a robust and preferential cytotoxic activity on quiescent lung CSCs (320). These findings opened the possibility to combine conventional chemotherapy with ABT-737 to kill otherwise resistant dormant CSCs, and thus prevent their relapse after reawakening (320).

We urge that more studies further explore dormancy regulation. These future studies will offer new possibilities for marker detection and metastatic prediction, opening a therapeutic window for prevention trials.

CONCLUDING REMARKS

This is an exciting moment for cancer research, with data bringing into sharp focus the complex factors and mechanisms that render the TME either metastasis-permissive or metastasis-suppressive, but we still have a long way to go. The ability to anticipate whether, when, and how dormant DCCs are reactivated could help make cancer curative intent a reality. The striking analogies between dormant DCCs and dormant CSCs, along with their co-evolution with the surrounding microenvironment, may provide the ground for developing therapies that consider dormancy as a whole process. This opportunity to rethink therapeutic strategies could be the way to eradicate and/or prevent lethal metastatic recurrence and would surely benefit from the possibility of monitoring dormancy over time through rigorous, non-invasive, and preferably low-cost approaches.

AUTHOR CONTRIBUTIONS

AS and RDM conceived the paper. AS wrote the first version of the manuscript and designed the figures with the help of MM, under the supervision of RDM. AS, MM, and CG prepared the tables, under the supervision of RDM. IV provided critical input to the preparation of the paper. All authors approved the final version of the article.

FUNDING

AS was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC, Start-Up 2016 #18418) and Ministero Italiano della Salute (RF_GR-2013-02357273). IV was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC, IG #20417) and a startup grant from the Italian Institute for Genomic Medicine (Candiolo, Turin, Italy) and Compagnia di San Paolo (Torino, Italy). RDM was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC, 5x1000 #9979).

REFERENCES

1. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer*. (2007) 7:834–46. doi: 10.1038/nrc2256
2. Hadfield G. The dormant cancer cell. *Br Med J*. (1954) 2:607–10. doi: 10.1136/bmj.2.4888.607
3. Linde N, Fluegen G, Aguirre-Ghiso JA. The relationship between dormant cancer cells and their microenvironment. *Adv Cancer Res*. (2016) 132:45–71. doi: 10.1016/bs.acr.2016.07.002
4. Manjili MH. Tumor dormancy and relapse: from a natural byproduct of evolution to a disease state. *Cancer Res*. (2017) 77:2564–9. doi: 10.1158/0008-5472.CAN-17-0068
5. Vitale I, Sistigu A, Manic G, Rudqvist NP, Trajanoski Z, Galluzzi L. Mutational and antigenic landscape in tumor progression and cancer immunotherapy. *Trends Cell Biol*. (2019) 29:396–416. doi: 10.1016/j.tcb.2019.01.003
6. Kleffel S, Schatton T. Tumor dormancy and cancer stem cells: two sides of the same coin? *Adv Exp Med Biol*. (2013) 734:145–79. doi: 10.1007/978-1-4614-1445-2_8
7. Talukdar S, Bhoopathi P, Emdad L, Das S, Sarkar D, Fisher PB. Dormancy and cancer stem cells: an enigma for cancer therapeutic targeting. *Adv Cancer Res*. (2019) 141:43–84. doi: 10.1016/bs.acr.2018.12.002
8. Maccalli C, De Maria R. Cancer stem cells: perspectives for therapeutic targeting. *Cancer Immunol Immunother*. (2015) 64:91–7. doi: 10.1007/s00262-014-1592-1
9. Batlle E, Clevers H. Cancer stem cells revisited. *Nat Med*. (2017) 23:1124–34. doi: 10.1038/nm.4409
10. Fiori ME, Villanova L, De Maria R. Cancer stem cells: at the forefront of personalized medicine and immunotherapy. *Curr Opin Pharmacol*. (2017) 35:1–11. doi: 10.1016/j.coph.2017.04.006
11. Turdo A, Veschi V, Gaggianesi M, Chinnici A, Bianca P, Todaro M, et al. Meeting the challenge of targeting cancer stem cells. *Front Cell Dev Biol*. (2019) 7:16. doi: 10.3389/fcell.2019.00016
12. Holmgren L, O'reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med*. (1995) 1:149–53. doi: 10.1038/nm0295-149
13. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature*. (2007) 450:903–7. doi: 10.1038/nature06309
14. Goddard ET, Bozic I, Riddell SR, Ghajar CM. Dormant tumour cells, their niches and the influence of immunity. *Nat Cell Biol*. (2018) 20:1240–9. doi: 10.1038/s41556-018-0214-0
15. Galluzzi L, Vitale I. Oncogene-induced senescence and tumour control in complex biological systems. *Cell Death Differ*. (2018) 25:1005–6. doi: 10.1038/s41418-018-0102-y
16. Chakradeo S, Elmore LW, Gewirtz DA. Is senescence reversible? *Curr Drug Targets*. (2016) 17:460–6. doi: 10.2174/1389450116666150825113500
17. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. *Cell*. (2017) 168:670–91. doi: 10.1016/j.cell.2016.11.037
18. Dillekas H, Rogers MS, Straume O. Are 90% of deaths from cancer caused by metastases? *Cancer Med*. (2019) 8:5574–6. doi: 10.1002/cam4.2474
19. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell*. (2011) 147:275–92. doi: 10.1016/j.cell.2011.09.024
20. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer*. (2003) 3:453–8. doi: 10.1038/nrc1098
21. Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell*. (2006) 127:679–95. doi: 10.1016/j.cell.2006.11.001
22. Welch DR, Hurst DR. Defining the hallmarks of metastasis. *Cancer Res*. (2019) 79:3011–27. doi: 10.1158/0008-5472.CAN-19-0458
23. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science*. (2011) 331:1559–64. doi: 10.1126/science.1203543
24. Massague J, Obenauf AC. Metastatic colonization by circulating tumour cells. *Nature*. (2016) 529:298–306. doi: 10.1038/nature17038
25. Giancotti FG. Mechanisms governing metastatic dormancy and reactivation. *Cell*. (2013) 155:750–64. doi: 10.1016/j.cell.2013.10.029
26. Luzzi KJ, Macdonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *Am J Pathol*. (1998) 153:865–73. doi: 10.1016/S0002-9440(10)65628-3
27. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature*. (2010) 467:1114–7. doi: 10.1038/nature09515
28. Bernards R, Weinberg RA. A progression puzzle. *Nature*. (2002) 418:823. doi: 10.1038/418823a
29. Ince TA, Richardson AL, Bell GW, Saitoh M, Godar S, Karnoub AE, et al. Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. *Cancer Cell*. (2007) 12:160–70. doi: 10.1016/j.ccr.2007.06.013
30. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature*. (2004) 432:332–7. doi: 10.1038/nature03096
31. Ding L, Ellis MJ, Li S, Larson DE, Chen K, Wallis JW, et al. Genome remodelling in a basal-like breast cancer metastasis and xenograft. *Nature*. (2010) 464:999–1005. doi: 10.1038/nature08989
32. Wu X, Northcott PA, Dubuc A, Dupuy AJ, Shih DJ, Witt H, et al. Clonal selection drives genetic divergence of metastatic medulloblastoma. *Nature*. (2012) 482:529–33. doi: 10.1038/nature10825
33. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, et al. Systemic spread is an early step in breast cancer. *Cancer Cell*. (2008) 13:58–68. doi: 10.1016/j.ccr.2007.12.003
34. Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambardzumyan D, Varmus H. Seeding and propagation of untransformed mouse mammary cells in the lung. *Science*. (2008) 321:1841–4. doi: 10.1126/science.1161621
35. Klein CA. Parallel progression of primary tumours and metastases. *Nat Rev Cancer*. (2009) 9:302–12. doi: 10.1038/nrc2627
36. Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, Mcallister F, et al. EMT and dissemination precede pancreatic tumor formation. *Cell*. (2012) 148:349–61. doi: 10.1016/j.cell.2011.11.025
37. Harper KL, Sosa MS, Entenberg D, Hosseini H, Cheung JF, Nobre R, et al. Mechanism of early dissemination and metastasis in $Her2^{+}$ mammary cancer. *Nature*. (2016) 540:588–92. doi: 10.1038/nature20609
38. Hosseini H, Obradovic MMS, Hoffmann M, Harper KL, Sosa MS, Werner-Klein M, et al. Early dissemination seeds metastasis in breast cancer. *Nature*. (2016) 540:552–8. doi: 10.1038/nature20785
39. Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, et al. The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature*. (2010) 467:1109–13. doi: 10.1038/nature09460
40. Gerlinger M, Rowan AJ, Horswell S, Math M, Larkin J, Endesfelder D, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. (2012) 366:883–92. doi: 10.1056/NEJMoa1113205
41. Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L, et al. Tumor self-seeding by circulating cancer cells. *Cell*. (2009) 139:1315–26. doi: 10.1016/j.cell.2009.11.025
42. Kurppa KJ, Liu Y, To C, Zhang T, Fan M, Vajdi A, et al. Treatment-induced tumor dormancy through YAP-mediated transcriptional reprogramming of the apoptotic pathway. *Cancer Cell*. (2020) *37*:104–22.e112. doi: 10.1016/j.ccell.2019.12.006
43. Almog N. Molecular mechanisms underlying tumor dormancy. *Cancer Lett*. (2010) 294:139–46. doi: 10.1016/j.canlet.2010.03.004

44. Korah R, Boots M, Wieder R. Integrin alpha5beta1 promotes survival of growtharrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. *Cancer Res.* (2004) 64:4514–22. doi: 10.1158/0008-5472.CAN-03-3853
45. Tivari S, Korah R, Lindy M, Wieder R. An *in vitro* dormancy model of estrogensensitive breast cancer in the bone marrow: a tool for molecular mechanism studies and hypothesis generation. *J Vis Exp.* (2015) *e*52672. doi: 10.3791/52672
46. Barkan D, El Touny LH, Michalowski AM, Smith JA, Chu I, Davis AS, et al. Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment. *Cancer Res.* (2010) 70:5706–16. doi: 10.1158/0008-5472.CAN-09-2356
47. Barkan D, Green JE. An *in vitro* system to study tumor dormancy and the switch to metastatic growth. *J Vis Exp.* (2011) 2614. doi: 10.3791/2914
48. Ghajar CM, Peinado H, Mori H, Matei IR, Evason KJ, Brazier H, et al. The perivascular niche regulates breast tumour dormancy. *Nat Cell Biol.* (2013) 15:807–17. doi: 10.1038/ncb2767
49. Marlow R, Honeth G, Lombardi S, Cariati M, Hessey S, Pipili A, et al. A novel model of dormancy for bone metastatic breast cancer cells. *Cancer Res.* (2013) 73:6886–99. doi: 10.1158/0008-5472.CAN-13-0991
50. Fang JY, Tan SJ, Wu YC, Yang Z, Hoang BX, Han B. From competency to dormancy: a 3D model to study cancer cells and drug responsiveness. *J Transl Med.* (2016) 14:38. doi: 10.1186/s12967-016-0798-8
51. Pavan Grandhi TS, Potta T, Nitiyanandan R, Deshpande I, Rege K. Chemomechanically engineered 3D organotypic platforms of bladder cancer dormancy and reactivation. *Biomaterials.* (2017) 142:171–85. doi: 10.1016/j.biomaterials.2017.07.008
52. Lee HR, Leslie F, Azarin SM. A facile *in vitro* platform to study cancer cell dormancy under hypoxic microenvironments using CoCl2. *J Biol Eng.* (2018) 12:12. doi: 10.1186/s13036-018-0106-7
53. Jia Q, Yang F, Huang W, Zhang Y, Bao B, Li K, et al. Low levels of Sox2 are required for melanoma tumor-repopulating cell dormancy. *Theranostics.* (2019) 9:424–35. doi: 10.7150/thno.29698
54. Narkhede AA, Crenshaw JH, Crossman DK, Shevde LA, Rao SS. An *in vitro* hyaluronic acid hydrogel based platform to model dormancy in brain metastatic breast cancer cells. *Acta Biomater.* (2020) 107:65–77. doi: 10.1016/j.actbio.2020.02.039
55. Clark AM, Wheeler SE, Young CL, Stockdale L, Shepard Neiman J, Zhao W, et al. A liver microphysiological system of tumor cell dormancy and inflammatory responsiveness is affected by scaffold properties. *Lab Chip.* (2016) 17:156–68. doi: 10.1039/C6LC01171C
56. Wheeler SE, Clark AM, Taylor DP, Young CL, Pillai VC, Stolz DB, et al. Spontaneous dormancy of metastatic breast cancer cells in an all human liver microphysiologic system. *Br J Cancer.* (2014) 111:2342–50. doi: 10.1038/bjc.2014.533
57. Khazali AS, Clark AM, Wells A. Inflammatory cytokine IL-8/CXCL8 promotes tumour escape from hepatocyte-induced dormancy. *Br J Cancer.* (2018) 118:566–76. doi: 10.1038/bjc.2017.414
58. Clark AM, Kumar MP, Wheeler SE, Young CL, Venkataramanan R, Stolz DB, et al. A model of dormant-emergent metastatic breast cancer progression enabling exploration of biomarker signatures. *Mol Cell Proteomics.* (2018) 17:619–30. doi: 10.1074/mcp.RA117.000370
59. Hassell BA, Goyal G, Lee E, Sontheimer-Phelps A, Levy O, Chen CS, et al. Human organ chip models recapitulate orthotopic lung cancer growth, therapeutic responses, and tumor dormancy *in vitro*. *Cell Rep.* (2017) 21:508–16. doi: 10.1016/j.celrep.2017.09.043
60. Sosnoski DM, Norgard RJ, Grove CD, Foster SJ, Mastro AM. Dormancy and growth of metastatic breast cancer cells in a bone-like microenvironment. *Clin Exp Metastasis.* (2015) 32:335–44. doi: 10.1007/s10585-015-9710-9
61. Vitetta ES, Tucker TF, Racila E, Huang YW, Marches R, Lane N, et al. Tumor dormancy and cell signaling. V. Regrowth of the BCL1 tumor after dormancy is established. *Blood.* (1997) 89:4425–36. doi: 10.1182/blood.V89.12.4425
62. Saudemont A, Quesnel B. In a model of tumor dormancy, long-term persistent leukemic cells have increased B7-H1 and B7.1 expression and resist CTL-mediated lysis. *Blood.* (2004) 104:2124–33. doi: 10.1182/blood-2004-01-0064
63. Naumov GN, Macdonald IC, Weinmeister PM, Kerkvliet N, Nadkarni KV, Wilson SM, et al. Persistence of solitary mammary carcinoma cells in a secondary site: a possible contributor to dormancy. *Cancer Res.* (2002) 62:2162–8.
64. Box GM, Eccles SA. Simple experimental and spontaneous metastasis assays in mice. *Methods Mol Biol.* (2011) 769:311–29. doi: 10.1007/978-1-61779-207-6_21
65. Montagner M, Bhome R, Hooper S, Chakravarty P, Qin X, Sufi J, et al. Crosstalk with lung epithelial cells regulates Sfrp2-mediated latency in breast cancer dissemination. *Nat Cell Biol.* (2020) 22:289–96. doi: 10.1038/s41556-020-0474-3
66. Wang H, Yu C, Gao X, Welte T, Muscarella AM, Tian L, et al. The osteogenic niche promotes early-stage bone colonization of disseminated breast cancer cells. *Cancer Cell.* (2015) 27:193–210. doi: 10.1016/j.ccell.2014.11.017
67. Izraely S, Sagi-Assif O, Klein A, Meshel T, Tsarfaty G, Pasmanik-Chor M, et al. The metastatic microenvironment: brain-residing melanoma metastasis and dormant micrometastasis. *Int J Cancer.* (2012) 131:1071–82. doi: 10.1002/ijc.27324
68. Lawson MA, Mcdonald MM, Kovacic N, Hua Khoo W, Terry RL, Down J, et al. Osteoclasts control reactivation of dormant myeloma cells by remodelling the endosteal niche. *Nat Commun.* (2015) 6:8983. doi: 10.1038/ncomms9983
69. Sakamoto S, Inoue H, Ohba S, Kohda Y, Usami I, Masuda T, et al. New metastatic model of human small-cell lung cancer by orthotopic transplantation in mice. *Cancer Sci.* (2015) 106:367–74. doi: 10.1111/cas.12624
70. Gao D, Nolan DJ, Mellick AS, Bambino K, Mcdonnell K, Mittal V. Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. *Science.* (2008) 319:195–8. doi: 10.1126/science.1150224
71. Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, et al. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell.* (2012) 149:656–70. doi: 10.1016/j.cell.2012.01.058
72. Lin WC, Rajbhandari N, Wagner KU. Cancer cell dormancy in novel mouse models for reversible pancreatic cancer: a lingering challenge in the development of targeted therapies. *Cancer Res.* (2014) 74:2138–43. doi: 10.1158/0008-5472.CAN-13-3437
73. Flores-Guzman F, Utikal J, Umansky V. Dormant tumor cells interact with memory $CD8^+$ T cells in RET transgenic mouse melanoma model. *Cancer Lett.* (2020) 474:74–81. doi: 10.1016/j.canlet.2020.01.016
74. Marshall JC, Collins JW, Nakayama J, Horak CE, Liewehr DJ, Steinberg SM, et al. Effect of inhibition of the lysophosphatidic acid receptor 1 on metastasis and metastatic dormancy in breast cancer. *J Natl Cancer Inst.* (2012) 104:1306–19. doi: 10.1093/jnci/djs319
75. Krall JA, Reinhardt F, Mercury OA, Pattabiraman DR, Brooks MW, Dougan M, et al. The systemic response to surgery triggers the outgrowth of distant immune-controlled tumors in mouse models of dormancy. *Sci Transl Med.* (2018) 10:eaan3464. doi: 10.1126/scitranslmed.aan3464
76. Giuliano M, Herrera S, Christiny P, Shaw C, Creighton CJ, Mitchell T, et al. Circulating and disseminated tumor cells from breast cancer patient-derived xenograft-bearing mice as a novel model to study metastasis. *Breast Cancer Res.* (2015) 17:3. doi: 10.1186/s13058-014-0508-5
77. Lawson DA, Bhakta NR, Kessenbrock K, Prummel KD, Yu Y, Takai K, et al. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. *Nature.* (2015) 526:131–5. doi: 10.1038/nature 15260
78. Williams ES, Rodriguez-Bravo V, Chippada-Venkata U, De Ia Iglesia-Vicente J, Gong Y, Galsky M, et al. Generation of prostate cancer patient derived xenograft models from circulating tumor cells. *J Vis Exp.* (2015) 53182. doi: 10.3791/53182
79. Page K, Uhr JW. Mathematical models of cancer dormancy. *Leuk Lymph.* (2005) 46:313–27. doi: 10.1080/10428190400011625
80. Wilkie KP. A review of mathematical models of cancer-immune interactions in the context of tumor dormancy. *Adv Exp Med Biol.* (2013) 734:201–34. doi: 10.1007/978-1-4614-1445-2_10
81. Wilkie KP, Hahnfeldt P. Mathematical models of immune-induced cancer dormancy and the emergence of immune evasion. *Interface Focus.* (2013) 3:20130010. doi: 10.1098/rsfs.2013.0010
82. Chen JC, Alvarez MJ, Talos F, Dhruv H, Rieckhof GE, Iyer A, et al. Identification of causal genetic drivers of human disease through

systems-level analysis of regulatory networks. *Cell*. (2014) 159:402–14. doi: 10.1016/j.cell.2014.09.021
83. Poleszczuk J, Hahnfeldt P, Enderling H. Evolution and phenotypic selection of cancer stem cells. *PLoS Comput Biol*. (2015) 11:e1004025. doi: 10.1371/journal.pcbi.1004025
84. Ahmad FK, Deris S, Othman NH. The inference of breast cancer metastasis through gene regulatory networks. *J Biomed Inform*. (2012) 45:350–62. doi: 10.1016/j.jbi.2011.11.015
85. Liang Y, Wu H, Lei R, Chong RA, Wei Y, Lu X, et al. Transcriptional network analysis identifies BACH1 as a master regulator of breast cancer bone metastasis. *J Biol Chem*. (2012) 287:33533–44. doi: 10.1074/jbc.M112.392332
86. Suhail Y, Cain MP, Vanaja K, Kurywchak PA, Levchenko A, Kalluri R, et al. Systems biology of cancer metastasis. *Cell Syst*. (2019) 9:109–27. doi: 10.1016/j.cels.2019.07.003
87. Kelly SE, Di Benedetto A, Greco A, Howard CM, Sollars VE, Primerano DA, et al. Rapid selection and proliferation of CD133+ cells from cancer cell lines: chemotherapeutic implications. *PLoS ONE*. (2010) 5:e10035. doi: 10.1371/journal.pone.0010035
88. Mathis SE, Alberico A, Nande R, Neto W, Lawrence L, Mccallister DR, et al. Chemo-predictive assay for targeting cancer stem-like cells in patients affected by brain tumors. *PLoS ONE*. (2014) 9:e105710. doi: 10.1371/journal.pone.0105710
89. Howard CM, Valluri J, Alberico A, Julien T, Mazagri R, Marsh R, et al. Analysis of chemopredictive assay for targeting cancer stem cells in glioblastoma patients. *Transl Oncol*. (2017) 10:241–54. doi: 10.1016/j.tranon.2017.01.008
90. Pradhan S, Sperduto JL, Farino CJ, Slater JH. Engineered *in vitro* models of tumor dormancy and reactivation. *J Biol Eng*. (2018) 12:37. doi: 10.1186/s13036-018-0120-9
91. Ranjan T, Howard CM, Yu A, Xu L, Aziz K, Jho D, et al. Cancer stem cell chemotherapeutics assay for prospective treatment of recurrent glioblastoma and progressive anaplastic glioma: a single-institution case series. *Transl Oncol*. (2020) 13:100755. doi: 10.1016/j.tranon.2020.100755
92. Kapalczynska M, Kolenda T, Przybyla W, Zajaczkowska M, Teresiak A, Filas V, et al. 2D and 3D cell cultures - a comparison of different types of cancer cell cultures. *Arch Med Sci*. (2018) 14:910–9. doi: 10.5114/aoms.2016.63743
93. Demicheli R, Retsky MW, Hrushesky WJ, Baum M. Tumor dormancy and surgery-driven interruption of dormancy in breast cancer: learning from failures. *Nat Clin Pract Oncol*. (2007) 4:699–710. doi: 10.1038/ncponc0999
94. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer*. (2005) 5:275–84. doi: 10.1038/nrc1590
95. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. (2008) 133:704–15. doi: 10.1016/j.cell.2008.03.027
96. Gupta PB, Fillmore CM, Jiang G, Shapira SD, Tao K, Kuperwasser C, et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell*. (2011) 146:633–44. doi: 10.1016/j.cell.2011.07.026
97. Plaks V, Kong N, Werb Z. The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell*. (2015) 16:225–38. doi: 10.1016/j.stem.2015.02.015
98. Oskarsson T, Batlle E, Massague J. Metastatic stem cells: sources, niches, and vital pathways. *Cell Stem Cell*. (2014) 14:306–21. doi: 10.1016/j.stem.2014.02.002
99. Aguirre-Ghiso JA, Estrada Y, Liu D, Ossowski L. ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). *Cancer Res*. (2003) 63:1684–95. doi: 10.1016/j.urolonc.2003.12.012
100. Kobayashi A, Okuda H, Xing F, Pandey PR, Watabe M, Hirota S, et al. Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone. *J Exp Med*. (2011) 208:2641–55. doi: 10.1084/jem.20110840
101. Schewe DM, Aguirre-Ghiso JA. ATF6alpha-Rheb-mTOR signaling promotes survival of dormant tumor cells *in vivo*. *Proc Natl Acad Sci USA*. (2008) 105:10519–24. doi: 10.1073/pnas.0800939105
102. Gaur P, Sceusi EL, Samuel S, Xia L, Fan F, Zhou Y, et al. Identification of cancer stem cells in human gastrointestinal carcinoid and neuroendocrine tumors. *Gastroenterology*. (2011) 141:1728–37. doi: 10.1053/j.gastro.2011.07.037
103. Shiozawa Y, Berry JE, Eber MR, Jung Y, Yumoto K, Cackowski FC, et al. The marrow niche controls the cancer stem cell phenotype of disseminated prostate cancer. *Oncotarget*. (2016) 7:41217–32. doi: 10.18632/oncotarget.9251
104. Pannuti A, Foreman K, Rizzo P, Osipo C, Golde T, Osborne B, et al. Targeting notch to target cancer stem cells. *Clin Cancer Res*. (2010) 16:3141–52. doi: 10.1158/1078-0432.CCR-09-2823
105. Abel EV, Kim EJ, Wu J, Hynes M, Bednar F, Proctor E, et al. The Notch pathway is important in maintaining the cancer stem cell population in pancreatic cancer. *PLoS ONE*. (2014) 9:e91983. doi: 10.1371/journal.pone.0091983
106. Kim JH, Park SY, Jun Y, Kim JY, Nam JS. Roles of Wnt target genes in the journey of cancer stem cells. *Int J Mol Sci*. (2017) 18:1604. doi: 10.3390/ijms18081604
107. Nguyen DX, Chiang AC, Zhang XH, Kim JY, Kris MG, Ladanyi M, et al. WNT/TCF signaling through LEF1 and HOXB9 mediates lung adenocarcinoma metastasis. *Cell*. (2009) 138:51–62. doi: 10.1016/j.cell.2009.04.030
108. Abravanel DL, Belka GK, Pan TC, Pant DK, Collins MA, Sterner CJ, et al. Notch promotes recurrence of dormant tumor cells following HER2/neutargeted therapy. *J Clin Invest*. (2015) 125:2484–96. doi: 10.1172/JCI74883
109. Shachaf CM, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, et al. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature*. (2004) 431:1112–7. doi: 10.1038/nature03043
110. Wu CH, Van Riggelen J, Yetil A, Fan AC, Bachireddy P, Felsher DW. Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation. *Proc Natl Acad Sci USA*. (2007) 104:13028–33. doi: 10.1073/pnas.0701953104
111. Civenni G, Malek A, Albino D, Garcia-Escudero R, Napoli S, Di Marco S, et al. RNAi-mediated silencing of Myc transcription inhibits stem-like cell maintenance and tumorigenicity in prostate cancer. *Cancer Res*. (2013) 73:6816–27. doi: 10.1158/0008-5472.CAN-13-0615
112. Yang A, Qin S, Schulte BA, Ethier SP, Tew KD, Wang GY. MYC inhibition depletes cancer stem-like cells in triple-negative breast cancer. *Cancer Res*. (2017) 77:66416650. doi: 10.1158/0008-5472.CAN-16-3452
113. Joensuu K, Hagstrom J, Leidenius M, Haglund C, Andersson LC, Sariola H, et al. Bmi-1, c-myc, and snail expression in primary breast cancers and their metastases–elevated Bmi-1 expression in late breast cancer relapses. *Virchows Arch*. (2011) 459:31–9. doi: 10.1007/s00428-011-1096-8
114. Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res*. (2006) 66:6063–71. doi: 10.1158/0008-5472.CAN-06-0054
115. Johnson RW, Finger EC, Olcina MM, Vilalta M, Aguilera T, Miao Y, et al. Erratum: induction of LIFR confers a dormancy phenotype in breast cancer cells disseminated to the bone marrow. *Nat Cell Biol*. (2016) 18:1260. doi: 10.1038/ncb3433
116. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death Differ*. (2015) 22:377–88. doi: 10.1038/cdd.2014.150
117. Sosa MS, Bragado P, Debnath J, Aguirre-Ghiso JA. Regulation of tumor cell dormancy by tissue microenvironments and autophagy. *Adv Exp Med Biol*. (2013) 734:73–89. doi: 10.1007/978-1-4614-1445-2_5
118. Vitale I, Manic G, Dandrea V, De Maria R. Role of autophagy in the maintenance and function of cancer stem cells. *Int J Dev Biol*. (2015) 59:95–108. doi: 10.1387/ijdb.150082iv
119. Vera-Ramirez L, Vodnala SK, Nini R, Hunter KW, Green JE. Autophagy promotes the survival of dormant breast cancer cells and metastatic tumour recurrence. *Nat Commun*. (2018) 9:1944. doi: 10.1038/s41467-018-04070-6
120. Nazio F, Bordi M, Cianfanelli V, Locatelli F, Cecconi F. Autophagy and cancer stem cells: molecular mechanisms and therapeutic applications. *Cell Death Differ*. (2019) 26:690702. doi: 10.1038/s41418-019-0292-y
121. Marcucci F, Stassi G, De Maria R. Epithelial-mesenchymal transition: a new target in anticancer drug discovery. *Nat Rev Drug Discov*. (2016) 15:311–25. doi: 10.1038/nrd.2015.13

122. Wei SC, Yang J. Forcing through tumor metastasis: the interplay between tissue rigidity and epithelial-mesenchymal transition. *Trends Cell Biol*. (2016) 26:111–20. doi: 10.1016/j.tcb.2015.09.009
123. Weidenfeld K, Schif-Zuck S, Abu-Tayeh H, Kang K, Kessler O, Weissmann M, et al. Dormant tumor cells expressing LOXL2 acquire a stem-like phenotype mediating their transition to proliferative growth. *Oncotarget*. (2016) 7:71362–77. doi: 10.18632/oncotarget.12109
124. Chaffer CL, Marjanovic ND, Lee T, Bell G, Kleer CG, Reinhardt F, et al. Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. *Cell*. (2013) 154:61–74. doi: 10.1016/j.cell.2013.06.005
125. De Cock JM, Shibue T, Dongre A, Keckesova Z, Reinhardt F, Weinberg RA. Inflammation triggers Zeb1-dependent escape from tumor latency. *Cancer Res*. (2016) 76:6778–84. doi: 10.1158/0008-5472.CAN-16-0608
126. Francescangeli F, Contavalli P, De Angelis ML, Careccia S, Signore M, Haas TL, et al. A pre-existing population of ZEB2^{+} quiescent cells with stemness and mesenchymal features dictate chemoresistance in colorectal cancer. *J Exp Clin Cancer Res*. (2020) 39:2. doi: 10.1186/s13046-019-1505-4
127. Hen O, Barkan D. Dormant disseminated tumor cells and cancer stem/progenitor-like cells: similarities and opportunities. *Semin Cancer Biol*. (2020) 60:157–65. doi: 10.1016/j.semcancer.2019.09.002
128. Francescangeli F, Patrizii M, Signore M, Federici G, Di Franco S, Pagliuca A, et al. Proliferation state and polo-like kinase1 dependence of tumorigenic colon cancer cells. *Stem Cells*. (2012) 30:1819–30. doi: 10.1002/stem.1163
129. Crea F, Nur Saidy NR, Collins CC, Wang Y. The epigenetic/noncoding origin of tumor dormancy. *Trends Mol Med*. (2015) 21:206–11. doi: 10.1016/j.molmed.2015.02.005
130. Vitale I, Manic G, De Maria R, Kroemer G, Galluzzi L. DNA damage in stem cells. *Mol Cell*. (2017) 66:306–19. doi: 10.1016/j.molcel.2017.04.006
131. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: an awakening field. *Nat Rev Cancer*. (2014) 14:611–22. doi: 10.1038/nrc3793
132. Yeh AC, Ramaswamy S. Mechanisms of cancer cell dormancy–another hallmark of cancer? *Cancer Res*. (2015) 75:5014–22. doi: 10.1158/0008-5472.CAN-15-1370
133. Gao XL, Zhang M, Tang YL, Liang XH. Cancer cell dormancy: mechanisms and implications of cancer recurrence and metastasis. *Onco Targets Ther*. (2017) 10:5219–28. doi: 10.2147/OTT.S140854
134. Jahanban-Esfahlan R, Seidi K, Manjili MH, Jahanban-Esfahlan A, Javaheri T, Zare P. Tumor cell dormancy: threat or opportunity in the fight against cancer. *Cancers*. (2019) 11:1207. doi: 10.3390/cancers11081207
135. Peinado H, Zhang H, Matei IR, Costa-Silva B, Hoshino A, Rodrigues G, et al. Pre-metastatic niches: organ-specific homes for metastases. *Nat Rev Cancer*. (2017) 17:302–17. doi: 10.1038/nrc.2017.6
136. Celia-Terrassa T, Kang Y. Metastatic niche functions and therapeutic opportunities. *Nat Cell Biol*. (2018) 20:868–77. doi: 10.1038/s41556-018-0145-9
137. Braun S, Vogl FD, Naume B, Janni W, Osborne MP, Coombes RC, et al. A pooled analysis of bone marrow micrometastasis in breast cancer. *N Engl J Med*. (2005) 353:793–802. doi: 10.1056/NEJMoa050434
138. Ghajar CM. Metastasis prevention by targeting the dormant niche. *Nat Rev Cancer*. (2015) 15:238–47. doi: 10.1038/nrc3910
139. Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood*. (2015) 125:2605–13. doi: 10.1182/blood-2014-12-570200
140. Capulli M, Hristova D, Valbret Z, Carys K, Arjan R, Maurizi A, et al. Notch2 pathway mediates breast cancer cellular dormancy and mobilisation in bone and contributes to haematopoietic stem cell mimicry. *Br J Cancer*. (2019) 121:157–71. doi: 10.1038/s41416-019-0501-y
141. Kahn M. Can we safely target the WNT pathway? *Nat Rev Drug Discov*. (2014) 13:513–32.
142. Buczacki SJA, Popova S, Biggs E, Koukorava C, Buzzelli J, Vermeulen L, et al. Itraconazole targets cell cycle heterogeneity in colorectal cancer. *J Exp Med*. (2018) 215:1891–912. doi: 10.1084/jem.20171385
143. Ren D, Dai Y, Yang Q, Zhang X, Guo W, Ye L, et al. Wnt5a induces and maintains prostate cancer cells dormancy in bone. *J Exp Med*. (2019) 216:428–49. doi: 10.1084/jem.20180661
144. Nemeth MJ, Topol L, Anderson SM, Yang Y, Bodine DM. Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation. *Proc Natl Acad Sci USA*. (2007) 104:15436–41. doi: 10.1073/pnas.0704747104
145. Keller JR, Mantel C, Sing GK, Ellingsworth LR, Ruscetti SK, Ruscetti FW. Transforming growth factor beta 1 selectively regulates early murine hematopoietic progenitors and inhibits the growth of IL-3-dependent myeloid leukemia cell lines. *J Exp Med*. (1988) 168:737–50. doi: 10.1084/jem.168.2.737
146. Yamazaki S, Iwama A, Takayanagi S, Eto K, Ema H, Nakauchi H. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood*. (2009) 113:1250–6. doi: 10.1182/blood-2008-04-146480
147. Bragado P, Estrada Y, Parikh F, Krause S, Capobianco C, Farina HG, et al. TGF-beta2 dictates disseminated tumour cell fate in target organs through TGF-beta-RIII and p38alpha/beta signalling. *Nat Cell Biol*. (2013) 15:1351–61. doi: 10.1038/ncb2861
148. Yumoto K, Eber MR, Wang J, Cackowski FC, Decker AM, Lee E, et al. Axl is required for TGF-beta2-induced dormancy of prostate cancer cells in the bone marrow. *Sci Rep*. (2016) 6:36520. doi: 10.1038/srep36520
149. Veschi V, Mangiapane LR, Nicotra A, Di Franco S, Scavo E, Apuzzo T, et al. Targeting chemoresistant colorectal cancer via systemic administration of a BMP7 variant. *Oncogene*. (2020) 39:987–1003. doi: 10.1038/s41388-019-1047-4
150. Gao H, Chakraborty G, Lee-Lim AP, Mo Q, Decker M, Vonica A, et al. The BMP inhibitor coco reactivates breast cancer cells at lung metastatic sites. *Cell*. (2012) 150:764–79. doi: 10.1016/j.cell.2012.06.035
151. Sansone P, Ceccarelli C, Berishaj M, Chang Q, Rajasekhar VK, Perna F, et al. Self-renewal of CD133(hi) cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer. *Nat Commun*. (2016) 7:10442. doi: 10.1038/ncomms10442
152. Ono M, Kosaka N, Tominaga N, Yoshioka Y, Takeshita F, Takahashi RU, et al. Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci Signal*. (2014) 7:ra63. doi: 10.1126/scisignal.2005231
153. Bliss SA, Sinha G, Sandiford OA, Williams LM, Engelberth DJ, Guiro K, et al. Mesenchymal stem cell-derived exosomes stimulate cycling quiescence and early breast cancer dormancy in bone marrow. *Cancer Res*. (2016) 76:5832–44. doi: 10.1158/0008-5472.CAN-16-1092
154. Bartosh TJ, Ullah M, Zeitouni S, Beaver J, Prockop DJ. Cancer cells enter dormancy after cannibalizing mesenchymal stem/stromal cells. (MSCs). *Proc Natl Acad Sci USA*. (2016) 113:E6447–56. doi: 10.1073/pnas.1612290113
155. Matsuo K, Irie N. Osteoclast-osteoblast communication. *Arch Biochem Biophys*. (2008) 473:201–9. doi: 10.1016/j.abb.2008.03.027
156. Lu X, Mu E, Wei Y, Riethdorf S, Yang Q, Yuan M, et al. VCAM-1 promotes osteolytic expansion of indolent bone micrometastasis of breast cancer by engaging alpha4beta1positive osteoclast progenitors. *Cancer Cell*. (2011) 20:701–14. doi: 10.1016/j.ccr.2011.11.002
157. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science*. (2018) 361:eaao4227. doi: 10.1126/science.aao4227
158. Kienast Y, Von Baumgarten L, Fuhrmann M, Klinkert WE, Goldbrunner R, Herms J, et al. Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med*. (2010) 16:116–22. doi: 10.1038/nm.2072
159. Cao Z, Ding BS, Guo P, Lee SB, Butler JM, Casey SC, et al. Angiocrine factors deployed by tumor vascular niche induce B cell lymphoma invasiveness and chemoresistance. *Cancer Cell*. (2014) 25:350–65. doi: 10.1016/j.ccr.2014.02.005
160. Price TT, Burness ML, Sivan A, Warner MJ, Cheng R, Lee CH, et al. Dormant breast cancer micrometastases reside in specific bone marrow niches that regulate their transit to and from bone. *Sci Transl Med*. (2016) 8:340ra373. doi: 10.1126/scitranslmed.aad4059
161. Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat Rev Cancer*. (2010) 10:138–46. doi: 10.1038/nrc2791
162. Fessler E, Dijkgraaf FE, De Sousa EMF, Medema JP. Cancer stem cell dynamics in tumor progression and metastasis: is the microenvironment to blame? *Cancer Lett*. (2013) 341:97–104. doi: 10.1016/j.canlet.2012.10.015
163. Charles N, Holland EC. The perivascular niche microenvironment in brain tumor progression. *Cell Cycle*. (2010) 9:3012–21. doi: 10.4161/cc.9.15.12710

164. Haas TL, Sciuto MR, Brunetto L, Valvo C, Signore M, Fiori ME, et al. Integrin alpha7 is a functional marker and potential therapeutic target in glioblastoma. *Cell Stem Cell*. (2017) *21*:35–50.e39. doi: 10.1016/j.stem.2017.04.009
165. Pallini R, Ricci-Vitiani L, Banna GL, Signore M, Lombardi D, Todaro M, et al. Cancer stem cell analysis and clinical outcome in patients with glioblastoma multiforme. *Clin Cancer Res*. (2008) 14:8205–12. doi: 10.1158/1078-0432.CCR-08-0644
166. Malanchi I, Santamaria-Martinez A, Susanto E, Peng H, Lehr HA, Delaloye JF, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. (2011) 481:85–9. doi: 10.1038/nature10694
167. Mcallister SS, Gifford AM, Greiner AL, Kelleher SP, Saelzler MP, Ince TA, et al. Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell*. (2008) 133:994–1005. doi: 10.1016/j.cell.2008.04.045
168. Ouyang G, Wang Z, Fang X, Liu J, Yang CJ. Molecular signaling of the epithelial to mesenchymal transition in generating and maintaining cancer stem cells. *Cell Mol Life Sci*. (2010) 67:2605–18. doi: 10.1007/s00018-010-0338-2
169. Oskarsson T, Acharyya S, Zhang XH, Vanharanta S, Tavazoie SF, Morris PG, et al. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nat Med*. (2011) 17:867–74. doi: 10.1038/nm.2379
170. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. *J Cell Sci*. (2010) 123:4195–200. doi: 10.1242/jcs.023820
171. Walker C, Mojares E, Del Rio Hernandez A. Role of extracellular matrix in development and cancer progression. *Int J Mol Sci*. (2018) 19:3028. doi: 10.3390/ijms19103028
172. Poltavets V, Kochetkova M, Pitson SM, Samuel MS. The role of the extracellular matrix and its molecular and cellular regulators in cancer cell plasticity. *Front Oncol*. (2018) 8:431. doi: 10.3389/fonc.2018.00431
173. Aguirre Ghiso JA, Kovalski K, Ossowski L. Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *J Cell Biol*. (1999) 147:89–104. doi: 10.1083/jcb.147.1.89
174. Aguirre-Ghiso JA. How dormant cancer persists and reawakens. *Science*. (2018) 361:1314–5. doi: 10.1126/science.aav0191
175. Cox TR, Bird D, Baker AM, Barker HE, Ho MW, Lang G, et al. Editor's note: LOX-mediated collagen cross-linking is responsible for fibrosis-enhanced metastasis. *Cancer Res*. (2019) 79:5124. doi: 10.1158/0008-5472.CAN-19-2419
176. El Touny LH, Vieira A, Mendoza A, Khanna C, Hoenerhoff MJ, Green JE. Combined SFK/MEK inhibition prevents metastatic outgrowth of dormant tumor cells. *J Clin Invest*. (2014) 124:156–68. doi: 10.1172/JCI70259
177. Simpkins F, Jang K, Yoon H, Hew KE, Kim M, Azzam DJ, et al. Dual Src and MEK inhibition decreases ovarian cancer growth and targets tumor initiating stem-like cells. *Clin Cancer Res*. (2018) 24:4874–86. doi: 10.1158/1078-0432.CCR-17-3697
178. Gao H, Chakraborty G, Zhang Z, Akalay I, Gadiya M, Gao Y, et al. Multi-organ site metastatic reactivation mediated by non-canonical discoidin domain receptor 1 signaling. *Cell*. (2016) 166:47–62. doi: 10.1016/j.cell.2016.06.009
179. Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ, et al. Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. *Cancer Res*. (2008) 68:6241–50. doi: 10.1158/0008-5472.CAN-07-6849
180. Shibue T, Weinberg RA. Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proc Natl Acad Sci USA*. (2009) 106:10290–5. doi: 10.1073/pnas.0904227106
181. Schrader J, Gordon-Walker TT, Aucott RL, Van Deemter M, Quaas A, Walsh S, et al. Matrix stiffness modulates proliferation, chemotherapeutic response, and dormancy in hepatocellular carcinoma cells. *Hepatology*. (2011) 53:1192–205. doi: 10.1002/hep.24108
182. Reid SE, Kay EJ, Neilson LJ, Henze AT, Serneels J, Mcghee EJ, et al. Tumor matrix stiffness promotes metastatic cancer cell interaction with the endothelium. *EMBO J*. (2017) 36:2373–89. doi: 10.15252/embj.201694912
183. Liu Y, Lv J, Liang X, Yin X, Zhang L, Chen D, et al. Fibrin stiffness mediates dormancy of tumor-repopulating cells via a Cdc42Driven Tet2 epigenetic program. *Cancer Res*. (2018) 78:3926–37. doi: 10.1158/0008-5472.CAN-17-3719
184. Barney LE, Hall CL, Schwartz AD, Parks AN, Sparages C, Galarza S, et al. Tumor cell-organized fibronectin maintenance of a dormant breast cancer population. *Sci Adv*. (2020) 6:eaaz4157. doi: 10.1126/sciadv.aaz4157
185. Baeriswyl V, Christofori G. The angiogenic switch in carcinogenesis. *Semin Cancer Biol*. (2009) 19:329–37. doi: 10.1016/j.semcancer.2009.05.003
186. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
187. Papetti M, Herman IM. Mechanisms of normal and tumor-derived angiogenesis. *Am J Physiol Cell Physiol*. (2002) 282:C947–70. doi: 10.1152/ajpcell.00389.2001
188. Folberg R, Hendrix MJ, Maniotis AJ. Vasculogenic mimicry and tumor angiogenesis. *Am J Pathol*. (2000) 156:361–81. doi: 10.1016/S0002-9440(10)64739-6
189. Naumov GN, Akslen LA, Folkman J. Role of angiogenesis in human tumor dormancy: animal models of the angiogenic switch. *Cell Cycle*. (2006) 5:1779–87. doi: 10.4161/cc.5.16.3018
190. Luo W, Wang Y. Hypoxia mediates tumor malignancy and therapy resistance. *Adv Exp Med Biol*. (2019) 1136:1–18. doi: 10.1007/978-3-030-12734-3_1
191. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, et al. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol Cell Biol*. (2009) 29:2570–81. doi: 10.1128/MCB.00166-09
192. Bayer C, Vaupel P. Acute versus chronic hypoxia in tumors: controversial data concerning time frames and biological consequences. *Strahlenther Onkol*. (2012) 188:616–27. doi: 10.1007/s00066-012-0085-4
193. Eales KL, Hollinshead KE, Tennant DA. Hypoxia and metabolic adaptation of cancer cells. *Oncogenesis*. (2016) 5:e190. doi: 10.1038/oncsis.2015.50
194. Michiels C, Tellier C, Feron O. Cycling hypoxia: a key feature of the tumor microenvironment. *Biochim Biophys Acta*. (2016) 1866:76–86. doi: 10.1016/j.bbcan.2016.06.004
195. Kang SY, Halvorsen OJ, Gravdal K, Bhattacharya N, Lee JM, Liu NW, et al. Prosaposin inhibits tumor metastasis via paracrine and endocrine stimulation of stromal p53 and Tsp-1. *Proc Natl Acad Sci USA*. (2009) 106:12115–20. doi: 10.1073/pnas.0903120106
196. Hendrix MJ, Seftor EA, Hess AR, Seftor RE. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat Rev Cancer*. (2003) 3:411–21. doi: 10.1038/nrc1092
197. Ricci-Vitiani L, Pallini R, Biffoni M, Todaro M, Invernici G, Cenci T, et al. Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. *Nature*. (2010) 468:824–8. doi: 10.1038/nature09557
198. Bao S, Wu Q, Sathornsumetee S, Hao Y, Li Z, Hjelmeland AB, et al. Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res*. (2006) 66:7843–8. doi: 10.1158/0008-5472.CAN-06-1010
199. Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, et al. A perivascular niche for brain tumor stem cells. *Cancer Cell*. (2007) 11:69–82. doi: 10.1016/j.ccr.2006.11.020
200. Beck B, Driessens G, Goossens S, Youssef KK, Kuchnio A, Caauwe A, et al. A vascular niche and a VEGF-Nrp1 loop regulate the initiation and stemness of skin tumours. *Nature*. (2011) 478:399–403. doi: 10.1038/nature10525
201. David L, Feige JJ, Bailly S. Emerging role of bone morphogenetic proteins in angiogenesis. *Cytokine Growth Factor Rev*. (2009) 20:203–12. doi: 10.1016/j.cytogfr.2009.05.001
202. Indraccolo S, Minuzzo S, Masiero M, Pusceddu I, Persano L, Moserle L, et al. Cross-talk between tumor and endothelial cells involving the Notch3Dll4 interaction marks escape from tumor dormancy. *Cancer Res*. (2009) 69:1314–23. doi: 10.1158/0008-5472.CAN-08-2791
203. Giuriato S, Ryeom S, Fan AC, Bachireddy P, Lynch RC, Rioth MJ, et al. Sustained regression of tumors upon MYC inactivation requires p53 or thrombospondin-1 to reverse the angiogenic switch. *Proc Natl Acad Sci USA*. (2006) 103:16266–71. doi: 10.1073/pnas.0608017103
204. Li Z, Bao S, Wu Q, Wang H, Eyler C, Sathornsumetee S, et al. Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. *Cancer Cell*. (2009) 15:501–13. doi: 10.1016/j.ccr.2009.03.018
205. Harris AL. Hypoxia–a key regulatory factor in tumour growth. *Nat Rev Cancer*. (2002) 2:38–47. doi: 10.1038/nrc704

206. Almog N, Ma L, Raychowdhury R, Schwager C, Erber R, Short S, et al. Transcriptional switch of dormant tumors to fast-growing angiogenic phenotype. *Cancer Res*. (2009) 69:836–44. doi: 10.1158/0008-5472.CAN-08-2590
207. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. (2011) 331:1565–70. doi: 10.1126/science.1203486
208. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol*. (2004) 22:329–60. doi: 10.1146/annurev.immunol.22.012703.104803
209. Kauffman HM, Mcbride MA, Delmonico FL. First report of the united network for organ sharing transplant tumor registry: donors with a history of cancer. *Transplantation*. (2000) 70:1747–51. doi: 10.1097/00007890-200012270-00014
210. Mackie RM, Reid R, Junor B. Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. *N Engl J Med*. (2003) 348:567–8. doi: 10.1056/NEJM200302063480620
211. Buell JF, Beebe TM, Trofe J, Gross TG, Alloway RR, Hanaway MJ, et al. Donor transmitted malignancies. *Ann Transplant*. (2004) 9:53–6.
212. Penn I. Malignant melanoma in organ allograft recipients. *Transplantation*. (1996) 61:274–8. doi: 10.1097/00007890-199601270-00019
213. Suranyi MG, Hogan PG, Falk MC, Axelsen RA, Rigby R, Hawley C, et al. Advanced donor-origin melanoma in a renal transplant recipient: immunotherapy, cure, and retransplantation. *Transplantation*. (1998) 66:655–61. doi: 10.1097/00007890-199809150-00020
214. Jimsheleishvili S, Alshareef AT, Papadimitriou K, Bregy A, Shah AH, Graham RM, et al. Extracranial glioblastoma in transplant recipients. *J Cancer Res Clin Oncol*. (2014) 140:801–7. doi: 10.1007/s00432-014-1625-3
215. Cozar JM, Aptsiauri N, Tallada M, Garrido F, Ruiz-Cabello F. Late pulmonary metastases of renal cell carcinoma immediately after post-transplantation immunosuppressive treatment: a case report. *J Med Case Rep*. (2008) 2:111. doi: 10.1186/1752-1947-2-111
216. Montgomery TR, Whitlock GF, Nohlgren JE, Lewis AM. What becomes of the patient with latent or occult carcinoma of the prostate. *J Urol*. (1961) 86:655–8. doi: 10.1016/S0022-5347(17)65239-X
217. Harach HR, Franssila KO, Wasenius VM. Occult papillary carcinoma of the thyroid. A "normal" finding in Finland. a systematic autopsy study. *Cancer*. (1985) 56:531–8.
218. Nielsen M, Thomsen JL, Primdahl S, Dyreborg U, Andersen JA. Breast cancer and atypia among young and middle-aged women: a study of 110 medicolegal autopsies. *Br J Cancer*. (1987) 56:814–9. doi: 10.1038/bjc.1987.296
219. Loeb LA, Harris CC. Advances in chemical carcinogenesis: a historical review and prospective. *Cancer Res*. (2008) 68:6863–72. doi: 10.1158/0008-5472.CAN-08-2852
220. Uhr JW, Pantel K. Controversies in clinical cancer dormancy. *Proc Natl Acad Sci USA*. (2011) 108:12396–400. doi: 10.1073/pnas.1106613108
221. Mahvi DA, Liu R, Grinstaff MW, Colson YL, Raut CP. Local cancer recurrence: the realities, challenges, and opportunities for new therapies. *CA Cancer J Clin*. (2018) 68:488–505. doi: 10.3322/caac.21498
222. Karrison TG, Ferguson DJ, Meier P. Dormancy of mammary carcinoma after mastectomy. *J Natl Cancer Inst*. (1999) 91:80–5. doi: 10.1093/jnci/91.1.80
223. Freedland SJ, Moul JW. Prostate specific antigen recurrence after definitive therapy. *J Urol*. (2007) 177:1985–91. doi: 10.1016/j.juro.2007.01.137
224. Mcnichols DW, Segura JW, Deweerd JH. Renal cell carcinoma: long-term survival and late recurrence. *J Urol*. (1981) 126:17–23. doi: 10.1016/S0022-5347(17)54359-1
225. Tsao H, Cosimi AB, Sober AJ. Ultra-late recurrence. (15 years or longer) of cutaneous melanoma. *Cancer*. (1997) 79:2361–70.
226. Davis TA, Maloney DG, Czerwinski DK, Liles TM, Levy R. Anti-idiotype antibodies can induce long-term complete remissions in non-Hodgkin's lymphoma without eradicating the malignant clone. *Blood*. (1998) 92:1184–90. doi: 10.1182/blood.V92.4.1184
227. Yilmaz M, Wang F, Loghavi S, Bueso-Ramos C, Gumbs C, Little L, et al. Late relapse in acute myeloid leukemia. (AML): clonal evolution or therapy-related leukemia? *Blood Cancer J*. (2019) 9:7. doi: 10.1038/s41408-019-0170-3
228. Farrar JD, Katz KH, Windsor J, Thrush G, Scheuermann RH, Uhr JW, et al. Cancer dormancy. VII. A regulatory role for CD8+ T cells and IFN-gamma in establishing and maintaining the tumor-dormant state. *J Immunol*. (1999) 162:2842–9.
229. Morecki S, Pugatsch T, Levi S, Moshel Y, Slavin S. Tumor-cell vaccination induces tumor dormancy in a murine model of B-cell leukemia/lymphoma. (BCL1). *Int J Cancer*. (1996) 65:204–8.
230. Granziero L, Krajewski S, Farness P, Yuan L, Courtney MK, Jackson MR, et al. Adoptive immunotherapy prevents prostate cancer in a transgenic animal model. *Eur J Immunol*. (1999) 29:1127–38.
231. Mahnke YD, Schwendemann J, Beckhove P, Schirrmacher V. Maintenance of long-term tumour-specific T-cell memory by residual dormant tumour cells. *Immunology*. (2005) 115:325–36. doi: 10.1111/j.1365-2567.2005.02163.x
232. Loeser S, Loser K, Bijker MS, Rangachari M, Van Der Burg SH, Wada T, et al. Spontaneous tumor rejection by cbl-b-deficient CD8+ T cells. *J Exp Med*. (2007) 204:879–91. doi: 10.1084/jem.20061699
233. Eyles J, Puaux AL, Wang X, Toh B, Prakash C, Hong M, et al. Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma. *J Clin Invest*. (2010) 120:2030–9. doi: 10.1172/JCI42002
234. Muller-Hermelink N, Braumuller H, Pichler B, Wieder T, Mailhammer R, Schaak K, et al. TNFR1 signaling and IFN-gamma signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis. *Cancer Cell*. (2008) 13:507–18. doi: 10.1016/j.ccr.2008.04.001
235. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. (2001) 410:1107–11. doi: 10.1038/35074122
236. Teng MW, Vesely MD, Duret H, Mclaughlin N, Towne JE, Schreiber RD, et al. Opposing roles for IL-23 and IL-12 in maintaining occult cancer in an equilibrium state. *Cancer Res*. (2012) 72:3987–96. doi: 10.1158/0008-5472.CAN-12-1337
237. Maccalli C, Volonte A, Cimminiello C, Parmiani G. Immunology of cancer stem cells in solid tumours. *A review Eur J Cancer*. (2014) 50:649–55. doi: 10.1016/j.ejca.2013.11.014
238. Maccalli C, Rasul KI, Elawad M, Ferrone S. The role of cancer stem cells in the modulation of anti-tumor immune responses. *Semin Cancer Biol*. (2018) 53:189–200. doi: 10.1016/j.semcancer.2018.09.006
239. Quesnel B. Tumor dormancy and immunoescape. *APMIS*. (2008) 116:685–94. doi: 10.1111/j.1600-0463.2008.01163.x
240. Beatty GL, Gladney WL. Immune escape mechanisms as a guide for cancer immunotherapy. *Clin Cancer Res*. (2015) 21:687–92. doi: 10.1158/1078-0432.CCR-14-1860
241. Bloy N, Garcia P, Laumont CM, Pitt JM, Sistigu A, Stoll G, et al. Immunogenic stress and death of cancer cells: contribution of antigenicity vs adjuvanticity to immunosurveillance. *Immunol Rev*. (2017) 280:165–74. doi: 10.1111/imr.12582
242. Weinhold KJ, Miller DA, Wheelock EF. The tumor dormant state. comparison of L5178Y cells used to establish dormancy with those that emerge after its termination. *J Exp Med*. (1979) 149:745–57. doi: 10.1084/jem.149.3.745
243. Matsuzawa A, Takeda Y, Narita M, Ozawa H. Survival of leukemic cells in a dormant state following cyclophosphamide-induced cure of strongly immunogenic mouse leukemia. (DL811). *Int J Cancer*. (1991) 49:303–9. doi: 10.1002/ijc.2910490227
244. Enderling H, Hahnfeldt P, Hlatky L, Almog N. Systems biology of tumor dormancy: linking biology and mathematics on multiple scales to improve cancer therapy. *Cancer Res*. (2012) 72:2172–5. doi: 10.1158/0008-5472.CAN-11-3269
245. Charoentong P, Finotello F, Angelova M, Mayer C, Efremova M, Rieder D, et al. Pan-cancer immunogenomic analyses reveal genotypeimmunophenotype relationships and predictors of response to checkpoint blockade. *Cell Rep*. (2017) 18:248–62. doi: 10.1016/j.celrep.2016.12.019
246. Pantel K, Schlimok G, Kutter D, Schaller G, Genz T, Wiebecke B, et al. Frequent down-regulation of major histocompatibility class I antigen expression on individual micrometastatic carcinoma cells. *Cancer Res*. (1991) 51:4712–5.

247. Pommier A, Anaparthy N, Memos N, Kelley ZL, Gouronnec A, Yan R, et al. Unresolved endoplasmic reticulum stress engenders immune-resistant, latent pancreatic cancer metastases. *Science*. (2018) 360:eaao4908. doi: 10.1126/science.aao4908
248. Agudo J, Park ES, Rose SA, Alibo E, Sweeney R, Dhainaut M, et al. Quiescent tissue stem cells evade immune surveillance. *Immunity*. (2018) *48*:271–85.e275. doi: 10.1016/j.immuni.2018.02.001
249. Bichsel CA, Wang L, Froment L, Berezowska S, Muller S, Dorn P, et al. Increased PD-L1 expression and IL-6 secretion characterize human lung tumor-derived perivascular-like cells that promote vascular leakage in a perfusable microvasculature model. *Sci Rep*. (2017) 7:10636. doi: 10.1038/s41598-017-09928-1
250. Dieterich LC, Ikenberg K, Cetintas T, Kapaklikaya K, Hutmacher C, Detmar M. Tumor-associated lymphatic vessels upregulate PDL1 to inhibit T-cell activation. *Front Immunol*. (2017) 8:66. doi: 10.3389/fimmu.2017.00066
251. Schmittnaegel M, Rigamonti N, Kadioglu E, Cassara A, Wyser Rmili C, Kiialainen A, et al. Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. *Sci Transl Med*. (2017) 9:eaak9670. doi: 10.1126/scitranslmed.aak9670
252. Saudemont A, Hamrouni A, Marchetti P, Liu J, Jouy N, Hetuin D, et al. Dormant tumor cells develop cross-resistance to apoptosis induced by CTLs or imatinib mesylate via methylation of suppressor of cytokine signaling 1. *Cancer Res*. (2007) 67:4491–8. doi: 10.1158/0008-5472.CAN-06-1627
253. Zamai L, Ponti C, Mirandola P, Gobbi G, Papa S, Galeotti L, et al. NK cells and cancer. *J Immunol*. (2007) 178:4011–6. doi: 10.4049/jimmunol.178.7.4011
254. Shimasaki N, Jain A, Campana D. NK cells for cancer immunotherapy. *Nat Rev Drug Discov*. (2020) 19:200–18. doi: 10.1038/s41573-019-0052-1
255. Malladi S, Macalinao DG, Jin X, He L, Basnet H, Zou Y, et al. Metastatic latency and immune evasion through autocrine inhibition of WNT. *Cell*. (2016) 165:45–60. doi: 10.1016/j.cell.2016.02.025
256. Ingangi V, Minopoli M, Ragone C, Motti ML, Carriero MV. Role of microenvironment on the fate of disseminating cancer stem cells. *Front Oncol*. (2019) 9:82. doi: 10.3389/fonc.2019.00082
257. Giles AJ, Reid CM, Evans JD, Murgai M, Vicioso Y, Highfill SL, et al. Activation of hematopoietic stem/progenitor cells promotes immunosuppression within the premetastatic niche. *Cancer Res*. (2016) 76:1335–47. doi: 10.1158/0008-5472.CAN-15-0204
258. Liu Y, Cao X. Immunosuppressive cells in tumor immune escape and metastasis. *J Mol Med*. (2016) 94:509–22. doi: 10.1007/s00109-015-1376-x
259. Kryczek I, Zou L, Rodriguez P, Zhu G, Wei S, Mottram P, et al. B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *J Exp Med*. (2006) 203:871–81. doi: 10.1084/jem.20050930
260. Kuang DM, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, et al. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med*. (2009) 206:1327–37. doi: 10.1084/jem.20082173
261. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. (2004) 10:942–9. doi: 10.1038/nm1093
262. Mcallister SS, Weinberg RA. The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. *Nat Cell Biol*. (2014) 16:717–27. doi: 10.1038/ncb3015
263. Kenkel JA, Tseng WW, Davidson MG, Tolentino LL, Choi O, Bhattacharya N, et al. An immunosuppressive dendritic cell subset accumulates at secondary sites and promotes metastasis in pancreatic cancer. *Cancer Res*. (2017) 77:4158–70. doi: 10.1158/0008-5472.CAN-16-2212
264. Priego N, Zhu L, Monteiro C, Mulders M, Wasilewski D, Bindeman W, et al. STAT3 labels a subpopulation of reactive astrocytes required for brain metastasis. *Nat Med*. (2018) 24:1024–35. doi: 10.1038/s41591-018-0044-4
265. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature*. (2015) 522:345–8. doi: 10.1038/nature14282
266. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasisinitiating breast cancer cells. *Nature*. (2015) 528:413–7. doi: 10.1038/nature16140
267. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol*. (2007) 25:267–96. doi: 10.1146/annurev.immunol.25.022106.141609
268. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, et al. Immune evasion in cancer: mechanistic basis and therapeutic strategies. *Semin Cancer Biol*. (2015) 35:S185–98. doi: 10.1016/j.semcancer.2015.03.004
269. Riethdorf S, Pantel K. Disseminated tumor cells in bone marrow and circulating tumor cells in blood of breast cancer patients: current state of detection and characterization. *Pathobiology*. (2008) 75:140–8. doi: 10.1159/000123852
270. Kowalik A, Kowalewska M, Gozdz S. Current approaches for avoiding the limitations of circulating tumor cells detection methods-implications for diagnosis and treatment of patients with solid tumors. *Transl Res*. (2017) *185*:58–84.e15. doi: 10.1016/j.trsl.2017.04.002
271. Zhang L, Ridgway LD, Wetzel MD, Ngo J, Yin W, Kumar D, et al. The identification and characterization of breast cancer CTCs competent for brain metastasis. *Sci Transl Med*. (2013) 5:180ra148. doi: 10.1126/scitranslmed.3005109
272. Larsen SB, Cowley CJ, Fuchs E. Epithelial cells: liaisons of immunity. *Curr Opin Immunol*. (2020) 62:45–53. doi: 10.1016/j.coi.2019.11.004
273. Lu X, Kang Y. Cell fusion as a hidden force in tumor progression. *Cancer Res*. (2009) 69:8536–9. doi: 10.1158/0008-5472.CAN-09-2159
274. Burkardt HJ. Standardization and quality control of PCR analyses. *Clin Chem Lab Med*. (2000) 38:87–91. doi: 10.1515/CCLM.2000.014
275. Pantel K, Schlimok G, Angstwurm M, Weckermann D, Schmaus W, Gath H, et al. Methodological analysis of immunocytochemical screening for disseminated epithelial tumor cells in bone marrow. *J Hematother*. (1994) 3:165–73. doi: 10.1089/scd.1.1994.3.165
276. Pantel K, Alix-Panabieres C. Bone marrow as a reservoir for disseminated tumor cells: a special source for liquid biopsy in cancer patients. *Bonekey Rep*. (2014) 3:584. doi: 10.1038/bonekey.2014.79
277. Aktas B, Tewes M, Fehm T, Hauch S, Kimmig R, Kasimir-Bauer S. Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res*. (2009) 11:R46. doi: 10.1186/bcr2333
278. Raimondi C, Gradilone A, Naso G, Vincenzi B, Petracca A, Nicolazzo C, et al. Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. *Breast Cancer Res Treat*. (2011) 130:449–55. doi: 10.1007/s10549-011-1373-x
279. Balic M, Lin H, Young L, Hawes D, Giuliano A, Mcnamara G, et al. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res*. (2006) 12:5615–21. doi: 10.1158/1078-0432.CCR-06-0169
280. Fluegen G, Avivar-Valderas A, Wang Y, Padgen MR, Williams JK, Nobre AR, et al. Phenotypic heterogeneity of disseminated tumour cells is preset by primary tumour hypoxic microenvironments. *Nat Cell Biol*. (2017) 19:120–32. doi: 10.1038/ncb3465
281. Bartkowiak K, Kwiatkowski M, Buck F, Gorges TM, Nilse L, Assmann V, et al. Disseminated tumor cells persist in the bone marrow of breast cancer patients through sustained activation of the unfolded protein response. *Cancer Res*. (2015) 75:5367–77. doi: 10.1158/0008-5472.CAN-14-3728
282. Hong Y, Fang F, Zhang Q. Circulating tumor cell clusters: What we know and what we expect.. *Int J Oncol*. (2016) 49:2206–16. doi: 10.3892/ijo.2016.3747
283. Riethdorf S, O'flaherty L, Hille C, Pantel K. Clinical applications of the cellsearch platform in cancer patients. *Adv Drug Deliv Rev*. (2018) 125:102–21. doi: 10.1016/j.addr.2018.01.011
284. Sai B, Xiang J. Disseminated tumour cells in bone marrow are the source of cancer relapse after therapy. *J Cell Mol Med*. (2018) 22:5776–86. doi: 10.1111/jcmm.13867
285. Lozar T, Gersak K, Cemazar M, Kuhar CG, Jesenko T. The biology and clinical potential of circulating tumor cells. *Radiol Oncol*. (2019) 53:131–47. doi: 10.2478/raon-2019-0024
286. Tjensvoll K, Nordgard O, Skjaeveland M, Oltedal S, Janssen E. A. M., Gilje, B. Detection of disseminated tumor cells in bone marrow predict late recurrences in operable breast cancer patients. *BMC Cancer*. (2019) 19:1131. doi: 10.1186/s12885-019-6268-y

287. Yang C, Xia BR, Jin WL, Lou G. Circulating tumor cells in precision oncology: clinical applications in liquid biopsy and 3D organoid model. *Cancer Cell Int*. (2019) 19:341. doi: 10.1186/s12935-019-1067-8
288. Mentis AA, Grivas PD, Dardiotis E, Romas NA, Papavassiliou AG. Circulating tumor cells as Trojan Horse for understanding, preventing, and treating cancer: a critical appraisal. *Cell Mol Life Sci*. (2020) 77:3671–90. doi: 10.1007/s00018-020-03529-4
289. Karczewski KJ, Snyder MP. Integrative omics for health and disease. *Nat Rev Genet*. (2018) 19:299–310. doi: 10.1038/nrg.2018.4
290. Sosa MS. Dormancy programs as emerging antimetastasis therapeutic alternatives. *Mol Cell Oncol*. (2016) 3:e1029062. doi: 10.1080/23723556.2015.1029062
291. Welcker M, Clurman BE. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer*. (2008) 8:83–93. doi: 10.1038/nrc2290
292. Takeishi S, Matsumoto A, Onoyama I, Naka K, Hirao A, Nakayama KI. Ablation of Fbxw7 eliminates leukemia-initiating cells by preventing quiescence. *Cancer Cell*. (2013) 23:347–61. doi: 10.1016/j.ccr.2013.01.026
293. Hidayat M, Mitsuishi Y, Takahashi F, Tajima K, Yae T, Miyahara K, et al. Role of FBXW7 in the quiescence of gefitinib-resistant lung cancer stem cells in EGFR-mutant non-small cell lung cancer. *Bosn J Basic Med Sci*. (2019) 19:355–67. doi: 10.17305/bjbms.2019.4227
294. Essers MA, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA, et al. IFNalpha activates dormant haematopoietic stem cells *in vivo*. *Nature*. (2009) 458:904–8. doi: 10.1038/nature07815
295. Saito Y, Uchida N, Tanaka S, Suzuki N, Tomizawa-Murasawa M, Sone A, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nat Biotechnol*. (2010) 28:275–80. doi: 10.1038/nbt.1607
296. Wang Q, Bu S, Xin D, Li B, Wang L, Lai D. Autophagy is indispensable for the self-renewal and quiescence of ovarian cancer spheroid cells with stem cell-like properties. *Oxid Med Cell Longev*. (2018) 2018:7010472. doi: 10.1155/2018/7010472
297. Shen S, Fan J, Cai B, Lv Y, Zeng M, Hao Y, et al. Vascular endothelial growth factor enhances cancer cell adhesion to microvascular endothelium *in vivo*. *Exp Physiol*. (2010) 95:369–79. doi: 10.1113/expphysiol.2009.050260
298. Shay G, Lynch CC, Fingleton B. Moving targets: emerging roles for MMPs in cancer progression and metastasis. *Matrix Biol*. (2015) 44–46:200–6. doi: 10.1016/j.matbio.2015.01.019
299. Nilsson M, Adamo H, Bergh A, Halin Bergstrom S. Inhibition of lysyl oxidase and lysyl oxidase-like enzymes has tumour-promoting and tumour-suppressing roles in experimental prostate cancer. *Sci Rep*. (2016) 6:19608. doi: 10.1038/srep19608
300. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*. (2011) 475:222–5. doi: 10.1038/nature10138
301. Germano G, Frapolli R, Belgiovine C, Anselmo A, Pesce S, Liguori M, et al. Role of macrophage targeting in the antitumor activity of trabectedin. *Cancer Cell*. (2013) 23:249–62. doi: 10.1016/j.ccr.2013.01.008
302. Wieland E, Rodriguez-Vita J, Liebler SS, Mogler C, Moll I, Herberich SE, et al. Endothelial Notch1 activity facilitates metastasis. *Cancer Cell*. (2017) 31:355–67. doi: 10.1016/j.ccell.2017.01.007
303. Naume B, Synnestvedt M, Falk RS, Wiedswang G, Weyde K, Risberg T, et al. Clinical outcome with correlation to disseminated tumor cell. (DTC) status after DTC-guided secondary adjuvant treatment with docetaxel in early breast cancer. *J Clin Oncol*. (2014) 32:3848–57. doi: 10.1200/JCO.2014.56.9327
304. June CH, O'connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science*. (2018) 359:1361–5. doi: 10.1126/science.aar6711
305. Mehta RS, Rezvani K. Chimeric antigen receptor expressing natural killer cells for the immunotherapy of cancer. *Front Immunol*. (2018) 9:283. doi: 10.3389/fimmu.2018.00283
306. Smith TT, Stephan SB, Moffett HF, Mcknight LE, Ji W, Reiman D, et al. *In situ* programming of leukaemia-specific T cells using synthetic DNA nanocarriers. *Nat Nanotechnol*. (2017) 12:813–20. doi: 10.1038/nnano.2017.57
307. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet*. (2005) 365:1687–717. doi: 10.1016/S0140-6736(05)66544-0
308. Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet*. (2013) 381:805–16. doi: 10.1016/S0140-6736(12)61963-1
309. Aguirre-Ghiso JA, Liu D, Mignatti A, Kovalski K, Ossowski L. Urokinase receptor and fibronectin regulate the ERK(MAPK) to p38(MAPK) activity ratios that determine carcinoma cell proliferation or dormancy *in vivo*. *Mol Biol Cell*. (2001) 12:863–79. doi: 10.1091/mbc.12.4.863
310. Carrio M, Arderiu G, Myers C, Boudreau NJ. Homeobox D10 induces phenotypic reversion of breast tumor cells in a three-dimensional culture model. *Cancer Res*. (2005) 65:7177–85. doi: 10.1158/0008-5472.CAN-04-1717
311. Chen A, Cuevas I, Kenny PA, Miyake H, Mace K, Ghajar C, et al. Endothelial cell migration and vascular endothelial growth factor expression are the result of loss of breast tissue polarity. *Cancer Res*. (2009) 69:6721–9. doi: 10.1158/0008-5472.CAN-08-4069
312. Catena R, Bhattacharya N, El Rayes T, Wang S, Choi H, Gao D, et al. Bone marrow-derived Gr1+ cells can generate a metastasis-resistant microenvironment via induced secretion of thrombospondin-1. *Cancer Discov*. (2013) 3:578–89. doi: 10.1158/2159-8290.CD-12-0476
313. Beliveau A, Mott JD, Lo A, Chen EI, Koller AA, Yaswen P, et al. Raf-induced MMP9 disrupts tissue architecture of human breast cells in threedimensional culture and is necessary for tumor growth *in vivo*. *Genes Dev*. (2010) 24:2800–11. doi: 10.1101/gad.1990410
314. Adam AP, George A, Schewe D, Bragado P, Iglesias BV, Ranganathan AC, et al. Computational identification of a p38SAPKregulated transcription factor network required for tumor cell quiescence. *Cancer Res*. (2009) 69:5664–72. doi: 10.1158/0008-5472.CAN-08-3820
315. Adomako A, Calvo V, Biran N, Osman K, Chari A, Paton JC, et al. Identification of markers that functionally define a quiescent multiple myeloma cell sub-population surviving bortezomib treatment. *BMC Cancer*. (2015) 15:444. doi: 10.1186/s12885-015-1460-1
316. Onder TT, Kara N, Cherry A, Sinha AU, Zhu N, Bernt KM, et al. Chromatin-modifying enzymes as modulators of reprogramming. *Nature*. (2012) 483:598–602. doi: 10.1038/nature10953
317. Welty CJ, Coleman I, Coleman R, Lakely B, Xia J, Chen S, et al. Single cell transcriptomic analysis of prostate cancer cells. *BMC Mol Biol*. (2013) 14:6. doi: 10.1186/1471-2199-14-6
318. Chery L, Lam HM, Coleman I, Lakely B, Coleman R, Larson S, et al. Characterization of single disseminated prostate cancer cells reveals tumor cell heterogeneity and identifies dormancy associated pathways. *Oncotarget*. (2014) 5:9939–51. doi: 10.18632/oncotarget.2480
319. Zeniou M, Feve M, Mameri S, Dong J, Salome C, Chen W, et al. Chemical library screening and structure-function relationship studies identify bisacodyl as a potent and selective cytotoxic agent towards quiescent human glioblastoma tumor stem-like cells. *PLoS ONE*. (2015) 10:e0134793. doi: 10.1371/journal.pone.0134793
320. Zeuner A, Francescangeli F, Contavalli P, Zapparelli G, Apuzzo T, Eramo A, et al. Elimination of quiescent/slow-proliferating cancer stem cells by Bcl-XL inhibition in nonsmall cell lung cancer. *Cell Death Differ*. (2014) 21:1877–88. doi: 10.1038/cdd.2014.105

Conflict of Interest: RDM is an advisory board member and research grant recipient from Hibercell, Inc. NY.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 May 2021
doi: 10.3389/fimmu.2021.656364

Aspects of the Tumor Microenvironment Involved in Immune Resistance and Drug Resistance

Khalil Khalaf[1,2]*, *Doris Hana*[1,2], *Jadzia Tin-Tsen Chou*[1,2], *Chandpreet Singh*[1,2], *Andrzej Mackiewicz*[1,2] *and Mariusz Kaczmarek*[1,2]

[1] Department of Cancer Diagnostics and Immunology, Greater Poland Cancer Center, Poznań, Poland, [2] Department of Cancer Immunology, Poznan University of Medical Sciences, Poznań, Poland

The tumor microenvironment (TME) is a complex and ever-changing "rogue organ" composed of its own blood supply, lymphatic and nervous systems, stroma, immune cells and extracellular matrix (ECM). These complex components, utilizing both benign and malignant cells, nurture the harsh, immunosuppressive and nutrient-deficient environment necessary for tumor cell growth, proliferation and phenotypic flexibility and variation. An important aspect of the TME is cellular crosstalk and cell-to-ECM communication. This interaction induces the release of soluble factors responsible for immune evasion and ECM remodeling, which further contribute to therapy resistance. Other aspects are the presence of exosomes contributed by both malignant and benign cells, circulating deregulated microRNAs and TME-specific metabolic patterns which further potentiate the progression and/or resistance to therapy. In addition to biochemical signaling, specific TME characteristics such as the hypoxic environment, metabolic derangements, and abnormal mechanical forces have been implicated in the development of treatment resistance. In this review, we will provide an overview of tumor microenvironmental composition, structure, and features that influence immune suppression and contribute to treatment resistance.

Keywords: TME (tumor microenvironment), HIF - hypoxia inducible factor, CAF, microRNA (miR), MDSC (myeloid-derived suppressor cells), tumor associated macrophage (TAM), Treg - regulatory T cell, TGF - β1

OPEN ACCESS

Edited by:
Sergio Rutella, Nottingham Trent University, United Kingdom

Reviewed by:
Lisa Sevenich, Georg Speyer Haus, Germany
Zong Sheng Guo, University of Pittsburgh, United States

***Correspondence:**
Khalil Khalaf
khalilkhalaf0216@gmail.com

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *20 January 2021*
Accepted: *27 April 2021*
Published: *27 May 2021*

Citation:
Khalaf K, Hana D, Chou JT-T, Singh C, Mackiewicz A and Kaczmarek M (2021) Aspects of the Tumor Microenvironment Involved in Immune Resistance and Drug Resistance. Front. Immunol. 12:656364. doi: 10.3389/fimmu.2021.656364

INTRODUCTION

Experimental observations of tumorigenesis show that tumor cells transition from being transformed and benign to an invasive malignant state. This process is the result of genome instability, in which cells lose their ability to fully differentiate and mature, resulting in the loss of contact inhibition (1).

Various studies have shown that a large majority of cancer-related deaths were attributed to distant metastasis (2, 3). Stephen Paget was the first to hypothesize on what he described as his "seed and soil" theory. In this hypothesis, tumor cells with metastatic potential (i.e. the seed) were inclined to migrate towards specific sites that nurtured and enhanced growth sites (i.e. the soil). This is the

earliest publication hypothesizing the importance of the "tumor microenvironment" (TME) in the development of metastases (4). It is known from extensive literature that the metastatic cascade starts with tumor cell dissociation from the cancer niche, followed by extravasation into capillary and lymphatic systems and along nerves, all the while evading immune surveillance. This process culminates in the invasion of distant sites (5). However, metastatic potential develops long before the tumor is ever detected. In the initial stages of primary tumor formation, the accumulation of both genetic and genomic instabilities lead to the development of phenotypic variants with metastatic capacity (6). Furthermore, these variants have the ability to resist apoptosis and circumvent immune defenses by using various soluble factors that are released by malignant and non-malignant tumor-supporting cells (7). These variants in combination with said soluble factors constitute what is now known as the TME.

Additionally, the TME induces chemotherapeutic resistance through acquired or *de novo* mechanisms. In acquired multi-drug resistance (MDR), the expression of ATP-binding cassettes (ABCs), oncogene activation, and tumor-suppressor gene deregulation are achieved *via* cellular crosstalk and cell-to-TME-matrix interaction. Previously-exposed cancer cells acquire phenotypic changes that lead to resistance to subsequent therapy (8). On the other hand, in *de novo* resistance, it has been shown that after exposure to therapy, stromal tissue within the TME provides refuge to a subpopulation of cancer cells and renders them chemo-resistant by inducing stemness (9).

The vast arsenal that is weaponized by the TME in the course of neoplastic disease is currently the topic of great research interest, and the available literature is daunting for researchers and practicing oncologists alike. This review aims to present an overview of the cells and structure of the TME, and its unique characteristics that induce drug resistance and metastasis that remain significant challenges in the treatment of cancer.

THE TUMOR MICROENVIRONMENT AND ITS ROLE IN IMMUNE SURVEILLANCE

Stem cells (SCs) are unspecified cells with the ability to differentiate into multiple cell types to maintain tissue homeostasis. They reside in a specific microenvironment called a stem cell niche, which consists of and is sustained by different soluble factors (10). Tissue homeostasis is balanced and maintained in a way that prevents SC depletion and overactive proliferation. This is achieved by choosing alternate fates: the SC is selected for senescence (i.e. death), or self-renewal (i.e. proliferation) through interactions with other cells and molecular signals within the microenvironment (11). Just like the stem cell niche of healthy tissues, the tumor microenvironment (TME) is very heterogeneous and is a complex component of solid tumors. The TME comprises a diverse cellular and acellular milieu in which cancer stem cells (CSCs) develop and thrive, and various stromal and immune cells are recruited to form and maintain this self-sustained environment (12). Stromal and tumor cell crosstalk has been recognized as crucial for the promotion of a well-organized TME, leading to effective immune evasion, ECM remodeling, and angiogenesis (7).

CELLS AND COMPONENTS OF THE TME INVOLVED IN SUPPRESSION OF THE ANTI-TUMOR RESPONSE

Stromal Cells

Vascular and Lymphatic Endothelial Cells

Neo-angiogenesis is promoted by both tumor and endothelial cells (ECs). Both vascular and lymphatic systems are implicated in early metastasis, with soluble VEGFA promoting vascular EC proliferation, while VEGFC, VEGFD, and VEGFR-3 promoting lymphatic EC proliferation (13, 14).

Tumor angiogenic vessels are either derived from endothelial progenitor cells or from existing vessels that propagate to feed growing tumors (15). The ECs present within the TME possess abnormal pericytes and pericyte coverage which enables leaks between tight junctions. This directly leads to the systemic circulation of tumor cells, i.e. presence of CTCs, thus increasing the tumor's metastatic potential (16). Hypoxia triggers stromal release of VEGF. Subsequent activation of VEGF-2 receptors on adjacent ECs promotes their migration to the region of hypoxia and production of hypoxia-inducible factors 1 (HIF-1) and 2 (HIF-2) (17) ultimately leading to EC proliferation, migration and maturation (18, 19). The result is tumor endothelial anergy, the cellular non-response to pro-inflammatory stimulation (i.e. IFN-γ, TNF-α, and IL-1). As vital gatekeepers of the TME, tumor endothelial cells (TECs) are the primary barrier to immune-stimulatory cells which promote the loss of anti-cancer immunity (20–23), TME-derived cytokines such as VEGF, ET1, FGF-2, and EGFL7 function to inhibit tumor endothelial ability to upregulate the expression of chemoattractants (i.e. CXCL7, CXCL10, IL-6, and CCL2) and adhesion molecules (ICAM1 and VCAM1), consequently promoting immunosuppression and tumor progression (20, 23–26). Additionally, TECs were shown to promote regulatory T cell (Treg) accumulation *via* up-regulation of the lymphatic and vasculature endothelial receptor 1 (CLEVER-1); an abundance of CLEVER-1-positive macrophages support immunosuppression. It has been reported that tumor-induced CLEVER-1 expression in both macrophages and endothelial cell populations was required to support the growth of melanoma, and that the chief driver was the diminished expression of vascular E- and P-selectin, and accumulation of Tregs and M2 macrophages in the tumors induced by CLEVER-1 (27, 28).

ECs can selectively upregulate T cell inhibitory receptors including: IDO1, TIM3, B7-H3, B7-H4, PD-L1 and PD-L2 (29–32) along with other soluble inhibitory molecules such as: TGF-β, IL-6, IL-10, and PGE2 (33–35), thus maintaining a constant inflammatory state within the TME. ECs may also express apoptosis-inducing molecules such as TNF-related

apoptosis-inducing ligand (TRAIL) and FasL which were shown to selectively extinguish effector T cells, sparing Tregs (36–40). Thus the tumor vasculature inhibits immune cell extravasation in the tumor bed and promotes the immunosuppressive state, and is one of the main modulators in immune resistance (41–43).

Mesenchymal Stem Cells

As important contributors to the TME, mesenchymal stem cells (MSCs) harvested from different tissues have demonstrated varying expression levels of factors that contribute to embryonic stem cell pluripotency, such as SOX-2, NANOG and OCT-4 (44, 45). MSCs are dispatched by a series of paracrine signaling pathways in response to injury, and either differentiate on-site to replenish damaged tissue with their cell multipotency (10, 46) or activate various trophic factors necessary to activate local SCs specific to the tissue (47), for the purposes of wound healing. The TME continuously recruits MSCs by generating constant inflammation, similar to that seen in wound healing, and is thus about to remodel itself perpetually (48, 49). Thus MSCs are able to populate the TME with other crucial cells such as pericytes and fibroblasts with their multipotency (50). In addition to the aforementioned, MSCs are involved in other cancer-promoting mechanisms. MSCs release specific molecules such as epidermal growth factors (EGFs) (51), IL-8/IL-6 cytokines (52) and CXCL1/2/12 chemokines (53) which directly act on cancer cells in a paracrine fashion and increase cellular proliferation by induction of phenotypic modification. In another immune suppressor mechanism, MSCs were shown to suppress both adaptive and innate immunity by directly inhibiting CD4 and CD8 T cell proliferation (54). A third mechanism includes stimulation of TLRs3/4 present on MSCs, inducing production of CXCL10, IL-8 and IL-6 which are crucial for T cell suppression (55). Furthermore, *via* adhesion to Th17 *via* CCL20, MSCs are capable of inducing T cell differentiation to Tregs thus suppressing both innate and adaptive immunity (56). MSCs also promote tumor revascularization by a) secreting various angiogenic factors such as EGF and VEGF, which are responsible for recruitment of ECs for vasculature maturation (57) or b) by converting into endothelial-like cells to modulate neo-angiogenesis (58). MSCs have also been shown to possess the ability to differentiate into tumor stromal progenitor cells, such as cancer-associated fibroblasts (CAFs), which further enhance the development and sustenance of the TME (59). MSCs have been demonstrated to be involved in the production of inflammatory chemokine CCL5, which is responsible for metastatic potential in breast cancer (60). MSCs are capable of impeding all immune responses through interactions with every cell in the immune system, directly or *via* soluble immune secretomes (40) such as:

prostaglandin E2 (PGE2) - PGE2 suppresses IL-2 formation and T cell function. The literature also suggests that PGE2 regulates the balance between different helper T cell (Th) configurations and responses, solely inhibiting Th1 IFN-γ production (61). PGE2-suppression of Th1 results from its ability to repress IL-12 production in dendritic cells (DCs), and monocytes (62, 63). Additionally, PGE2 is required for the development of myeloid-derived suppressor cells (MDSCs) (64) and tumor-associated macrophages (TAMs) (65). MDSCs express high levels of COX2, a major source of PGE2. The positive feedback between COX2 and PGE2 promotes MDSC stability, and leads to the production of additional MDSC-associated suppressive mediators (64). HIF-1-α also mediates and likely initiates a signaling cascade in PGE2-mediated MDSC development (66).

Indoleamine 2,3-dioxegynase (IDO) - Cells expressing IDO can suppress immunity by catabolizing tryptophan (Trp) and other indole compounds (67). Potent IDO inducers IFN-I and IFN-II are produced at sites of inflammation. IDO is also expressed by DCs, resulting in DC conversion to tolerogenic antigen-presenting cells (APCs) that suppress effector T cells (Teff) whilst promoting Tregs. Non-catalytic signaling induces TGF-β release by a subset of DCs, leading to tolerance. Tolerogenic IDO promotes tumorigenesis by allowing cancer cells to evade immune surveillance. Some cells express IDO1 genes which deplete Trp and generate bioactive catabolites such as kynurenines (Kyn). This is sensed by a population of immune cells, leading to suppression of innate and adaptive immunity (68).

Nitric oxide (NO) - a pleiotropic and short-lived radical which has pathophysiological functions. Produced by MSCs, NO is responsible for mediating T cell-dependent immunosuppression (69). MSCs express compounds such as arginase, β2 integral, Gr-1 granulocyte marker, and inducible nitric oxide synthase (iNOS) which converts L-arginine into urea and L-ornithine (70). This hints at a potential synergy between arginase and iNOS which would result in superoxide (O^{2-}). O^{2-} then may react with NO to produce peroxinitrite ($ONOO^-$) as well as other reactive nitrogen intermediates which induce T cell apoptosis (71). Another immunosuppressive mechanism is the high NO-concentration impairment of IL-2-R-induced signaling. This leads to the activation of Janus kinases 1 (JAK1) and 2 (JAK2), with signal transducer and activator of transcription factor 5 (STAT5) (72).

Cancer-Associated Fibroblasts

Cancer-associated fibroblasts (CAFs) produce key proteins such as periostin and Tenascin-C which are necessary for tumor support and metastasis (73, 74). Their expression in the TME changes the predominant cell type in the stromal tissue as well as the modulator of the ECM. It has been shown that CAFs placed with normal prostate cells *in vitro* induces rapid cell growth and alters prostate cell histology (75). The histological changes may be the result of CAFs' ability to induce epithelial-to-mesenchymal transition (EMT) *via* upregulation of TGF-β, which modifies cellular cytoskeleton architecture, cyclin-dependent kinases, and decreases the potency of immune surveillance (76). This subsequently enables cellular migration and invasion, and induces the development of pluripotent tumor cells (77, 78). This is evident with the demonstration that growth factors such as CCL2 and hepatocyte growth factor (HGF) induced CSC renewal and stemness of cancer cells in both breast (79) and hepatocellular carcinoma (80). Another mechanism of stemness is the upregulation of the NF-κB

signaling pathway. This prompts continuous secretion of pro-inflammatory cytokines such as IL-6 and IL-8; this constant inflammatory milieu induces EMT (81). The importance of IL-6 has been previously elucidated. Increased expression of IL-6 in myeloma cells induces activation of the JAK2-STAT3 pathway (82) and increases expression of Bcl-Xc which correlates with resistance to therapy (83). In early TME development, the ECM is reconstructed in a stiffened manner (84); the elastin component of the ECM is cross-linked with collagen in the presence of lysyl oxidase (LOX) and these are both produced by CAFs (85). CAFs are also responsible for the secretion of fibroblast growth factor-2 (FGF-2), an essential signaling molecule responsible for angiogenesis (86), and expresses stromal-derived factor-1 (SDF-1) which induces metastasis in breast cancer by acting as a chemotactic factor for circulating ECs (87). The role of Chi3L1, a non-enzymatic chitinase-3 like-protein 1, has also been studied. Regulated by the ECM, it binds to heparin, hyaluronic acid, and chitin, and is synthesized by a variety of cells including tumor cells, fibroblast-like cells, smooth muscle cells, chondrocytes, macrophages, neutrophils, and synoviocytes (88). Genetic targeting of CAF-derived Chi3L1 in fibroblasts has attenuated recruitment and reprogrammed macrophages to an M2 phenotype, which promotes a Th1 phenotype in the TME (89). Additionally, the TAM polarization to the M2 phenotype was shown to be induced by high expression of TGF-β (90). In an *ex vivo* model of oral squamous cell carcinoma, CAFs promoted the development of an M2-like phenotype from CD14 myeloid cells after induction by IL-10, TGF-β, and ARG1. This ultimately suppresses T cell proliferation (91). With their reciprocal interactions, TAMs and CAFs are central immunosuppressive players in the TME. Notably, CAFs recruit macrophages through the expression of stromal cell-derived factor 1 (SDF-1/CXCL12). SDF-1 magnifies M2 polarization of macrophages mirroring high production of immunosuppressive cytokine IL-10 (92–94).

Pericytes

Arising from differentiated mesenchymal precursors, pericytes are recruited when cancer cells overexpress platelet-derived growth factor beta (PDGF-β) (95) in both healthy and neoplastic tissues alike. They exhibit many tumor-supporting mechanisms including the release of EC-attracting soluble factors, which rapidly induces revascularization of the TME (96). In addition to their angiogenic properties, pericytes express the cluster of differentiation (CD) markers of MSCs. Their potential for multipotency contributes to metastatic processes by generating other stromal cells for the TME (97). Furthermore, pericytes have been shown to induce immune suppression through secretion of various soluble factors including prostaglandin E2 (PG-E2), TGF-β and nitric oxide (98). Pericytes are capable of regulating T cell trafficking and modulation. Pericytes of the TME were shown to express PD-L1 and PD-L2, responsible for T cell exhaustion (99). Retinal pericytes, too, exhibit immunosuppressive properties. When pericytes were cultured with activated T cells, production of IFN-γ and TNF-α decreased. Pericytes coexpressing CD248, CD90, and PDGFR isolated from human gliomas were able to inhibit cytotoxic T cell (CTL) proliferation, and thus induce immunosuppression within the TME (98, 100). Additionally, pericytes from normal brain tissue and malignant gliomas secrete immunosuppressive factors such as: PGE2, TGF-β, and NO, previously shown to inhibit anti-tumor response and suppressed mitogen-activated T cell activity (98). Pericytes produce growth factors, chemokines, cytokines, and adhesion molecules which regulate the microenvironmental ability to evade immune surveillance.

Cancer Stem Cells

The majority of cancer cells arise from cancer stem cells (CSCs) that express surface markers similar to that of stem cells (SCs), such as CD44, CD90 and CD133. It is uncertain whether CSCs arise from non-SCs or from somatic SCs (101, 102). The tumorigenic potential of CSCs was shown when leukemia-initiating SCs from AML patients were transplanted into severe combined immunodeficiency (SCID) mice, which later developed AML (103). In another study, CSCs and a non-CSC counterpart were injected into immunodeficient mice; only the CSC-injected mice were able to repopulate parental tumor cells (104). The theory of CSC is further supported by their discovery in breast, brain, colon, hematopoietic and lung cancer (101, 105). As the architects of the TME, CSCs are able to self-renew and drive the pathophysiologic mechanisms of carcinogenesis aided by various non-cancerous cells (101). CSCs possess both plasticity and immunomodulatory features capable of evading immune surveillance, thus they are the most distinguished malignant cell unit implicated in primary cancer or in resistance to immunotherapy. Bidirectional release of cytokines, cell-to-cell communication *via* extracellular vesicles, and fusion of CSCs with fusogenic stromal cells are mechanistic immunomodulatory properties of CSCs. Recent studies suggest that CSCs are pivotal players in immune escape: due to their immunomodulating nature, they are capable of cellular dormancy whilst evading immunosurveillance (106, 107). The tumor niche consists of intratumor immune cell populations which interact with CSCs and affect their functional status (108, 109). Undergoing cell-to-cell fusion (a process which generates tumor cell hybrids under pathological conditions) with various sorts of microenvironmental fusogenic cells such as: fibroblasts, macrophages, MDSCs and MSCs, the tumor niche contributes to the formation of aberrant cells that possess SC-like properties and are correlated with tumor initiation, progression, and metastatic potential (110, 111). CSC-related immune escape mechanisms are further complicated by epigenetic perturbations (112). Epigenetic modifications of differentiated cancer cells and CSCs can lead to expression modifications in immune-related genes. This domino effect impacts antigen presentation, processing, and immune evasion. For example, re-expression may be possible through demyelinating agents, allowing for immunotherapeutic applications (113). CSCs contribute to metastasis and tumor heterogeneity, implying their capacity for resistance to chemo-, radio-, and immunotherapies, and more besides (114). The principal limitation of efficacious anti-CSC treatment is the challenge in recognizing CSC-characteristic biomarkers.

Immune Cells

With regards to carcinogenesis, immune cells possess dual action dependent upon various chemokine expressions within the TME. It has been previously shown that Tregs, M2 macrophages and T-helper 2 cells (Th2) support tumorigenesis while NK cells, antigen-presenting cells (APCs), cytotoxic T cells (Tcs, CTLs) and M1 macrophages are protective against tumor development. High expression of chemokines such as CXC (1-16), with their respective CXC receptors (CXC-R), attracts various cancer-supporting immune cells that have been shown to be responsible for poorer prognosis in colorectal cancer (115).

Macrophages possess critical phagocytic properties in the adaptive and innate systems. Tumor-associated macrophages (TAMs) are derived from CCR2 inflammatory monocytes, and are classified as either pro-inflammatory (M1) anti-cancer cells through the production of IL-1 and tumor-necrosis factor alpha (TNF-α) (116) or anti-inflammatory (M2) cancer-supporting cells through the production of immunosuppressive cytokines such as IL-10 (117). M2 macrophages have been linked to progression in colon, renal cell and breast carcinomas (118–120) *via* multiple mechanisms. Primarily, anti-inflammatory cytokines and chemokines cause immune suppression by inhibiting T cells and NK cells (121, 122); chemokines CCL5, CCL20 and CCL22 recruit Tregs and activate their inhibitory actions *via* production of IL-10 and TGF-β1 (123). Secondly, angiogenesis is induced by the release of signaling protein WNT7B, which targets ECs for stimulation of VEGF (124). This produces another major angiogenic factor called pro-matrix metalloproteinase 9 (proMMP9) (125). M2 macrophages also facilitate carcinogenesis and metastasis through the production of CCL18 (126) and the nuclear factor- κB/FAK pathway (127) leading to induction, migration, invasion, and the EMT. Lastly, TNF is a product of both activated macrophages and the cells of the TME; in addition to being an anti-cancer cytokine, it has been implicated in the inflammatory process necessary for tumor growth (128). The role of STAT3 as a mediator between TAMs and tumor cells has been elucidated, showing that STAT3 activation inhibited Th1 subtype differentiation by blocking the expression of immune-stimulatory mediators (129).

Like TAMs, tumor associated neutrophils (TANs) demonstrate two subtypes: the N1 TAN phenotype which possesses anti-tumor action, and the N2 TAN phenotype which has tumor-support activity (130). Sustained inflammation induces an IL-8-dependent neutrophil chemotaxis within the TME (131). As previously described, TGF-β was shown to be highly expressed within the TME, inducing a generalized immunosuppressive state; additionally it was shown to polarize TANs into the N2 phenotype (130). N2 TANs sustain inflammation within the TME by releasing genotoxic elements such as NO and ROS (131). Tumor models have shown that N2-TAN-mediated immune suppression was achieved through various mechanisms: 1) production of TNF-α and NO to induce T cell apoptosis (132); 2) inhibition of T cell proliferation through modulation of PD-1/PD-L1 signaling and release of arginase (133); 3) N2-TAN expression of TGF-β, and 4) production of CCL17, shown to recruit Tregs to further induce an immunosuppressive state (134).

T cells, part of the adaptive immune system, prevent tumor growth through lytic action and the production of IFN-γ-dependent cell-cycle arrest (135). After lysis, the cell component is phagocytosed and expressed on APCs, exposing them to maturing lymphocytes and resulting in tumor suppression (136). Tregs impede the immune response by expressing various cytokines against anti-tumor cells. It has been shown that when the Treg-to-CD8 ratio is high in hepatic (137) and breast carcinoma (138), this results in uncontrolled progression and worse prognosis. Th2 is yet another cell responsible for promoting the necessary inflammatory state within the TME, and it has since been proposed as an agent in tumor progression. Countering the anti-tumor activity of Th1, Th2 has been associated with poorer prognosis when detected (128). Its differentiation is driven by thymic stromal lymphopoietin (TSLP), an IL-17-like cytokine produced in response to TNF-α and IL-1-β from TAMs and TME stromal cells (139).

APCs are innate cells which process and display antigens bound to major histocompatibility complexes (MHCs) to naïve T cells to induce cytotoxicity. They are categorized into professional (dendritic cells; DCs) and non-professional (fibroblast) APCs. It has been previously shown that because of the presence of IL-6 and granulocyte-colony stimulating factors (G-CSF), APCs of the TME lack the co-stimulatory receptor B7 and cannot stimulate T cell cytotoxicity. This alters the differentiation of APC to mature cells (140). Additionally, various signals within the TME induces differentiation of granulocytes to immunosuppressive cells such as TAMs and tumor-associated neutrophils (TANs) (141).

NK cells are an important innate component responsible for destroying tumor cells and preventing the progression of tumorigenesis. In the immunocompetent, NK cells select out the APCs with improper expression of MHC-I and retain a pool of competent APCs (142). However NK activation is greatly inhibited within the TME due to excess production of TGF-β and other anti-inflammatory cytokines and chemokines (143). Microarray analysis of extra-tumoral and intra-tumoral NK cells in the lung tumor microenvironment demonstrated upregulation of cytotoxic gene expression, and intra-tumoral NK cells were associated with better prognosis (144).

B cells are most common in draining lymph nodes. They have been shown to infiltrate tumor margins and have been associated with proper antibody response in ovarian and breast carcinomas (145, 146). On the other hand, B cells have been shown to differentiate into another tumor-associated cell. An IL-10-secreting B cell named Breg (147) promoted metastasis of breast cancer (148) and it has been shown to be implicated in inflammation-induced squamous cell carcinoma through the secretion of TNF-α in animal models (149). It should be noted that this B cell was non-infiltrating – that is, present only in the surrounding tissue – thus further studies are warranted to determine if they behave the same way in human cancers.

Little is known of myeloid-derived suppressor cells (MDSCs). They are identified as immature myeloid cells that are

upregulated in cancer and other inflammatory processes (150). Their phenotype is variable and their characterization is difficult. MDSCs can also differentiate into TAMs, as they both possess immunosuppressive markers such as CD115 and F4/80 (151). It has been shown that MDSCs are able to directly suppress CD8 cells by producing nitric oxide synthase-2 (NOS-2) and arginase (ARG-1) (71). Another immunosuppressive mechanism exhibited by MDSCs is their positive effect on T cell differentiation into cancer-supporting Tregs (152).

Dendritic cells (DCs), the so-called professional APCs, are among the first cells to appear during inflammatory states. Varying subsets of DC maturation have been observed in the TME (153); this typically comprises of only a few mature DCs and is associated with better prognosis (154). Generally, the previously described immunosuppressive states impair DC maturation and activation (155). As stated, the DC maturational stage is crucial for normal function. Multiple subsets have been identified including anti-tumor classical DCs with high CD8 and NK cell-activation activity (156); while plasmacytoid DCs (157, 158) and monocyte-differentiating DCs have either immune-supportive or immune-suppressive actions (153, 159). The known immune suppressor PD-L1 is highly expressed within the TME. Tumor derived factors directly increase the expression of PD-L1 in DCs and MDSCs, further inhibiting anti-tumor immunity (160).

Cancer-Associated Adipocytes

Adipocytes are known, key contributors to the TME and are proposed to be involved in the metastatic process, angiogenesis, and resistance to apoptosis (161). Cancer-associated adipocytes (CAAs) are a broad grouping of the following: intratumoral adipocytes, peritumoral adipocytes, recruited adipocytes, and *de novo* differentiation of MSCs into adipocytes or adipocyte-like cells that store large amounts of energy-rich lipids (162). It has been shown that mature adipocytes incubated with breast cancer cells induced phenotypic change of adipocytes into fibroblast-like cells that contributed to the expansion of CAFs, well-known immune suppressors (163). CAAs can influence the TME through direct contact with adjacent cells or in a paracrine manner through the production of adipokines, hormones and proinflammatory cytokines (i.e. CCL6, CCL2, CCL5, MMP, VEGF, TNF-α, insulin and leptin, to name a few) to facilitate cancer invasion and immune resistance (164). CAAs were shown to possess dysfunctional proinflammatory features that support the TME (165). CCL2 and CCL5 released from CAAs were shown to recruit and promote M2 polarization of macrophages (166). Furthermore, the high concentration of TNF-α and IL-6 mediated JAK2/STAT3 pathway activation to induce phenotypic change into breast cancer cells with SC properties (164). The important adipokine leptin was also shown to make use of the JAK-STAT3 pathway to induce cancer stemness and evade immune surveillance (167). In cachectic mice, phenotypic change in white adipocytes with overexpression of uncoupling protein 1 (UP-1) induced their differentiation into brown adipocytes with fibroblastic characteristics (168). Furthermore, signaling proteins within the TME (i.e. IL-6, exosomal contents, and parathyroid hormone related peptide PTHrP) were shown to promote phenotypic variations into brown adipocytes (169). Because PD-L1 is strongly expressed on brown adipocytes, PTHrP has been linked to tumor invasion and metastasis. Phenotypic variations leading to the differentiation from white to brown adipose tissue appears to be another immunosuppressive mechanism (170).

Extracellular Matrix

The extracellular matrix (ECM) contributes the largest component of the TME and is composed of proteins such as collagen, proteoglycans, hyaluronic acid and laminins (171). The ECM is crucial for the maintenance of the TME and the induction of metastasis. Aside from acting as a physical cellular scaffold, it is responsible for cellular adhesion and migration out of the TME. It stores various soluble factors such as angiogenic factors and chemokines that induce a continuous inflammatory state, resulting in expansion of the cellular repertoire (172). The continuous inflammatory state exacerbates the conversion of stromal fibroblasts into myofibroblasts (173) which in turn deposit large amounts of growth factors and ECM proteins, inducing contraction and increasing stiffness (174). Newly-deposited ECM proteins are acted upon by CAF enzymes such as LOX to further stiffen the ECM; stored growth factors are subsequently released to amplify the circuitry between the tumor cells and its ECM. This eventually contributes to metastasis and ensures ECM resistance to treatment (175). The ECM can influence the recruitment of immune cells into the TME. For instance, the ECM can drive PI3K/AKT (pro-survival pathway) activation, which facilitates CSC immune evasion (176). ECM proteins can also recruit immunosuppressive cells such as Tregs and TAMs which were shown to promote CSC survival while blocking anti-tumorigenic immune cell (i.e. CTL) recruitment (177–179). The ECM is capable of impairing the proliferation and activation of T cells, which are responsible for eliminating CSCs (180). The composition of the ECM also plays a crucial role in modulating the state of tumor infiltrating immune cells. For example, M2 polarization of macrophages is achieved in a periostin-rich or stiff collagen-rich ECM (181). After recruitment, CSC survival signaling pathways such as Src, STAT3/SOX2, Hedgehog, and NF-κB are activated by the M2 macrophages, leading to inhibition of T cell proliferation and activation through type I collagen-dependent fusion of LAIR receptors while sequestering T cell proliferation growth factors (177). In addition to the aforementioned, neutrophils and TAMs are capable of selectively recognizing the EMC in order to promote cancer growth as they are recruited to the microenvironment (182, 183). This implies the ability of the ECM to modulate immune surveillance in the CSC microenvironment.

An increase in metabolic stress and hypoxia leads to poor diffusion in ECM-rich tumors, ultimately up-regulating immunosuppressive factors such as: CCL22, CCL18, TGF-β, IL-10, VEGF-B, and PGE2 (184–186). TGF-β in particular acts as a suppressor of CD8 CTLs and NK cells in the TME by attracting Tregs and functioning as an M2-polarizing agent for macrophages (186–188). Both of these phenomena negatively regulate infiltration and activity of CTLs (189). In addition, T cells are suppressed by VEGF-A which recruits Tregs that express NRP1 (a coreceptor of VEGF) (190, 191).

Micro-RNA Deregulation

Micro-RNAs (miRNAs, miRs) are an endogenously-expressed class of non-coding single-stranded RNA fragments that are involved in gene expression modulation. By targeting mRNA at the post-transcriptional level, they may act as tumor suppressors or oncogenes (192). When deregulated they are associated with tumorigenesis and metastatic development (193). Oncogenic miR-21 overexpression in CAFs has been associated with tumor aggressiveness *via* induction of angiogenesis and treatment resistance (194, 195). MiR-155 and miR-210 over-secretion in cancer cells has been shown to induce the transition of MSCs and normal fibroblasts to CAFs, thus reinforcing the TME (196, 197). Overexpression of miR-17-to-92 may lead to downregulation of tumor suppressor genes, much like with oncogenes, inhibiting apoptosis *via* various pathways (198). Other types of miRNAs include the tumor suppressors comprising of miR-126, whose main function is to suppress MSC recruitment in the TME by inhibiting SDF-1 and CCL2. When downregulated, miR-126 has been shown to promote breast cancer metastasis by inducing fibroblast recruitment and EMT (199). MiRNAs represents another hurdle to consider when evaluating TME defenses. Cancer cell-derived immune modulatory miRNAs regulate a multitude of immune components such as CTLs, Tregs, NK cells, DCs, and MDSCs *via* intracellular communication (i.e. micro vesicles and exosomes). Cancer-derived miRNAs have been implicated in various mechanisms to induce immune evasion. This is achieved through the modulation of expression profiles using histone modification and DNA methylation (200). It has been shown that these epigenetic pathways occur simultaneously and act on each other, i.e. DNA methylation- or histone acetylation-induced deregulation of miRNAs, and vice versa (201, 202).

miRNAs: Modulating Antigen Processing and Presentation in Cancer

A number of miRNAs interrupt MHC-I and antigen-processing machinery (APM) components in cancer cells (**Table 1**). A study of nasopharyngeal cancer cells indicated that miR-9 targeted a multitude of APM constituents such as β2-microglobulin, low molecular weight polypeptide subunits LMP10, LMP9, LMP8, and transporter associated with antigen processing 1 (TAP1). MiR-9 has the potential to downregulate MHC-I molecules (i.e. HLA-H, HLA-B, HLA-C, and HLA-F) and its overexpression in cancer cells enhances immune tolerance in the TME (203). MiR-346 is an endoplasmic reticulum (ER) stress-associated miRNA which regulates the immune response by indirectly suppressing the IFN by targeting adenylate uridylate-rich elements (AREs) on the 3'-UTR region of mRNA transcripts resulting in termination; or by directly phosphorylating TAP1, resulting in interference of MHC-I signaling pathways (206, 207). Likewise, miR-125a-5p in esophageal adenocarcinoma cells bind to the 3'-UTR of TAP2 mRNA resulting in interference with antigen presentation (204). Proteomic screening of miR-27 decreased cell surface expression of MHC-I expression, promoting cancer progression; miR-27-a-induced MHC-I downregulation depended on calreticulin suppression (an essential calcium-binding protein which regulates gene transcription) (208).

miRNAs Targeting HLA-G

This non-classical MHC-I molecule has immune inhibitory function, and it can be hijacked by cancer cells to escape immune attack. When HLA-G binds to NK cells and CTLs, the effector cell cytotoxicity is suppressed (209). HLA-G expression is elevated in cancers such as endometrial, breast, melanoma, gastric, hepatocellular, lung, and colorectal carcinoma (210, 211). The increase in HLA-G expression correlates to the loss of regulatory HLA-G-targeting miRNAs such as miR-152, miR-148a, and miR-148b (212, 213). For instance, the oncogenic estrogenic G-protein-coupled estrogen receptor 1 (GPER) signaling pathway is known to decrease miR-148 levels in breast cancer cells, contributing to cancer immune evasion (214).

miRNAs Associated With Immune Checkpoint Ligands

Immune checkpoint signaling is determined by factors including pre-existing inflammation of the oncogenic signaling pathway. Studies indicate that an increase in PD-L1 expression on numerous cancer cells was achieved by a loss of miR-138, miR-34a, miR-191-5p, miR-148-3p, miR-873, miR-479-5p, miR-195-5p, and miR-3609. A decrease in miR-383 was shown to profoundly elevate PD-L1 expression on cervical cancer cells (215–220). In contrast, PD-L1 expression is promoted by miR-18a *via* SOX6, WNK2, and PTEN signaling pathways. After induction of PD-L1 expression, various

TABLE 1 | Cancer antigen processing and presentation, regulated by miRNAs.

Cancer cell type	miRNA	miRNA target	Reference
Nasopharyngeal cancer	miR-9	β2-microglobulin	(203)
Nasopharyngeal cancer	miR-9	LMP9/10	(203)
Nasopharyngeal cancer	miR-9	LMP8	(203)
Lung cancer	miR-451		
Nasopharyngeal cancer	miR-9	TAP1	(203)
Esophageal adenocarcinoma	miR-125a-5p	TAP2	(204)
- Nasopharyngeal cancer	miR-9	MHC-I	(203–205)
- Esophageal adenocarcinoma	miR-148a-3p		
- Colorectal cancer	miR-27a		

LMP, low molecular weight polypeptide subunit; TAP, transporter associated with antigen processing; MHC-I, major histocompatibility complex class I.

pathways (Wnt/beta-catenin, ERK, and PI3K-AKT) were activated, ultimately leading to PD-L1 transcription (221).

Phenotypic Variations Induced by miRNAs

MHC-I chain-related molecule A and B (MICA, MICB) (222), and UL16-binding protein (ULBP) are stress-induced ligands which are recognized by the presence of NKG2D present on CTLs and NK cells (223). NKG2D is responsible for maintaining cancer immune surveillance, and is downregulated in cancer cells, resulting in cancer cell immune escape at the post-transcriptional level (224). Alternatively, it has been shown that various miRNAs directly target the ULBP2 3'-UTR, and overexpression of these miRNAs lead to downregulation of ULBP expression. Such miRNAs include miR-34a, miR-34c in malignant melanoma and miR-519a-3p in breast cancer (202, 225).

MiRNA mimics can inhibit receptor expression hereby diminishing tumor cell recognition by NK cells. MiRNAs function at the post-transcriptional level of gene expression, and both miRNAs and IFN-γ downregulate expression of the MICA ligand. MiRNA targets MICA/B mRNA by directly binding to the 3'UTR of the target gene, causing mRNA degradation or translation repression. MiRNAs that target MICA include miR-93, miR-106b, miR-106a, miR-373, miR-20a in hepatocellular carcinoma (HCC), miR-519a-3p, miR-20a in breast cancer, and miR-125b in multiple myeloma (202, 226, 227). MiRNAs that target MICB include miR-20a in breast cancer (228), and miR-302c and miR-520c in multiple cancers (228, 229). This leads to immune escape of malignant cells.

miRNAs Relative to Cancer Cell Metabolites

Tryptophan (Trp) is an example of a metabolite responsible for maintaining the function of tumor infiltrating lymphocytes (TILs). The rate limiting enzyme of Trp metabolism, converting Trp to 3-hydroxyanthranilic acid and kynurenine, is IDO1 (230). Increase in IDO1 expression with concurrent decrease in Trp leads to dysfunctional Teffs, permitting cancer immune evasion (231). The downregulation of miR-218 and subsequent elevation of IDO1 has been shown to safeguard cervical cancer cells from immune attack (232).

Cancer Cell-Derived miRNAs Which Regulate Immune Evasion Through Vehicles or Exosomes

Cancer-derived miRNAs are capable of exhibiting extracellular bio-activities through microvesicles or exosomes as well as modulate the expression profile within cancer cells (233). Cancer-derived miRNAs can be transferred *via* exosome to several TILs in order to mold an immunosuppressive microenvironment. CAFs are immune cells regulated by cancer cell-derived exosomal miRNAs. They can be reprogrammed by various miRNAs to induce tumor progression (234). MiRNAs released into the TME by CAFs function as paracrine stimuli for the activation of adjacent fibroblasts and cancer cells. Exogenous overexpression of miRNAs leads to fibroblast-to-CAF-like cell conversion, resulting in immune suppression (235). Some examples of CAF-derived miRNAs include miR-21 and miR-1247-3p in HCC, and miR-27a in gastric cancer (236–238).

Notably, MDSCs are another class of immune cells which are regulated by cancer cell-derived exosomal miRNAs; miR-17-5p (breast cancer) and miR-20a (in several cancers) (239, 240) promote the STAT3-mediated suppressive function of MDSCs. Additionally, miR-21 and miR-155 show associations with STAT3 activation through the phosphatase and tensin homolog (PTEN) target, along with SHIP1, leading to MDSC expansion in both granulocytic and monocytic subpopulations (241).

TAMs are also immune cells derived from exosomal miRNAs, and can be activated through two pathways: M1 (classical pathway), and M2 (alternative pathway); two perform different regulatory functions in the TME (242). Several miRNAs engage in the polarization into M2 macrophages, which inhibits immune surveillance. For instance, miR-21 regulates TAM through IFN-γ/STAT1 and PTEN to promote M2 polarization, increasing tumor cell migration while decreasing PD-L1 expression and M1 polarization (243, 244). Additionally, miR-324 in colon cancer targets CUEDC2, which regulates TAM to increase pro-inflammatory cytokine production as well as further increase tumorigenesis (245).

Exosomes

These extracellular micro-vesicles contain components of proteins, lipids and genetic materials of the parent cell (246), and are potent signaling molecules within the TME. Exosomes arising from both malignant and non-malignant cells have been shown to be involved in tumorigenesis, therapy resistance, and immune resistance (247).

Homotypic transfer of exosome refers to signal transfer between cancer cells. Glioblastoma cell exosomes were shown to induce change in wild-type cells *via* transfer of the oncogenic protein epidermal growth factor receptor 3 protein (EGFR-v-III) (248). Similarly, it has been shown that exosomes from breast cancer cell lines and breast cancer patients, which contain miRNA machinery, were able to induce malignant transformation in normal cells (249). Another study showed that exosomes arising from pancreatic adenocarcinoma were able to modify the NOTCH-1 pathway and inhibit cellular death (250). Homotypic exosome transfer promotes cancer progression *via* the oncogenic pathway.

Heterotypic transfer of exosome, as previously described with regards to tumor growth and dissemination, is widely dependent upon its TME. Cellular crosstalk between the TME and either internal or external components is crucial for TME survival; this is achieved through multiple signaling networks such as paracrine and juxtacrine pathways (251). A study was conducted to spatially separate the TME. The complexity of the system was observed, with the authors demonstrating a vast cellular heterogeneity that consisted of six interacting layers of cells (252). Heterotypic transfer of exosome not only supports tumor growth but also elicits cellular resistance to various therapies as well as the harsh conditions within the TME (247).

Cancer Cells

Aside from the immunosuppressive TME, cancer cells themselves when exposed to CTLs were shown to evade immune surveillance by modifying intrinsic mechanisms. These include expression

downregulation of tumor associated antigens (TAAs), expression upregulation of PD-L1/2, and mutation induction within the antigen-binding machinery (β2-macroglobulin and HLA) and extrinsic pro-apoptotic genes such as CASP8 (253, 254). In addition, it was recently shown that clonal expansion of TAAs strongly correlated with the intensity of the immunogenic response (255). In analyzing the tumor genomic landscape, two mechanisms for TAA loss were observed:

1-immune-mediated elimination of TAAs presented by immune cells, followed by outgrowth of the remainder following "Darwinian evolutionary theory";

2- acquisition of one or more genetic alterations, resulting in TAA loss and subsequent expansion of resistant clones (256). In determining how the EMT may contribute to immune escape, a study demonstrated that after prolonged exposure of breast carcinoma cells to CTLs, expression to TNF-α or *via* stable expression of SNAIL was increased. Protection from CTL-mediated lysis was linked to the activation of the autophagy pathway, which led to the survival of cells through dormancy (257). Impairment of CTL-mediated lysis was evident in another study in which breast carcinoma cells elicited increased TGF-β expression by silencing the Wnt1-inducible signaling pathway protein 2 (WISP2), which resulted in stemness (258, 259). Autophagy was not evident in the resistant phenotype; however inhibition of TGF-βwas able to induce EMT reversal thus rendering cancer cells more sensitive to CTLs (260). This suggests that chief developmental pathways utilizing TGF-β are fundamental in mediating immune resistance to CTLs. It is evident that along the EMT spectrum, several mesenchymal cancer cell variants have the potential to engage further mechanisms of resistance.

Tumor hypoxia is a significant parameter, as a driver of the EMT, tumor immune escape, and heterogeneity (261). Cells derived from a lung adenocarcinoma model were induced by hypoxia, and demonstrated a shift towards mesenchymal phenotypes. Only some cells underwent the EMT thus promoting cancer cell heterogeneity (262). Hypoxic stress leads to the emergence of cancer subclones, and analysis of these cells showed an increased tendency to resist CTL-mediated lysis. Of note, the resistance mechanism is suspected to be independent of E-cadherin-CD103 interaction. This is because TGF-β inhibition minimized cellular resistance to CTL-mediated killing without causing any changes to the E-cadherin expression in mesenchymal cancer cells (262).

CTLs primarily utilize the perforin/granzyme pathway to demolish target cells. When the perforin pathway is activated, further counter-mechanisms such as Fas or TRAIL are engaged at the cancer cell surface to induce T cell apoptosis (263). The pancreatic carcinoma model was used and given an EMT inducer in the form of the novel tumor antigen Brachyury. The cancer cells showed decreased susceptibility to CTL-mediated killing compared with control. Target cancer cells were co-cultured with CTLs, and poor killing was observed under experimental conditions. This was due to defective caspase-dependent apoptotic cell death despite immune antigenicity (264, 265).

Additionally, defects in the APM – correlated to immune-proteasome deficiency – was found to be common among cancers with a greater mesenchymal profile, and ultimately affected T cell-mediated cytotoxicity (266). Manipulation of cell-to-cell interactions and immunological synapses (IS) has been linked to immune resistance.

The IS involves interactions between immune killer cells (NK cells and CTLs) with their APCs or targeted cancer cells necessary to achieve maturation and production of TNF-α and IFN-γ, and their lytic functions (267–269) IS formation in T cells is regulated by cytoskeletal elements (i.e. actin), interaction of MHC-TCR, and the integration of integrin-based signals, generated when integral molecules (lymphocyte function-associated antigen 1, LFA-1) on the T cell interact with ICAM-1. Integrins undergo conformational changes through phosphorylation cascades (i.e. phosphotyrosine kinase activation linking integrins to the actin cytoskeleton) during peptide-MHC/TCR ligation. The actin cytoskeleton polymerizes at the edges of the active synapses, causing an increase in synaptic diameter size and immune cell flattening (270). This phenomenon leads to the emergence of T cell receptor (TCR) microclusters. These clusters merge at the center of the IS zone and are referred to as the central supramolecular cluster. In contrast, microclusters found at the periphery of the synapse join to form a highly contractile zone called the peripheral supramolecular activation cluster (271).

Mechanical forces brought about at the synapse *via* intercellular adhesion also play a role in rearranging the actin cytoskeleton and regulating adhesion-based signals. IS and its relationship with NK cells abide by similar rules, differing only in that NK cells express 2B4, DNAM1 and NKG2D receptors, rather than TCR. These receptors also regulate signaling activity and the changes in the integrin-actin network at different points of NK cell cytotoxicity. Numerous genetic aberrations have been shown to alter various stages in CTL and NK cell cytotoxicity, F-actin/microtubule networking, and cellular recognition which ultimately leads to NK or T cell disorders, resulting immunodeficiency (272, 273). These examples highlight the role of the operational IS in an appropriate and effective immune response.

The establishment of the IS and activation cascades relies on heterophilic interactions between ICAM-1 and integrins on target cells; the loss of ICAM-1 can be expected to impede IS formation. Moreover, manipulation of the actin network through changes in mechanical forces renders a significant effect on the IS and the lytic commitment (274, 275). The discomposure of the actin network in certain cells will either render them more resistant or more susceptible to CTL-mediated lysis (276).

See **Figure 1** for a diagrammatic summary of the major pathways that promote immune resistance, and immune and treatment resistance.

CHARACTERISTICS OF THE TME AND ITS EFFECTS ON TREATMENT RESISTANCE

The Tachyphylactic TME

Epigenetics: The Link to Treatment Resistance

To unleash, hijack, and restrict cellular plasticity, CSCs play a chief and fundamental role in epigenetics. In cancers, one of the

most habitually mutated gene classes are epigenetic regulators, resulting in this characteristic uncontrolled cellular self-renewal. Epigenetic regulator mutations lead to oncogenic cellular reprogramming during cancer initiation. CSCs are either promoted or inhibited by the epigenetic mechanisms that integrate the cell-extrinsic (microenvironmental signaling), or cell-intrinsic (subclonal mutations) effects that establish intratumoral heterogeneity. Over time, CSCs generate self-renewing subclones with diverse fitness, whilst environmental changes are able to act on their genetic heterogeneity and modulate their phenotype. Further discussion on the CSC mechanistic roles and implications now focuses on how cellular plasticity can be affected by manipulation of DNA methylation and chromatin. In addition to the previously described role of miRNA, the following sections will shed light on epigenetic DNA methylation and histone modification leading to the development of CSCs, followed by the role of CSCs in drug resistance (277).

Pathways Involved in CSC Development

Wnt/β-Catenin Signaling Pathway

β-catenin is transcription co-activator regulated by the WNT gene family, and is mainly involved in embryonic development, adult homeostasis, and, if highly expressed, various cancers (278, 279). Physiologically, the absence of WNT signaling keeps β-catenin at low levels through the ubiquitin-proteasome system (UPS). β-catenin is recruited into a destruction complex consisting of the adenomatous polyposis coli gene (APC gene) and Axin. This promotes the phosphorylation of β-catenin by glycogen synthase kinase 3β(GSK-3 β) and casein kinase 1 (CK1), which tags β-catenin and subsequently goes through UPS. Stabilization of β-catenin occurs with Wnt ligand binding to Frizzled receptors, allowing the degradation complex to be inactivated *via* low density lipoprotein receptor-related protein 5/6 (LDR5/6) and Disheveled. β-catenin accumulates and translocates into the nucleus where it couples with T cell factor/lymphoid enhancer factor (TCF/LEF) transcription

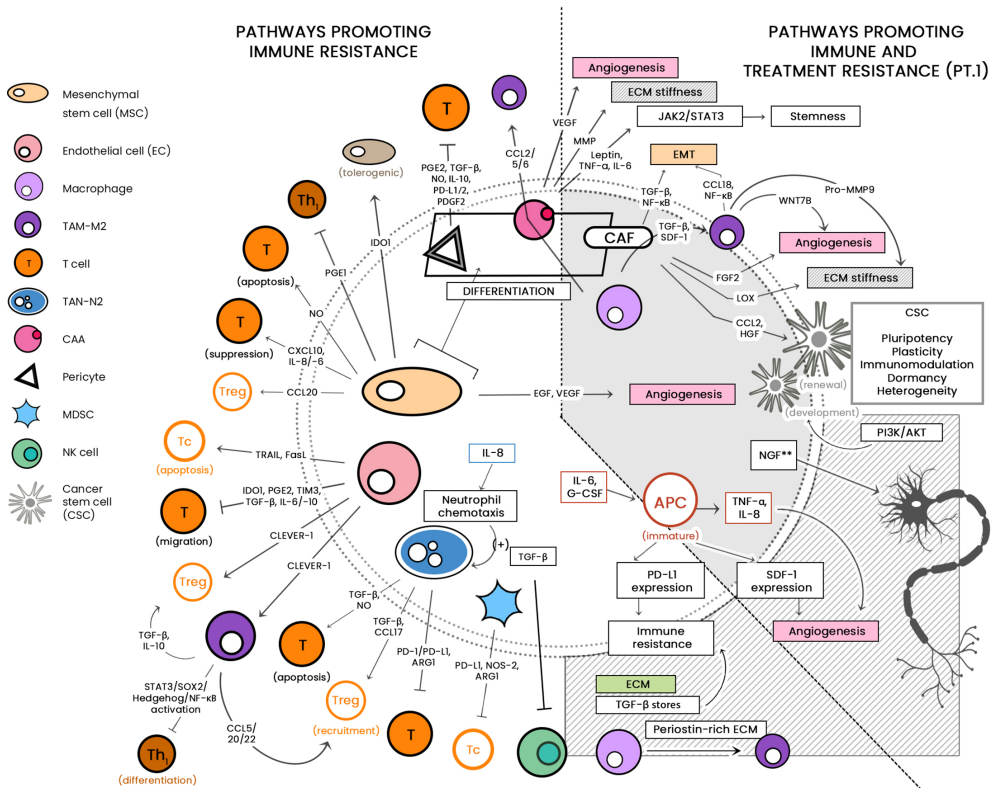


FIGURE 1 | The large cellular repertoire of the tumor microenvironment (TME) is depicted in this diagram. Through the release of soluble factors, the presented cellular entities are seen to be involved in: 1) immune suppression, by either inducing apoptosis or inhibiting anti-tumor activity; and 2) both immune/drug resistance by stiffening the extracellular matrix, inducing epithelial-to-mesenchymal transition (EMT) and induction of stemness. CCL, C-C motif chemokine ligand; PG-E2, Prostaglandin E2; TGF-ß, Transforming growth factor beta; NO, nitric oxide; IL, interleukin; IDO-1, indoleamine 2,3-dioxygenase 1; TRAIL, TNF-related apoptosis-inducing ligand; Fas-L, Fas-ligand; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; CLEVER-1, lymphatic endothelial and vascular endothelial receptor-1; PD-1/-L1, programmed cell death protein 1/ligand 1; Arg-1, arginase; NOS, nitric oxide synthase; JAK/STAT, Janus kinase/signal transducer and activator of transcription; ECM, extracellular matrix; CSC, cancer stem cell; PI3K/AKT, phosphatidylinositol 3 kinase/protein kinase B; NGF, neurotrophic growth factor; TNF-α, tumor necrosis factor alpha; SDF-1, stromal-derived factor-1; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor. **associated with treatment resistance; mechanism as-yet-unknown.

factors to induce transcription of WNT target genes, Cyclin D-1 (CCND1), c-MYC, and Jun. β-catenin plays a crucial role in the self-renewal and differentiation of CSCs (278, 280, 281). The anomalous activation of Wnt/β-catenin is either through genetic alterations such as mutations in CTNNB1, the APC gene and AXIN genes, or through epigenetic modulation (282–284).

In breast and colorectal cancers, aberrant Wnt/β-catenin pathway activation is carried out by DNA methylation in the promoter region and silencing of multiple Wnt inhibitors such as Wnt inhibitory factor 1 (WIF-1), AXIN2, Secreted frizzled-related protein 1 (SFRP-1), and Dickkopf-related protein 1 (DKK1) (285–287).

Histone modifications are also implemented in the deregulation of the Wnt/β-catenin pathway in cancer. Decreased acetylation of H3K16 and increased H3K27 trimethylation, along with the recruitment of Sirtuin 1 (SirT1), enhancer of zeste homolog 2 (EZH2) and suppressor of zeste 12 protein homolog (Suz12) (components of polycomb repressor complex 2, PCR2) to the DKK1 promoter inhibits the expression of the DKK1 Wnt antagonist (288). Bivalent histone modifications, activating H3K4me3 and repressing H3K27me3 histone marks at its locus, are implemented in colorectal cancer by regulating Disheveled-binding antagonist of β-catenin 3 (DACT3). In turn, DACT3 expression in colorectal cancer lines is decreased, with overexpression of Wnt/β-catenin and CSC induction (289).

Hedgehog-Signaling Pathway

As an important mediator of embryogenesis and tissue homeostasis, Hedgehog (Hh) signaling has been shown to maintain SC and regulate the proliferation of progenitor cells (290). In the absence of the Sonic Hedgehog ligand (SHH), inhibition of Smoothened (Smo) protein by Patched receptor (PTCH-1) activates kinesin family member 7 (Kif7) and suppressor of fused homolog (SUFU), resulting in sequestration of Gli proteins which function as transcription factors. Moreover, upon binding of SHH to PTCH-1, Smo activates Hh signaling by releasing Gli protein back to the nucleus and exerting transcription of target genes (291). The implication of Hh mutation-induced signaling alterations in SCs has been well-documented in medulloblastoma and basal cell carcinoma. The upregulation of SHH within hair follicles or the interfollicular dermis in basal cell carcinoma was shown to contribute to tumorigenesis (292, 293). Moreover, granule neuron progenitors, identified as the medulloblastoma cell of origin, were seen to possess high levels of Hh signaling activity (294). In addition to genetic mutations, epigenetic factors were also seen to impact Hh-signaling. The chromatin remodeling protein SNF5 was seen to directly alter Hh signaling by interacting with Gli, resulting in the downregulation of PTCH-1 and resultant loss of the Hh inactivation feedback loop (295). Furthermore, it has been shown that hypomethylation of the SHH promoter allowed NF-κB to bind to the promoter, resulting in higher expression of SHH in gastric and breast cancer cells (296). Overexpression of SHH has been linked to CSC renewal and cancer aggressiveness (297).

Notch Signaling Pathway

The Notch signaling pathway is a highly-conserved cell signaling system that plays a major role in the regulation of embryonic development. It also regulates cellular proliferation and differentiation amongst a vast range of cell types and stages of cell maturation. Its cell-dependent signaling consists of the binding of ligands Jagged-1/-2 or Delta1-4, which triggers cleavage of the Notch intracellular domain (NICD) by γ-secretase, followed by release into the cytoplasm (298). This allows for modulation of SC differentiation and self-renewal, crucial for the survival and maintenance of neural stem cells (NSCs) (299).

In multiple myeloma, epigenetic histone acetylation causes overexpression of Jagged-2 ligand (300). Histone acetylation is governed by histone deacetylase (HDAC), and the recruitment of HDACs to the promoter regions is usually carried out by nuclear co-repressor silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). In multiple myeloma, decreased levels of SMRT reduces HDAC recruitment to the Jagged-2 promoter, which in turn increases histone acetylation and increases Notch ligand transcription, ultimately resulting in overactivation of the Notch signaling pathway.

Serine-threonine kinase receptor-associated protein (STRAP) promotes tumorigenesis and stemness by stabilizing intracellular fragment of NOTCH3 (ICN3), notable in colorectal cancer. STRAP inhibits histone methylation of H3k27 at the HES5 and HES1 promoters, leading to gene overactivation and inducing treatment resistance (301).

Cancer Stem Cells: Drivers of Therapy Resistance

CSCs and EMT-induced heterogeneity convey resistance to chemotherapeutic agents such as cisplatin, gemcitabine, and 5-fluorouracil (5-FU) (302, 303). Pancreatic cell lines exhibiting resistance to gemcitabine expressed high ZEB1 and low E-cadherin, thus acquiring great migratory ability (304). Tumor cell response to therapy may largely be due to epigenetic modulations.

With the enhanced expression of drug efflux transporters such as multidrug resistance-associated protein 1 (MRP1) and ATP-binding cassette sub-family G member 2 (ABCG2), drug resistance is increasingly observed (305–307). Transporter expression is regulated by various pathways and mechanisms, and deregulation results in protein enrichment and drug efflux. Notch signaling upregulates MRP1 expression and is responsible for CSC drug resistance (308, 309). The modification of histones (decreased HDAC1, and increased H3K4 tri-methylation, H3S10 phosphorylation, and histone H3 acetylation) leads to upregulation of ABCG2 expression. Along with decreased H3K9 tri-methylation, this allows for chromatin remodeling protein Brahma-related gene 1 (Brg1) and RNA polymerase II to gain access to the promotor, ultimately activating ABCG2 transcription (310). As a result of aberrant epigenetic modifications, physiologic SCs are susceptible to deregulation that facilitates tumor progression and invasion. Epigenetic regulation of signaling pathways is thus a potential target for anti-CSC therapy.

Heterogeneity is omnipresent in mammalian cells, and fundamental with regard to CSCs (311). The complicated picture of CSC heterogeneity involves dynamic cell populations capable of undergoing spontaneous state transitions; spontaneous switches from non-SCs to stem-like cells was observed in a study of breast cancer cells where plasticity was regulated by ZEB1 (312, 313).

CSC heterogeneity and plasticity in various cancers varies from patient to patient, but phenotypically distinct CSC markers may be identified depending on the tumor genotype (311, 314). Non-CSCs and CSCs in breast cancer exhibited a dynamic equilibrium that was maintained by cytokine-mediated crosstalk among marked populations. This suggests that cancers have reversible phenotypic plasticity and do not solely depend on genetic variation (315, 316). Colorectal cancer studies have provided compelling evidence demonstrating CSC plasticity and tumor progression. The Wnt target gene LGR5 acts as a functional CRC marker. Anti-cancer drug therapy resulted in the conversion of LGR5+ into LGR5- cells, while in the absence of the drug, LGR5- reverted back to LGR5+ (317). CSCs have been shown to overcome DNA damage induced by radio/chemotherapy. Furthermore, they acquire resistance through overactivation of DNA repair mechanisms such as the expression of excision nucleotide repair protein ERCC1 and overexpression of cell cycle checkpoints (318, 319). CSCs have also been shown to inactivate cell cycle gene expression as well as apoptosis-inducing genes such as p53 and c-MYC, creating so-called "undruggable phenotypes" (320). The activation of autophagy pathways after exposure to cytotoxic agents induces apoptosis; unfortunately, this mechanism is a double-edged sword and has been shown to instead enable CSCs and a heterogeneous subpopulation of cancer cells to tolerate the cytotoxic agents and TME-induced stress. These cells enter a state of dormancy and degrade key transcription factors (i.e. p53) to prolong cellular survival until TME conditions become favorable for growth and proliferation (321, 322).

Hypoxia: The Master Regulator of Cellular Heterogeneity

Hypoxia develops as a result of malignant cell overgrowth relative to their angiogenic requirements. To elicit cellular viability and progression, tumor-associated cells increase secretion of hypoxia-inducible factors (HIFs), mainly HIF-1-α and HIF-2-α, which in turn regulates angiogenesis in a chaotic manner (323). Under normal conditions, HIF-1-α is kept in check by hydroxylase enzymes which are dependent upon intracellular oxygen concentration. They are ubiquitinated and degraded after tumor suppressor protein von Hippel-Lindau complex formation. In hypoxic conditions, hydroxylation is diminished resulting in the overexpression of HIFs (324). The resultant chaotic blood vessel formation leads to irregular oxygen delivery and decrease in oxygen perfusion leads to necrosis (325). Drug distribution varies greatly between well-perfused and hypoxic areas, and effective cancer therapy requires efficient tumoral penetration; this patchy blood vessel distribution unfortunately results in tumor cell survival (326). HIF-1-α has been shown to upregulate various transcription factors (i.e. ZEB1/2, TWIST and SNAIL) that reduce E-cadherin expression, which results in EMT (327). Additionally, HIF-1-α activates focal adhesion kinase and steroid receptor coactivator (FAK-Src) which also decreases E-cadherin, promoting the EMT and VEGF-dependent angiogenesis and drug resistance by formation of SC-like phenotypic variants resistant to chemotherapy (328, 329). Intra-tumoral hypoxia induces a harsh environment that is crucial for cellular heterogeneity. The reprogramming of cellular phenotypes and metabolism drives adaptation and enhances signaling pathways leading to treatment resistance. As such, it is associated with poor prognosis (330). In a recent study, different patients with the same cancer type were shown to possess different inter- and intra-tumoral phenotypes. Very low oxygen concentrations correlates with an increase in mutational load in individual cells, and in varying the degree of hypoxia in each patient, alternations in tumor suppressors and oncogenes as such Myc, PTEN, and TP53 was elicited (331). HIF-1-α was shown to be a key player in the regulation of multiple metabolic pathways (i.e. amino acid metabolism, lipid metabolism, glycogenesis, and the TCA cycle) which ensures cancer cell sustenance and resistance to treatment (332, 333). A robust understanding of the HIF-1-α expression pathomechanism is required before we may implement effective therapeutic regimens.

Metabolism of the TME

Lactate Metabolism

Metabolic reprogramming occurs when it is necessary to increase cellular proliferation under hypoxic conditions. It has been shown that cancer cells increase metabolism of reactive oxygen species, lactate, lipids, amino acids, glutamine and glucose (334). Under normoxic conditions, normal cells general energy through oxidative phosphorylation, while cancer cells employ lactate metabolism and glycolysis. It was previously shown that tumor cell production of lactate occurs via: 1) glycolysis using lactate dehydrogenase (LDH), which converts pyruvate into lactate, bypassing the TCA cycle; and 2) glutaminolysis which forms various metabolites, including lactate and pyruvate, allowing the cell to hijack the TCA cycle and utilize glucose-derived metabolism for better efficiency (335, 336). As glucose concentration within the TME is scanty, numerous tumor types (i.e. lung adenocarcinoma, pancreatic adenocarcinoma, and more) have shown very high expression of lactate dehydrogenase which is known to induce the EMT (337). Furthermore, a high-lactate TME has been shown to reprogram TME cells. The high lactate environment prevents the proliferation of cytotoxic and effector T cells while promoting immunosuppressive Tregs (338); it has also been shown to induce M2 polarization of TAMs, subsequently leading to recruitment of other Tregs to enhance the protection of the TME. High lactate content promotes survival of hypoxic cells by inducing angiogenesis (339). Glutaminolysis provides a source of nitrogen, carbon, and energy to fuel the stromal and cancer cells (340). A recent study pointed out the importance of glutamine metabolism, demonstrating that breast cancer cells used the pyruvate metabolite within the TME to effect ECM remodeling, inducing cancer cell stemness and resistance to anti-tumor agents (341, 342). The role of lactate in treatment resistance has been well documented. After irradiation of non-small cell lung cancer (NSCLC), mice xenografts showed resistance within six weeks (343). The importance of lactate as a key molecule in resistance mechanisms has been further elucidated in epidermal growth factor receptor (EGFR) and tyrosine kinase- (TK) targeted therapies. These treatment modalities prompted cancer cell lactate production, which directed TME cells to produce hepatocyte

growth factor (HGF), ultimately resulting in EMT and resistance (344). Lactate metabolism was shown to increase DNA repair mechanisms by exploiting DNA-dependent protein kinases (DNA-PK), rendering cells resistant to cisplatin and doxorubicin (345).

Lipid Metabolism

Most neoplasms of organs and tissues are associated with adipocytes. The high rate of cellular proliferation demands abundant fuel *via* a process called lipid metabolic reprogramming. Lipid metabolism reprogramming has been correlated with resistance to conventional chemotherapeutic agents. Lipid and lipoproteins result from either catabolic processes or *de novo* synthesis (346). *De novo* fatty acid synthesis – lipogenesis – is controlled by the upregulation of lipogenic enzymes, and several crucial lipogenic enzymes such as fatty acid synthase (FASN), acetyl co-A carboxylase, stearoyl-CoA-desaturase-1 (SCD-1) and ATP citrate lyase are highly expressed in most neoplastic cells (347). High lipogenic enzyme concentration is correlated to invasion and worse prognosis (348). Upregulation of the prominent enzyme FASN is complex. It may be mediated by various growth factors, such as EGFR, HER2, steroid hormone receptors-androgen receptors, estrogen receptors and progesterone receptors; release is induced by the harsh conditions of the TME, or may result from post-translational miRNA modifications (349). Another key contributor in lipogenesis is SCD1, which is upregulated by growth factors (i.e. EGFR, PDGF, TGF-β) within the TME, and has been associated with treatment resistance and worse prognosis (350, 351). Various studies showed that inhibiting FASN and SCD1 action in lipogenesis led to tumor regression and improved responsiveness to prior therapeutic resistance (352). Another means by which various cancers may derive energy metabolites is *via* lipolysis. Overexpression of fatty acid-binding protein-4 (FABP-4), which induces lipolysis, has been shown to contribute to rapid tumor growth, metastasis in ovarian cancer and resistance to carboplatin (353). CAAs provide cancer cells with exogenous free fatty acids through cancer cell phenotypic expression of surface fatty acid translocase (CD36) through the fatty acid beta-oxidation (FAO) pathway (162). The CD36+ subpopulation have been shown to be more aggressive and resistant to treatment (354). In another study of radiotherapy-resistant breast cancer cells and breast cancer SCs, carnitine-palmitoyl-transferase-1a-and-2 (CPT1a/2), a known contributor to the FAO pathway was shown to be highly expressed. When CPT was knocked out by genetic editing techniques, this rendered previously-resistant cells sensitive to radiotherapy (355). The TME demonstrates atypical lipid metabolism for cell membrane formation and production of energy (356). Lipid metabolism has been linked to cancer growth, recurrence (357) and CD8 T cell exhaustion *via* the upregulation of programmed-cell death protein-1 (PD-1) (358) resulting in post-chemotherapy evasion of immune surveillance. The derangements of lipid metabolism are especially crucial for CSCs as the high ectopic metabolism of lipids has been linked to CSC formation, self-renewal and pluripotency (359). In obese breast cancer patients, sustained elevation of IL-6 and FGF-2 was observed. Obese mouse breast cancer xenografts also showed resistance to anti-VEGF therapy; the pathomechanism is hypothesized to be the constant release of proinflammatory cytokines by adipocytes. IL-6 and FGF-2 blockade restored sensitivity of cancer cells to anti-VEGF therapy (360). The association between drug resistance and lipid metabolism reprogramming has been well-documented in the literature (**Table 2**).

Reactive Oxygen Species Metabolism

Reactive oxygen species (ROS) elevation is closely related to cancer severity due to its influence over tumor immunity, tumorigenesis, and cellular reprogramming (369). Under hypoxic conditions, HIFs are activated by local mitochondrial ROS, and are therefore implicated in angiogenesis (370). ROS are produced by various cells within the TME, inducing activation of the KRAS pathway and promoting tumorigenesis (369). ROS were shown to play a critical role in the activation of TAMs, MDSCs and CAFs, enhancing their immunosuppressive roles (123, 371). Therapy resistance remains the most challenging barrier in cancer treatment. The pioneer in cancer metabolism, Otto Warburg first observed that cancer cells rely on glycolysis rather than oxidative phosphorylation, and this shift from oxidative to reductive metabolism is now termed the "Warburg effect" (336). Although glycolysis is considered an inefficient mode of energy production, ATP can be provided to cells at a faster and safer rate compared to the TCA cycle, which induces more stress *via* ROS formation (372). Upregulation of the glycolytic pathway aids cellular proliferation by shunting metabolites (glycine, serine, alanine) and nucleotides to the pentose phosphate pathway (PPP) (373). Transketolase, a key enzyme in the PPP was shown to increase pyrimidine synthesis and induce resistance to gemcitabine (374). ROS are the consequence of aerobic metabolism, and the major sources are peroxisomes, mitochondria and NADPH oxidase. Under physiologic conditions, redox homeostasis with low levels of ROS is maintained through fluctuations in generation and elimination processes, as an elevation in ROS is detrimental and leads to cell death. In cancer cells, metabolic derangement and oncogenic signaling induces high production of ROS (375, 376). Mitochondria are susceptible to ROS-induced oxidative damage, which usually results in elevation of NADPH oxidase expression. This in turn favors glycolysis and decreases the intrinsic production of ROS (377, 378).

- ROS-mediated maintenance of glycolysis: Pyruvate kinase (PK) is a rate limiting enzyme of the glycolytic pathway, and appears in two isoforms termed M1 and M2. PKM1 has high kinase activity and is present in physiologic conditions whereas PKM2 exhibits low pyruvate kinase activity which prevents its entrance in the TCA cycle; PKM2 is highly expressed in cancer cells (379). PKM2 was shown to activate HIF-1-α-related genes (i.e. LDHA, SLC2A1) after hypoxia-induced anti-angiogenic therapy (380). Furthermore, the low activity of PKM2 induces glutathione reduction in order to counter the effects of ROS accumulation after ROS-producing therapies (381). Another important glycolytic enzyme termed the "housekeeping gene", glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) is upregulated in tumors and is associated with cancer aggressiveness (382). GAPDH maintains glycolysis by redirecting metabolites to the PPP in order to induce an increase in NADPH. A study showed that changes in glucose concentration enhanced NADPH oxidase-dependent ROS production, leading to resistance

TABLE 2 | Pharmaceutical agents or medical interventions for which a TME-regulated resistance mechanism has been described.

Cancer type	Pharmaceutical agent or intervention	Mechanism of action	Resistance mechanism	Reference
LIPOGENIC				
Breast cancer (*in vitro*)	Tamoxifen	Inhibition of Estrogen Receptor	Alterations within the cholesterol pathway were prominent in all resistant cell lines	(361)
NSCLC (*in vivo*)	Gefitinib	Inhibits EGFR	SCD-1 upregulation induced resistance to gefitinib by promoting the EGFR-signalling pathway. Inhibition of SCD-1 rendered the cells responsive to gefitinib	(362)
AML (*in vitro*)	Mitoxantrone	Inhibitor of Topoisomerase II	Cellular visualization showed an increase in lipid droplet accumulation. Genetic analysis from sensitive and resistant cell lines showed that resistant cell lines had significantly higher mitochondrial activity and oxidative phosphorylation (OXPHOS) indicating an increase in fatty acid synthesis. OXPHOS inhibitors reversed cellular resistance	(363)
HNSCC (*in vitro*)	Radiation therapy	Double-strand DNA breaks	Glucose uptake was shown to be high in cells, and decrease in mitochondrial OXPHOS was apparent. Resistance was achieved through increased expression of fatty acid synthase (FAS). Combination treatment with FAS inhibitors induced cytotoxicity to resistant cells	(364)
LIPOLYTIC				
AML (*in vivo*)	Cytarabine	Nucleoside analog	An increase in fatty acid beta-oxidation (FAO) was observed, with high mitochondrial OXPHOS and CD36 expression. Targeting the FAO-OXPHOS-CD36 axis rendered the cells sensitive to conventional therapy.	(365)
Multiple cancer models	Anti-angiogenic therapy	Inhibitor of VEGF-R	VEGF inhibitors induced lipid metabolic reprogramming by increasing free fatty acid levels through increased CPT-1 expression, thus causing resistance. Inhibition of CPT-1 re-sensitizes previously resistant cells to anti-VEGF.	(366)
Breast cancer (*in vitro*)	Paclitaxel	Anti-microtubule	Activation of the JAK/STAT3 pathway confers resistance to breast cancer and breast cancer stem cell lines. Inhibition of JAK/STAT3 led to inhibition of CPT-1b and abolished CSC self-renewal capabilities.	(367)
Melanoma (*in vitro/ vivo*)	Inhibitors of BRAF/MEK	Selectively inhibits mitogen activated protein kinases	To acquire resistance, cells switch from the glycolytic pathway to oxidative respiration by peroxisomal FAO. Knockdown of peroxisome key enzymes (acyl-CoA oxidase-1) or treatment with peroxisomal FAO inhibitor resulted in a durable anti-tumor response.	(368)

NSCLC, non-small cell lung cancer; AML, acute myeloid leukemia; HNSCC, squamous cell carcinoma of the head and neck; SDT-1, Stearoyl-CoA-desaturase-1; CPT-1, carnitine-palmitoyl-transferase-1a; CSC, cancer stem cell.

to doxorubicin (383). Upregulation of glycolysis has been shown to enhance DNA repair mechanisms after chemo- or radiation therapy (384). Inhibition of the glycolytic pathway re-sensitized cells to previously resistant drugs (385, 386).

- ROS-mediated activation of oncogenic signals:

Adenosine monophosphate protein kinase (AMPK), a key element of tumor suppression that prevents the Warburg effect was shown to possess tumor-supporting actions, inducing metabolic variations to sustain the ROS-damaged cellular mechanism (387). Apart from its angiogenic functions, HIF-1-α induces the expression of glycolysis-associated genes (i.e. GLUT1/3, hexokinases, and PKM2) to maintain glycolysis and inhibit the TCA cycle (388). The "guardian of the genome", p53, functions to maintain genome integrity after DNA-induced damage. It has also been shown that p53 acts as a negative regulator of the Warburg effect (389). ROS-induced damage impairs p53 activity and prevents apoptosis (390). Furthermore, ROS metabolism has been linked to treatment-associated metabolic disturbances (391). Chemo- and radiation therapy induce cancer cell death *via* ROS production, and ROS production has been shown to induce the activation of oncogenic signaling pathways (NF-κB and PI3/Akt) which ensures cell survival against the ROS onslaught (392). Well-documented drug efflux mechanisms induce MDR (ABC transporters, i.e. P-glycoprotein) (393, 394). Eventually, TME cells acclimate to ROS and become resistant to ROS-eliciting drugs by producing antioxidants or increasing efflux of cytotoxic agents (395, 396).

Acidic TME

As a result of hypoxia and high lactate, TME niches are acidic. This harsh environment induces oncogene activation, and cellular metabolism shifts to adapt (397). Compared to normal cells, cancer cells possess a high intracellular pH which promotes proliferation and inhibits apoptosis, and maintains a low extracellular pH in a "reversed pH gradient" (398, 399). The acidic niche acts synergistically with the effects of lactate by inducing TAM M2 polarization, and inhibiting the cytotoxicity of infiltrating T cells (400). These effects support cellular development (401) and regulate immune surveillance. The acidic niche has been shown to induce invasiveness and the EMT in melanoma (402), neuroblastoma (399, 403) and breast carcinoma cells (404). The pH gradient between intra- and extracellular spaces forms a physical barrier to weak-base chemotherapy, preventing proper drug uptake and distribution through physiological resistance or "ion trapping phenomenon". Ionization of weak-base agents within the acidic extracellular environment prevents them from traversing this barrier (405, 406).

In contrast, weak acids exhibit high intracellular permeability. For example, paclitaxel, a non-ionizable agent, was not impeded by this physiologic barrier, showing how the ion trapping hypothesis may be relevant in future treatment modalities (407). This has prompted researchers to alkalinize appropriate modalities or treatment combinations prior to administration. Low pH brought about by hypoxia and low perfusion was shown

to induce epigenetic modifications, mainly in p53, preventing apoptosis and increasing activity of P-glycoprotein in order to induce MDR (408, 409). It has been previously reported that the acidic TME was involved in cellular protection against irradiation (410). In an investigation of radio- and/or chemo-resistance, a study showed that the acidic niche functions to induce cellular dormancy by arresting the cell cycle at G2/M phase (411). Finally, another mechanism of treatment resistance depends on the genomic instability generated by acidic milieu, which induces phenotypic variations that lead to cellular stemness (412).

Immune Micro-Environment Variability Between Primary and Secondary Tumors

As previously described, the vast cellular repertoire within the TME contributes to immune suppression. Secretion of soluble factors within the TME prevents active immune surveillance from entering the tumor; these are known as "cold tumors" – low-immune infiltrates that enhance proliferation, migration and invasion. 90% of cancer-related deaths occur in the metastatic stage because of the inefficient localization of micro-metastatic niches and therapeutic failure (413). A study utilized deep learning was conducted to detect micro-metastatic niches, and this innovative technique enabled metastatic analysis of mice with metastatic lung, pancreatic and breast cancers that may potentially be treatable. Antibody-targeted treatment applied to visible metastatic nodules was also distributed to the micro-niches in close vicinity. This approach provides the means to identify micro-niches distributed throughout the body for the purposes of improving treatment efficacy (414). The TME-induced heterogeneity was more evident in another study that showed discrepancies in the cellular and immune repertoire within primary and metastatic lesions. This suggests yet another therapeutic resistance mechanism (415). In addition to immune suppression, the microenvironmental repertoire of immune cells has been implicated in treatment resistance. As stated earlier, a large portion of the TME consists of bone marrow-derived myeloid cells which are modulated by both physical and biochemical signals that cause them to differentiate. Myeloid cells include TAMs, TANs and MDSCs which were all shown to induce chemo- and radio-therapeutic resistance through a variety of mechanisms.

TAMs are the predominant myeloid cell type within the TME, and their differentiation into the M2 phenotype is an important factor in treatment resistance. An influx of TAMs is observed after the initiation of therapy (416). TAMs were shown to be key players in chemotherapy resistance, producing various inflammatory mediators such as TNF-α, MMP, cathepsin and TGF-β. They are also commonly described as EMT transducers, degrading and synthesizing denser ECM, which ultimately leads to treatment resistance (416, 417). Another resistance mechanism is *via* TAM production of signaling factors such as FGF-2, IL-8 and VEGF for angiogenesis (418, 419). TAMs were shown to sustain an elevated level STAT3 activation, which has been associated with chemo- and radiotherapy resistance. Elevated STAT3 inhibits apoptosis *via* upregulation of anti-apoptotic proteins bcl-2 and IAP (420). Similarly, TAM overexpression of EGFs such as milk fat globule EGF-8 (MFG-E8) was shown to induce overactivation of the Sonic Hedgehog and STAT3 pathways in CSCs, resulting in treatment resistance to cisplatin (418, 421).

As previously described, microenvironmental recruitment of TANs results in a high likelihood of N2 polarization. In addition to their immune modulatory effects, they have been implicated in ECM remodeling and the EMT through the secretion of proteins such as HGF, MMP and oncostatin-M (422, 423). Similar to TAMs, TANs also induced angiogenesis, promoting treatment resistance *via* secretion of Bv8, MMP9 and VEGF (424). Additionally, HCC xenografts showed an increase in TAN activity and an increase in the expression of chemokines such as CCL2 and CCL17, which serve to attract Tregs and macrophages to the TME, thus inducing resistance to sorafenib. Pharmacologic inhibition of the PI3K-AKT pathway was shown to decrease levels of CCL2 and CCL17 chemokines and re-sensitize cells to sorafenib (425).

MDSCs are a major determinant of immunogenicity. Through the production of TGF-β, MDSCs induce polarization of TAMs and TANs into their respective tumor-supporting subtypes (150). IL-10 oversecretion by MDSCs was shown to inhibit anti-tumor activity by preventing macrophage activation and DC maturation (426, 427). The receptor tyrosine kinase inhibitor sunitinib malate was shown to not only reduce the suppressive function of MDSCs, but also decrease the expression of Fox-p3, TGF-β and IL-10, inducing a significant increase in anti-tumor activity (428). An *in vivo* study of multiple myeloma xenografts showed neutrophil accumulation in the bone marrow in the course of disease. The accumulation of MDSCs and Tregs is thought to be a result of cancer expression of stem-cell factor ckit ligand (429). The resistance of multiple myeloma to melphalan and doxorubicin is due to the immunosuppressive actions of MDSCs, mediated by soluble factors, and it is hypothesized that targeting the MDSCs would enhance chemotherapeutic efficacy in this cancer (430). Furthermore, reprogramming the TME immune repertoire induces a better anti-tumor activity and more robust response to chemotherapy (431).

As described above, CAFs are one of the key mediators of ECM stiffness and myeloid cell differentiation. CAFs differentiate from various stromal cells of the TME. Despite advancements in oncological treatments, the prognosis of solid tumors such as HCC and pancreatic ductal adenocarcinoma remains poor. Firstly, CAF-dependent secretions promote ECM rigidity, which prevents effective drug penetration. Secondly, CAF-derived miRNAs previously shown to induce immune suppression were also shown to induce treatment resistance. Ovarian cancer cells showed downregulation of programmed cell death 4 (PDCD-4) in the presence of CAF-derived overexpression of miR-182. MiRNA-182 alterations of PDCD-4 expression rendered the cancer cells resistant to chemotherapy (432). Cisplatin-based therapy was administered to patients with esophageal cancer; high levels of CAF-derived miR-27a were subsequently observed. MiR-27a-dependent transformation of fibroblasts into CAFs resulted in optimal production of TGF-β, and is thought to be the mechanism of therapy resistance. Inhibition of TGF-β subsequently re-sensitized the cells to cisplatin (433). Thirdly, CAF-derived exosomal release promotes cancer aggressiveness and therapy resistance. This occurs when the EMT is induced by

modulating Wnt-PCP autocrine signaling, which is further involved in cellular polarity *via* JNK and ROCK pathways (434). CAF-derived exosomes were shown to induce therapy resistance in breast cancer cells *via* juxtacrine and paracrine signaling of STAT-1 and NOTCH-3 pathways (435). Additionally, STAT-1 and NOTCH-3 have been associated with the maintenance of cancer cell stemness, which has been further associated with oxaliplatin and 5-FU resistance in colorectal cancer (436). Within the TME, CAFs were shown to hyperactivate the Wnt/β pathway, which in turn induces the expression of ABC and P-glycoprotein (437, 438). Overactivation of the Wnt pathway not only results in chemo- and radiotherapy resistance, it has also been shown to reduce intracellular ROS through the overexpression of COX-2 and aldehyde dehydrogenase (439, 440).

Although the molecular interplay between treatment resistance and immune suppression is not yet fully elucidated, these novel resistance mechanisms induced by TAMs, TANs, MDSCs and CAFs may present the future for targeted therapy.

Mechanical TME

The importance of ECM remodeling as a result of mechanical changes has been well established. Multiple studies demonstrate that tension accumulated in the TME induces an increase in metabolism for: 1) rapid proliferation (441); 2) mobility and structural changes that regulate invasion (442), and 3) immune evasion, acquired epigenetic modification by miRNA, and stress-induced signaling that induces resistance to therapy, which collectively constitute the most threatening aspect of cancer cell dormancy (443, 444). It has been shown that physical signals can alter cellular behavior beyond the traits of CSCs (445). The TME – with dense interstitial matrix, abnormal blood and lymphatic vessels, and increased stromal pressure – is physically distinct from normal tissue (446). Physical signals of the TME include increased matrix stiffness, solid stress and interstitial fluid pressure. Operating in tandem, these physical factors contribute to treatment resistance (445, 447).

Increased ECM Stiffness

ECM composition determines its rigidity. As described previously, the ECM provides crucial biochemical and structural support for the TME and is comprises of two components: 1) polysaccharides, which assemble into proteoglycans; this forms a gel-like structure in which fibrillar proteins embed; and 2) fibrillar proteins (fibronectins, laminins, collagen and elastin) which function as ligands for cell adhesion molecules (448). ECM proteins are produced by mesenchymal cells and the constant restructuring of the ECM is modulated by hormones, growth factors, cytokines and extracorporeal factors which influence homeostasis, repair mechanisms and morphology (449). A key aspect of ECM remodeling focuses on mesenchymal cell (i.e. fibroblast)-mediated proteolysis and re-synthesis, which is dependent on the activity of MMPs and LOX, respectively. During re-synthesis, CAFs express high levels of LOX which cross-links collagen and elastin thus increasing the rigidity of the ECM. Another stiffening mechanism, previously described, is the constant inflammatory state of the TME that induces fibroblastic transformation into myelofibroblasts. The level of desmoplastic reconstruction is positively associated with treatment resistance and worse prognosis (450). A meta-analysis showed that the level of ECM stiffness was positively correlated with cancer cell genomic instability. Three hypotheses were proposed by the authors: 1) stiffness induces DNA damage during cellular migration; 2) tumor invasion of a densely-packed environment results in the selection of more aggressive phenotypes; and 3) stiffness enhances proliferation (451). It was hypothesized that a shift from the physiologic basement membrane to a collagen-rich, dense and rigid ECM is a key factor in therapy resistance (452).

Cellular stiffness within the TME is exerted *via* transmembrane proteins, mainly integrins. Integrins exhibit dual function when exposed to stress:

1. as messengers that interact with intracellular-signaling pathways (kinases such as FAK/Src, MAPK, ROCK, JNK) and anti-apoptotic oncogenes (e.g. the YAP/TAZ/HIPPO pathway) delivering mechanical signals from surrounding cells to the transcription apparatus of the nucleus (453, 454). This activates integrin and kinase overexpression, inducing phenotypic variation and EMT (455);
2. physically connects to actin components of the cytoskeleton *via* linker proteins (e.g. vinculin, α-actinin and talin), signaling molecules (FAK, Src), and adapter proteins (Paxillin, senescent cell-antigen-like containing domain 1, PINCH-1) to modify cytoskeletal contractile forces and thereby inducing the EMT (456, 457).

The modification of the nuclear envelope, with regard to cancer cell progression has been described in a number of studies (458). The nuclear envelope mainly consists of the nuclear pore complex and lamins, which link the nuclear and cellular cytoskeletons, and both were shown to be greatly modulated by cancer cells (458). A mechano-sensor, the nuclear envelope converts and transmits signals to the nucleus, thus dictating nuclear deformability (459). This parameter in turn regulates cellular plasticity and invasion of dense tissue (460). Nucleus-cytoskeleton interactions influence nuclear stiffness, which impacts chromatin rearrangement, transcription of previously repressed genes, and change in cellular polarity. These interactions are shown to support resistance to therapy and facilitate the metastatic process (461).

Multiple cancer models have established how ECM rigidity influences chemotherapeutic resistance and cancer proliferation. Breast cancer is resistant to sorafenib, a result that is positively correlated with collagen concentration and degree of stiffness. Furthermore, triple negative breast cancer (TNBC) cells exhibit resistance as a result of overexpression of β1-integrin dependent activation of the JNK pathway (462). Moreover, another study cultured TNBC cells in varying degrees of ECM stiffness before exposing them to doxorubicin, and doxorubicin efficacy is seen to be negatively correlated with ECM stiffness. Nuclear translocation of YAP in those cells appears to be the primary driver of the EMT (463). Another well-studied entity is HCC which presents with extensive fibrosis. HCC resistance to paclitaxel, cisplatin, 5-FU and sorafenib is shown to be positively correlated with ECM

stiffness (462, 464). High stiffness-ECM was seen to induce HCC dormancy, with expression of SC markers such as CD133, CXCR4 and NANOG (465). Furthermore, ECM stiffness has been shown to mediate HCC stemness and resistance to oxaliplatin. The resistance mechanism appears to depend on integrin expression in response to ECM-mediated stiffness, which in turn upregulates phosphorylation of the Akt/mTOR pathway that is crucial for self-renewal (466). These investigations show how ECM stiffness mediates treatment resistance, utilizing a cascade of signals that originate from cell-cell and cell-ECM connections, and are a potential target to mitigate treatment resistance.

Growth-Induced Solid Stress

Solid stress arises from mechanical (shear, compressive, and tensile) forces exerted by the elastic and solid components of the TME. Rapid cellular proliferation, infiltration, and ECM deposition leads to the ready accumulation of solid stress in the TME, and becomes a significant barrier to effective drug delivery. Furthermore, solid stress collapses vessels and initiates cellular dormancy; after conventional treatments deplete sensitive cancer cells, these dormant cells with stemness are reawakened and nourished by the blood vessels (467–469). Solid stress induces hypoxic failure of chemo- and radiotherapy delivery, while hypoxia-mediated HIF-1-α has the capacity to induce EMT and encourage the development of cells with SC phenotypes (470). Moreover, a study showed that solid stress induced the upregulation of ECM adhesion molecules and the formation of "leader cells". These are capable of coordinating cellular migration, resulting in cellular invasion and metastasis (471). A boost in leader cell phenotypes has been observed following exposure to conventional treatments (472). Demonstrating high transcriptional plasticity, leader cells have been shown to possess CSC-like properties with resistance to chemo- and radiotherapy (473).

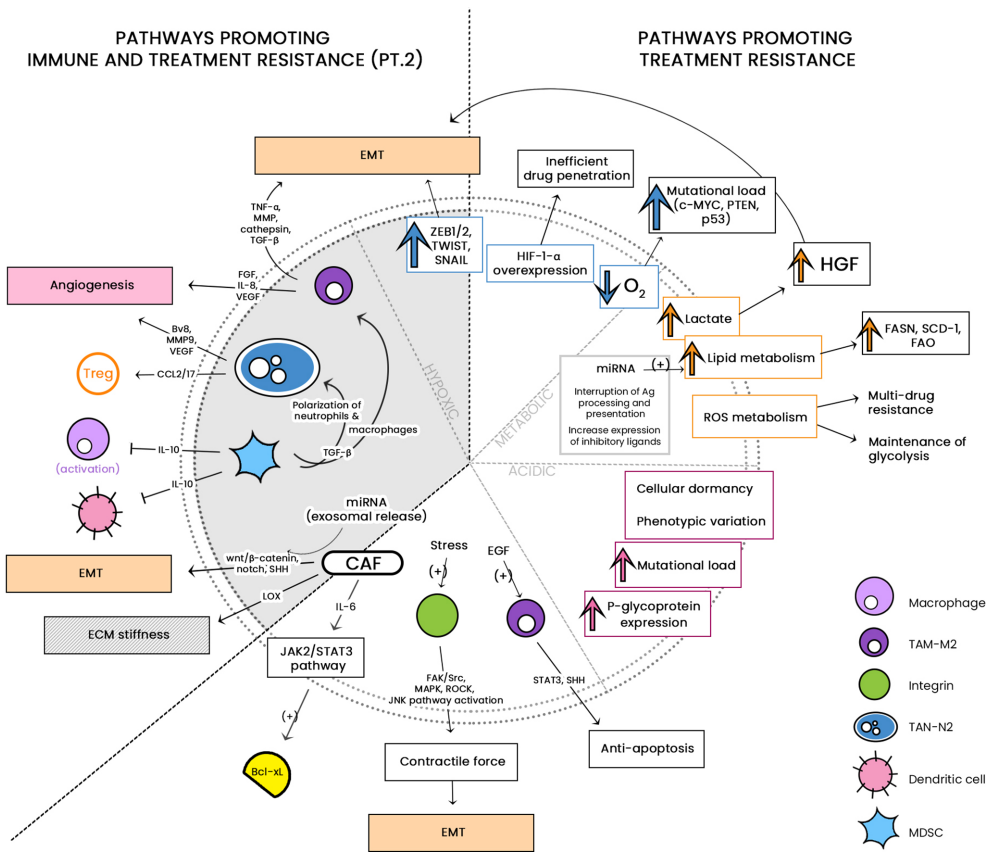


FIGURE 2 | Alterations in the tumor microenvironment (TME) induce modifications in metabolic pathways and mechanical stress. These alterations have been shown to induce drug resistance by amplifying cell-to-cell and cell-to-ECM crosstalk, activating protective pathways and inducing phenotypic variations, in addition to biochemical signaling and resistance to apoptosis. Moreover, soluble factors released by tumor-supporting immune cells have been shown to induce both immune and drug resistance *via* induction of epithelial-to-mesenchymal transition (EMT), extracellular matrix (ECM) stiffness and angiogenesis. TGF-ß, Transforming growth factor- ß; LOX, lysyl oxidase; TNF- α, tumor necrosis factor alpha; ROS, reactive oxygen species; ECM, extracellular matrix; HGF, hepatocyte growth factor; HIF-1-α, hypoxia inducible factor-1-alpha; VEGF, vascular endothelial growth factor; CCL, C-C motif chemokine ligand; MAPK, mitogen activated protein kinase; EMT, epithelial-to-mesenchymal transition; IL, interleukin; JNK, c-Jun-N-terminal kinase; JAK/STAT, Janus kinase/Signal transducer and activator of transcription; ROCK, Rho-associated protein kinases; FAK/Src, Focal adhesion kinase/src family kinase; FASN, fatty acid synthase; Bv8, prokineticin 1; FGF, fibroblast growth factor; FAO, fatty acid oxidation; SCD-1, stearoyl-CoA-desaturase-1; MMP, matrix metalloproteinase.

Interstitial Fluid Pressure

High interstitial fluid pressure (IFP) is also dependent on ECM stiffness. This is caused by hypoxia-induced angiogenesis and impaired vessel function, which has been associated with resistance to targeted therapy, chemo-, and radiation therapy (474, 475). IFP increase in tumors has not been fully explained but it is thought to occur after leakage in defective vessels, followed by high protein deposition, contributing to ECM rigidity (476). This was particularly apparent in pancreatic ductal adenocarcinoma, where it was observed that high hyaluronan content collapsed vessels and decreased cytotoxic drug distribution. This mechanical resistance was reversed after enzymatic breakdown of the stroma (477).

The mechanical changes within tumors render otherwise effective chemo- and radiotherapeutic approaches ineffective. In addition to the physical barrier to therapy, the stiffened ECM was shown to induce the EMT and the development of cellular dormancy. As was described in these three mechanisms of resistance, early combination therapies targeting the cancer type and aspects of the physical blockade could increase efficacy and prevent the development of therapy-resistant variants.

TME Innervation

It has been well established that cancer invasion can occur into or around nerve routes *via* perineural invasion (PNI) (478); this has been associated with pain and poor prognosis (479). It has been shown that PNI induces the release of factors necessary for tumor growth (480), and consequently, the cells of the TME were seen to induce an adrenergic neuronal cell phenotype which supports metastasis in pulmonary (481), ovarian (482) and pancreatic cancers (483, 484). Prostate cancer studies have shown that cancer cells express neurotrophic growth factor (NGF), which attracts nerve fibers toward the TME to promote tumor invasion and metastasis (485). Additionally, denervation has been shown to suppress tumorigenesis, further denoting the importance of innervation (486). Although apparently significant, neurotransmitter concentration in serum was not sufficiently elevated, and it is now thought that perhaps this increased concentration is diverted towards the TME. Additionally, it has been shown that astrocytomas were able to resist treatment modalities by forming a tight microenvironment covered by a microtubular network resistant to radiotherapy (487), and expressing phenotypic changes within the TME that induced stemness, which resulted in chemotherapeutic resistance (488).

See **Figure 2** for a diagrammatic summary of the major pathways that promote treatment resistance, and immune and treatment resistance.

CONCLUSION

Within the complex microenvironment of the TME, immune progenitors are encouraged to differentiate into regulatory T cells, M2 macrophages and MDSCs, amongst others, rather than fulfilling their tumor-suppressive roles as fully mature immune cells. The interaction between cellular components and soluble factors of the TME efficiently nurtures immune evasion and suppression, drug resistance, and promotes malignancy. Cellular crosstalk *via* both paracrine and juxtracrine signaling coordinates key elements that define cancer stemness, extracellular matrix remodeling, and the recruitment of non-malignant tumor-supporting cells. In addition to immune resistance, therapy resistance within the TME is achieved through various physical and biochemical factors that induce the EMT and modulate epigenetic changes that result in the formation of CSCs. In this review, it is evident that landmark research has elucidated these dysfunctional immune components with increasing clarity. Many of these components are now targets of promising drug therapies currently undergoing investigation, and these ground-breaking new discoveries continue to pave the way for new treatment modalities in the fight against cancer.

AUTHOR CONTRIBUTIONS

Conceptualisation, KK; Investigation and Resources, KK, DH, JC, CS; Writing - Original Draft Preparation, KK, DH, JC, CS; Writing - Review and Editing, KK, JC, MK; Visualisation, KK, JC, MK; Graphics, JC, KK; Supervision, AM, MK; and Project Administration, AM, MK. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic Alterations During Colorectal-Tumor Development. *N Engl J Med* (1988) 319:525–32. doi: 10.1056/NEJM198809013190901
2. Chaffer CL, Weinberg RA. A Perspective on Cancer Cell Metastasis. *Science* (2011) 331:1559–64. doi: 10.1126/science.1203543
3. Dillekås H, Rogers MS, Straume O. Are 90% of Deaths From Cancer Caused by Metastases? *Cancer Med* (2019) 8:5574–6. doi: 10.1002/cam4.2474
4. Paget S. The Distribution of Secondary Growths In Cancer Of the Breast. *Lancet* (1889) 133:571–3. doi: 10.1016/S0140-6736(00)49915-0
5. Seyfried TN, Huysentruyt LC. On the Origin of Cancer Metastasis. *Crit Rev Oncog* (2013) 18:43–73. doi: 10.1615/CritRevOncog.v18.i1-2.40
6. Fidler IJ, Talmadge JE. Evidence That Intravenously Derived Murine Pulmonary Melanoma Metastases can Originate From the Expansion of a Single Tumor Cell. *Cancer Res* (1986) 46:5167–71.
7. Hanahan D, Coussens LM. Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. *Cancer Cell* (2012) 21:309–22. doi: 10.1016/j.ccr.2012.02.022
8. Castells M, Thibault B, Delord J-P, Couderc B. Implication of Tumor Microenvironment in Chemoresistance: Tumor-Associated Stromal Cells Protect Tumor Cells From Cell Death. *Int J Mol Sci* (2012) 13:9545–71. doi: 10.3390/ijms13089545
9. Dauer P, Nomura A, Saluja A, Banerjee S. Microenvironment in Determining Chemo-Resistance in Pancreatic Cancer: Neighborhood Matters. *Pancreatology* (2017) 17:7–12. doi: 10.1016/j.pan.2016.12.010
10. Hernández R, Jiménez-Luna C, Perales-Adán J, Perazzoli G, Melguizo C, Prados J. Differentiation of Human Mesenchymal Stem Cells Towards Neuronal Lineage: Clinical Trials in Nervous System Disorders. *Biomol Ther* (2020) 28:34–44. doi: 10.4062/biomolther.2019.065
11. Martinez-Agosto JA, Mikkola HKA, Hartenstein V, Banerjee U. The Hematopoietic Stem Cell and its Niche: A Comparative View. *Genes Dev* (2007) 21:3044–60. doi: 10.1101/gad.1602607

12. Kidd S, Spaeth E, Watson K, Burks J, Lu H, Klopp A, et al. Origins of the Tumor Microenvironment: Quantitative Assessment of Adipose-Derived and Bone Marrow-Derived Stroma. *PLoS One* (2012) 7:e30563. doi: 10.1371/journal.pone.0030563
13. Achen MG, McColl BK, Stacker SA. Focus on Lymphangiogenesis in Tumor Metastasis. *Cancer Cell* (2005) 7:121–7. doi: 10.1016/j.ccr.2005.01.017
14. Chen J-C, Chang Y-W, Hong C-C, Yu Y-H, Su J-L. The Role of the VEGF-C/VEGFRs Axis in Tumor Progression and Therapy. *Int J Mol Sci* (2012) 14:88–107. doi: 10.3390/ijms14010088
15. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
16. Carmeliet P, Jain RK. Principles and Mechanisms of Vessel Normalization for Cancer and Other Angiogenic Diseases. *Nat Rev Drug Discov* (2011) 10:417–27. doi: 10.1038/nrd3455
17. Wong BW, Kuchnio A, Bruning U, Carmeliet P. Emerging Novel Functions of the Oxygen-Sensing Prolyl Hydroxylase Domain Enzymes. *Trends Biochem Sci* (2013) 38:3–11. doi: 10.1016/j.tibs.2012.10.004
18. Skuli N, Liu L, Runge A, Wang T, Yuan L, Patel S, et al. Endothelial Deletion of Hypoxia-Inducible factor-2alpha (Hif-2alpha) Alters Vascular Function and Tumor Angiogenesis. *Blood* (2009) 114:469–77. doi: 10.1182/blood-2008-12-193581
19. Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber H-P, et al. Loss of HIF-1Alpha in Endothelial Cells Disrupts a Hypoxia-Driven VEGF Autocrine Loop Necessary for Tumorigenesis. *Cancer Cell* (2004) 6:485–95. doi: 10.1016/j.ccr.2004.09.026
20. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, et al. Endothelin B Receptor Mediates the Endothelial Barrier to T Cell Homing to Tumors and Disables Immune Therapy. *Nat Med* (2008) 14:28–36. doi: 10.1038/nm1699
21. Kambayashi T, Laufer TM. Atypical MHC Class II-Expressing Antigen-Presenting Cells: Can Anything Replace a Dendritic Cell? *Nat Rev Immunol* (2014) 14:719–30. doi: 10.1038/nri3754
22. Goveia J, Rohlenova K, Taverna F, Treps L, Conradi L-C, Pircher A, et al. An Integrated Gene Expression Landscape Profiling Approach to Identify Lung Tumor Endothelial Cell Heterogeneity and Angiogenic Candidates. *Cancer Cell* (2020) 37::21–36.e13. doi: 10.1016/j.ccell.2019.12.001
23. De Sanctis F, Ugel S, Facciponte J, Facciabene A. The Dark Side of Tumor-Associated Endothelial Cells. *Semin Immunol* (2018) 35:35–47. doi: 10.1016/j.smim.2018.02.002
24. Flati V, Pastore LI, Griffioen AW, Satijn S, Tomato E, D'Alimonte I, et al. Endothelial Cell Anergy Is Mediated by bFGF Through the Sustained Activation of P38-MAPK and NF-κb Inhibition. *Int J Immunopathol Pharmacol* (2006) 19:761–73. doi: 10.1177/039463200601900406
25. Delfortrie S, Pinte S, Mattot V, Samson C, Villain G, Caetano B, et al. Egfl7 Promotes Tumor Escape From Immunity by Repressing Endothelial Cell Activation. *Cancer Res* (2011) 71:7176–86. doi: 10.1158/0008-5472.CAN-11-1301
26. Nagl L, Horvath L, Pircher A, Wolf D. Tumor Endothelial Cells (Tecs) as Potential Immune Directors of the Tumor Microenvironment – New Findings and Future Perspectives. *Front Cell Dev Biol* (2020) 8:766. doi: 10.3389/fcell.2020.00766
27. Shetty S, Weston CJ, Oo YH, Westerlund N, Stamataki Z, Youster J, et al. Common Lymphatic Endothelial and Vascular Endothelial Receptor-1 Mediates the Transmigration of Regulatory T Cells Across Human Hepatic Sinusoidal Endothelium. *J Immunol* (2011) 186:4147–55. doi: 10.4049/jimmunol.1002961
28. Hollmén M, Figueiredo CR, Jalkanen S. New Tools to Prevent Cancer Growth and Spread: A 'Clever' Approach. *Br J Cancer* (2020) 123:501–9. doi: 10.1038/s41416-020-0953-0
29. Krambeck AE, Thompson RH, Dong H, Lohse CM, Park ES, Kuntz SM, et al. B7-H4 Expression in Renal Cell Carcinoma and Tumor Vasculature: Associations With Cancer Progression and Survival. *Proc Natl Acad Sci* (2006) 103:10391–6. doi: 10.1073/pnas.0600937103
30. Riesenberg R, Weiler C, Spring O, Eder M, Buchner A, Popp T, et al. Expression of Indoleamine 2,3-Dioxygenase in Tumor Endothelial Cells Correlates With Long-Term Survival of Patients With Renal Cell Carcinoma. *Clin Cancer Res* (2007) 13:6993–7002. doi: 10.1158/1078-0432.CCR-07-0942
31. Huang D, Ding Y, Zhou M, Rini BI, Petillo D, Qian C-N, et al. Interleukin-8 Mediates Resistance to Antiangiogenic Agent Sunitinib in Renal Cell Carcinoma. *Cancer Res* (2010) 70:1063–71. doi: 10.1158/0008-5472.CAN-09-3965
32. Mazanet MM, Hughes CCW. B7-H1 Is Expressed by Human Endothelial Cells and Suppresses T Cell Cytokine Synthesis. *J Immunol* (2002) 169:3581–8. doi: 10.4049/jimmunol.169.7.3581
33. Mulligan JK, Young MRI. Tumors Induce the Formation of Suppressor Endothelial Cells In Vivo. *Cancer Immunol Immunother* (2009) 59:267. doi: 10.1007/s00262-009-0747-y
34. Casós K, Siguero L, Fernández-Figueras M-T, León X, Sardá M-P, Vila L, et al. Tumor Cells Induce COX-2 and mPGES-1 Expression in Microvascular Endothelial Cells Mainly by Means of IL-1 Receptor Activation. *Microvasc Res* (2011) 81:261–8. doi: 10.1016/j.mvr.2011.01.006
35. Taflin C, Favier B, Baudhuin J, Savenay A, Hemon P, Bensussan A, et al. Human Endothelial Cells Generate Th17 and Regulatory T Cells Under Inflammatory Conditions. *Proc Natl Acad Sci* (2011) 108:2891–6. doi: 10.1073/pnas.1011811108
36. Sata M, Walsh K. Tnfα Regulation of Fas Ligand Expression on the Vascular Endothelium Modulates Leukocyte Extravasation. *Nat Med* (1998) 4:415–20. doi: 10.1038/nm0498-415
37. Yu JS, Lee PK, Ehtesham M, Samoto K, Black KL, Wheeler CJ. Intratumoral T Cell Subset Ratios and Fas Ligand Expression on Brain Tumor Endothelium. *J Neurooncol* (2003) 64:55–61. doi: 10.1007/BF02700020
38. Motz GT, Santoro SP, Wang L-P, Garrabrant T, Lastra RR, Hagemann IS, et al. Tumor Endothelium FasL Establishes a Selective Immune Barrier Promoting Tolerance in Tumors. *Nat Med* (2014) 20:607–15. doi: 10.1038/nm.3541
39. Lanitis E, Irving M, Coukos G. Targeting the Tumor Vasculature to Enhance T Cell Activity. *Curr Opin Immunol* (2015) 33:55–63. doi: 10.1016/j.coi.2015.01.011
40. Wang M, Yuan Q, Xie L. Mesenchymal Stem Cell-Based Immunomodulation: Properties and Clinical Application. *Stem Cells Int* (2018) 2018:e3057624. doi: 10.1155/2018/3057624
41. Ley K, Kansas GS. Selectins in T-cell Recruitment to Non-Lymphoid Tissues and Sites of Inflammation. *Nat Rev Immunol* (2004) 4:325–36. doi: 10.1038/nri1351
42. Weber C, Fraemohs L, Dejana E. The Role of Junctional Adhesion Molecules in Vascular Inflammation. *Nat Rev Immunol* (2007) 7:467–77. doi: 10.1038/nri2096
43. Ala A, Dhillon AP, Hodgson HJ. Role of Cell Adhesion Molecules in Leukocyte Recruitment in the Liver and Gut. *Int J Exp Pathol* (2003) 84:1–16. doi: 10.1046/j.1365-2613.2003.00235.x
44. Greco SJ, Liu K, Rameshwar P. Functional Similarities Among Genes Regulated by OCT4 in Human Mesenchymal and Embryonic Stem Cells. *Stem Cells Dayt Ohio* (2007) 25:3143–54. doi: 10.1634/stemcells.2007-0351
45. Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I, et al. Embryonic Stem Cell Marker Expression Pattern in Human Mesenchymal Stem Cells Derived From Bone Marrow, Adipose Tissue, Heart and Dermis. *Stem Cell Rev Rep* (2009) 5:378–86. doi: 10.1007/s12015-009-9094-9
46. Hocking AM, Gibran NS. Mesenchymal Stem Cells: Paracrine Signaling and Differentiation During Cutaneous Wound Repair. *Exp Cell Res* (2010) 316:2213–9. doi: 10.1016/j.yexcr.2010.05.009
47. Saud B, Malla R, Shrestha K. A Review on the Effect of Plant Extract on Mesenchymal Stem Cell Proliferation and Differentiation. *Stem Cells Int* (2019) 2019:7513404. doi: 10.1155/2019/7513404
48. Komarova S, Kawakami Y, Stoff-Khalili MA, Curiel DT, Pereboeva L. Mesenchymal Progenitor Cells as Cellular Vehicles for Delivery of Oncolytic Adenoviruses. *Mol Cancer Ther* (2006) 5:755–66. doi: 10.1158/1535-7163.MCT-05-0334
49. Ren C, Kumar S, Chanda D, Chen J, Mountz JD, Ponnazhagan S. Therapeutic Potential of Mesenchymal Stem Cells Producing Interferon-Alpha in a Mouse Melanoma Lung Metastasis Model. *Stem Cells Dayt Ohio* (2008) 26:2332–8. doi: 10.1634/stemcells.2008-0084
50. Jacobetz MA, Chan DS, Neesse A, Bapiro TE, Cook N, Frese KK, et al. Hyaluronan Impairs Vascular Function and Drug Delivery in a Mouse Model of Pancreatic Cancer. *Gut* (2013) 62:112–20. doi: 10.1136/gutjnl-2012-302529
51. Yan X, Fu C, Chen L, Qin J, Zeng Q, Yuan H, et al. Mesenchymal Stem Cells From Primary Breast Cancer Tissue Promote Cancer Proliferation and

Enhance Mammosphere Formation Partially Via EGF/EGFR/Akt Pathway. *Breast Cancer Res Treat* (2012) 132:153–64. doi: 10.1007/s10549-011-1577-0
52. Liu S, Ginestier C, Ou SJ, Clouthier SG, Patel SH, Monville F, et al. Breast Cancer Stem Cells Are Regulated by Mesenchymal Stem Cells Through Cytokine Networks. *Cancer Res* (2011) 71:614–24. doi: 10.1158/0008-5472.CAN-10-0538
53. Rhodes LV, Antoon JW, Muir SE, Elliott S, Beckman BS, Burow ME. Effects of Human Mesenchymal Stem Cells on ER-Positive Human Breast Carcinoma Cells Mediated Through ER-SDF-1/CXCR4 Crosstalk. *Mol Cancer* (2010) 9:295. doi: 10.1186/1476-4598-9-295
54. Xu G, Zhang L, Ren G, Yuan Z, Zhang Y, Zhao RC, et al. Immunosuppressive Properties of Cloned Bone Marrow Mesenchymal Stem Cells. *Cell Res* (2007) 17:240–8. doi: 10.1038/cr.2007.4
55. Liotta F, Angeli R, Cosmi L, Filì L, Manuelli C, Frosali F, et al. Toll-Like Receptors 3 and 4 are Expressed by Human Bone Marrow-Derived Mesenchymal Stem Cells and Can Inhibit Their T-Cell Modulatory Activity by Impairing Notch Signaling. *Stem Cells Dayt Ohio* (2008) 26:279–89. doi: 10.1634/stemcells.2007-0454
56. Ghannam S, Pène J, Moquet-Torcy G, Torcy-Moquet G, Jorgensen C, Yssel H. Mesenchymal Stem Cells Inhibit Human Th17 Cell Differentiation and Function and Induce a T Regulatory Cell Phenotype. *J Immunol Baltim Md 1950* (2010) 185:302–12. doi: 10.4049/jimmunol.0902007
57. Chen L, Tredget EE, Wu PYG, Wu Y. Paracrine Factors of Mesenchymal Stem Cells Recruit Macrophages and Endothelial Lineage Cells and Enhance Wound Healing. *PLoS One* (2008) 3:e1886. doi: 10.1371/journal.pone.0001886
58. Oswald J, Boxberger S, Jørgensen B, Feldmann S, Ehninger G, Bornhäuser M, et al. Mesenchymal Stem Cells Ban be Differentiated Into Endothelial Cells In Vitro. *Stem Cells Dayt Ohio* (2004) 22:377–84. doi: 10.1634/stemcells.22-3-377
59. Spaeth EL, Dembinski JL, Sasser AK, Watson K, Klopp A, Hall B, et al. Mesenchymal Stem Cell Transition to Tumor-Associated Fibroblasts Contributes to Fibrovascular Network Expansion and Tumor Progression. *PLoS One* (2009) 4:e4992. doi: 10.1371/journal.pone.0004992
60. Aldinucci D, Colombatti A. The Inflammatory Chemokine CCL5 and Cancer Progression. *Mediators Inflammation* (2014) 2014:292376. doi: 10.1155/2014/292376
61. Betz M, Fox BS. Prostaglandin E2 Inhibits Production of Th1 Lymphokines But Not of Th2 Lymphokines. *J Immunol Baltim Md 1950* (1991) 146:108–13.
62. van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA. Prostaglandin-E2 Is a Potent Inhibitor of Human Interleukin 12 Production. *J Exp Med* (1995) 181:775–9. doi: 10.1084/jem.181.2.775
63. Kaliński P, Hilkens CM, Snijders A, Snijdewint FG, Kapsenberg ML. IL-12-Deficient Dendritic Cells, Generated in the Presence of Prostaglandin E2, Promote Type 2 Cytokine Production in Maturing Human Naive T Helper Cells. *J Immunol Baltim Md 1950* (1997) 159:28–35.
64. Obermajer N, Muthuswamy R, Lesnock J, Edwards RP, Kalinski P. Positive Feedback Between PGE2 and COX2 Redirects the Differentiation of Human Dendritic Cells Toward Stable Myeloid-Derived Suppressor Cells. *Blood* (2011) 118:5498–505. doi: 10.1182/blood-2011-07-365825
65. Heusinkveld M, de Vos van Steenwijk PJ, Goedemans R, Ramwadhdoebe TH, Gorter A, Welters MJP, et al. M2 Macrophages Induced by Prostaglandin E2 and IL-6 From Cervical Carcinoma Are Switched to Activated M1 Macrophages by CD4+ Th1 Cells. *J Immunol Baltim Md 1950* (2011) 187:1157–65. doi: 10.4049/jimmunol.1100889
66. Jung Y-J, Isaacs JS, Lee S, Trepel J, Neckers L. Il-1beta-mediated Up-Regulation of HIF-1alpha Via an NFkappaB/COX-2 Pathway Identifies HIF-1 as a Critical Link Between Inflammation and Oncogenesis. *FASEB J* (2003) 17:2115–7. doi: 10.1096/fj.03-0329fje
67. Munn DH, Mellor AL. Indoleamine 2,3 Dioxygenase and Metabolic Control of Immune Responses. *Trends Immunol* (2013) 34:137–43. doi: 10.1016/j.it.2012.10.001
68. Mellor AL, Munn DH. Creating Immune Privilege: Active Local Suppression That Benefits Friends, But Protects Foes. *Nat Rev Immunol* (2008) 8:74–80. doi: 10.1038/nri2233
69. Hobbs AJ, Higgs A, Moncada S. Inhibition of Nitric Oxide Synthase as a Potential Therapeutic Target. *Annu Rev Pharmacol Toxicol* (1999) 39:191–220. doi: 10.1146/annurev.pharmtox.39.1.191
70. Bronte V, Zanovello P. Regulation of Immune Responses by L-Arginine Metabolism. *Nat Rev Immunol* (2005) 5:641–54. doi: 10.1038/nri1668
71. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-Arginine Metabolism in Myeloid Cells Controls T-Lymphocyte Functions. *Trends Immunol* (2003) 24:302–6. doi: 10.1016/s1471-4906(03)00132-7
72. Bingisser RM, Tilbrook PA, Holt PG, Kees UR. Macrophage-Derived Nitric Oxide Regulates T Cell Activation Via Reversible Disruption of the Jak3/STAT5 Signaling Pathway. *J Immunol Baltim Md 1950* (1998) 160:5729–34.
73. Kikuchi Y, Kashima TG, Nishiyama T, Shimazu K, Morishita Y, Shimazaki M, et al. Periostin Is Expressed in Pericryptal Fibroblasts and Cancer-Associated Fibroblasts in the Colon. *J Histochem Cytochem* (2008) 56:753–64. doi: 10.1369/jhc.2008.951061
74. De Wever O, Nguyen Q-D, Van Hoorde L, Bracke M, Bruyneel E, Gespach C, et al. Tenascin-C and SF/HGF Produced by Myofibroblasts In Vitro Provide Convergent Pro-Invasive Signals to Human Colon Cancer Cells Through RhoA and Rac. *FASEB J* (2004) 18:1016–8. doi: 10.1096/fj.03-1110fje
75. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-Associated Fibroblasts Direct Tumor Progression of Initiated Human Prostatic Epithelium. *Cancer Res* (1999) 59:5002–11. doi: 10.1186/bcr138
76. Kuperwasser C, Chavarria T, Wu M, Magrane G, Gray JW, Carey L, et al. Reconstruction of Functionally Normal and Malignant Human Breast Tissues in Mice. *Proc Natl Acad Sci* (2004) 101:4966–71. doi: 10.1073/pnas.0401064101
77. Ribatti D, Tamma R, Annese T. Epithelial-Mesenchymal Transition in Cancer: A Historical Overview. *Transl Oncol* (2020) 13:100773. doi: 10.1016/j.tranon.2020.100773
78. Zhou B, Chen W-L, Wang Y-Y, Lin Z-Y, Zhang D-M, Fan S, et al. A Role for Cancer-Associated Fibroblasts in Inducing the Epithelial-to-Mesenchymal Transition in Human Tongue Squamous Cell Carcinoma. *J Oral Pathol Med* (2014) 43:585–92. doi: 10.1111/jop.12172
79. Tsuyada A, Chow A, Wu J, Somlo G, Chu P, Loera S, et al. Ccl2 Mediates Cross-Talk Between Cancer Cells and Stromal Fibroblasts That Regulates Breast Cancer Stem Cells. *Cancer Res* (2012) 72:2768–79. doi: 10.1158/0008-5472.CAN-11-3567
80. Lau EYT, Lo J, Cheng BYL, Ma MKF, Lee JMF, Ng JKY, et al. Cancer-Associated Fibroblasts Regulate Tumor-Initiating Cell Plasticity in Hepatocellular Carcinoma Through c-Met/FRA1/HEY1 Signaling. *Cell Rep* (2016) 15:1175–89. doi: 10.1016/j.celrep.2016.04.019
81. Gascard P, Tlsty TD. Carcinoma-Associated Fibroblasts: Orchestrating the Composition of Malignancy. *Genes Dev* (2016) 30:1002–19. doi: 10.1101/gad.279737.116
82. Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, et al. Constitutive Activation of Stat3 Signaling Confers Resistance to Apoptosis in Human U266 Myeloma Cells. *Immunity* (1999) 10:105–15. doi: 10.1016/s1074-7613(00)80011-4
83. Inohara N, Ding L, Chen S, Núñez G. Harakiri, a Novel Regulator of Cell Death, Encodes a Protein That Activates Apoptosis and Interacts Selectively With Survival-Promoting Proteins Bcl-2 and Bcl-X(L). *EMBO J* (1997) 16:1686–94. doi: 10.1093/emboj/16.7.1686
84. Bonnans C, Chou J, Werb Z. Remodelling the Extracellular Matrix in Development and Disease. *Nat Rev Mol Cell Biol* (2014) 15:786–801. doi: 10.1038/nrm3904
85. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling. *Cell* (2009) 139:891–906. doi: 10.1016/j.cell.2009.10.027
86. Pietras K, Pahler J, Bergers G, Hanahan D. Functions of Paracrine Pdgf Signaling in the Proangiogenic Tumor Stroma Revealed by Pharmacological Targeting. *PLoS Med* (2008) 5:e19. doi: 10.1371/journal.pmed.0050019
87. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal Fibroblasts Present in Invasive Human Breast Carcinomas Promote Tumor Growth and Angiogenesis Through Elevated SDF-1/CXCL12 Secretion. *Cell* (2005) 121:335–48. doi: 10.1016/j.cell.2005.02.034
88. Zhao T, Su Z, Li Y, Zhang X, You Q. Chitinase-3 Like-Protein-1 Function and its Role in Diseases. *Signal Transduct Target Ther* (2020) 5:1–20. doi: 10.1038/s41392-020-00303-7
89. Monteran L, Erez N. The Dark Side of Fibroblasts: Cancer-Associated Fibroblasts as Mediators of Immunosuppression in the Tumor

Microenvironment. *Front Immunol* (2019) 10:1835. doi: 10.3389/fimmu.2019.01835
90. Allavena P, Sica A, Garlanda C, Mantovani A. The Yin-Yang of Tumor-Associated Macrophages in Neoplastic Progression and Immune Surveillance. *Immunol Rev* (2008) 222:155–61. doi: 10.1111/j.1600-065X.2008.00607.x
91. Takahashi H, Sakakura K, Kudo T, Toyoda M, Kaira K, Oyama T, et al. Cancer-Associated Fibroblasts Promote an Immunosuppressive Microenvironment Through the Induction and Accumulation of Protumoral Macrophages. *Oncotarget* (2017) 8:8633–47. doi: 10.18632/oncotarget.14374
92. Comito G, Giannoni E, Segura CP, Barcellos-de-Souza P, Raspollini MR, Baroni G, et al. Cancer-Associated Fibroblasts and M2-Polarized Macrophages Synergize During Prostate Carcinoma Progression. *Oncogene* (2014) 33:2423–31. doi: 10.1038/onc.2013.191
93. Scherz-Shouval R, Santagata S, Mendillo ML, Sholl LM, Ben-Aharon I, Beck AH, et al. The Reprogramming of Tumor Stroma by HSF1 Is a Potent Enabler of Malignancy. *Cell* (2014) 158:564–78. doi: 10.1016/j.cell.2014.05.045
94. Feig C, Jones JO, Kraman M, Wells RJB, Deonarine A, Chan DS, et al. Targeting CXCL12 From FAP-Expressing Carcinoma-Associated Fibroblasts Synergizes With Anti-PD-L1 Immunotherapy in Pancreatic Cancer. *Proc Natl Acad Sci USA* (2013) 110:20212–7. doi: 10.1073/pnas.1320318110
95. Yang W, Wetterskog D, Matsumoto Y, Funa K. Kinetics of Repression by Modified p53 on the PDGF Beta-Receptor Promoter. *Int J Cancer* (2008) 123:2020–30. doi: 10.1002/ijc.23735
96. Agrawal V, Maharjan S, Kim K, Kim N-J, Son J, Lee K, et al. Direct Endothelial Junction Restoration Results in Significant Tumor Vascular Normalization and Metastasis Inhibition in Mice. *Oncotarget* (2014) 5:2761–77. doi: 10.18632/oncotarget.1942
97. Courtney J-M, Sutherland BA. Harnessing the Stem Cell Properties of Pericytes to Repair the Brain. *Neural Regener Res* (2019) 15:1021–2. doi: 10.4103/1673-5374.270301
98. Ochs K, Sahm F, Opitz CA, Lanz TV, Oezen I, Couraud P-O, et al. Immature Mesenchymal Stem Cell-Like Pericytes as Mediators of Immunosuppression in Human Malignant Glioma. *J Neuroimmunol* (2013) 265:106–16. doi: 10.1016/j.jneuroim.2013.09.011
99. Pober JS. Tellides G. Participation of Blood Vessel Cells in Human Adaptive Immune Responses. *Trends Immunol* (2012) 33:49–57. doi: 10.1016/j.it.2011.09.006
100. Tu Z, Li Y, Smith DS, Sheibani N, Huang S, Kern T, et al. Retinal Pericytes Inhibit Activated T Cell Proliferation. *Invest Ophthalmol Vis Sci* (2011) 52:9005–10. doi: 10.1167/iovs.11-8008
101. Beck B, Blanpain C. Unravelling Cancer Stem Cell Potential. *Nat Rev Cancer* (2013) 13:727–38. doi: 10.1038/nrc3597
102. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJA. Opinion: The Origin of the Cancer Stem Cell: Current Controversies and New Insights. *Nat Rev Cancer* (2005) 5:899–904. doi: 10.1038/nrc1740
103. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A Cell Initiating Human Acute Myeloid Leukaemia After Transplantation Into SCID Mice. *Nature* (1994) 367:645–8. doi: 10.1038/367645a0
104. Zhou B-BS, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB. Tumour-Initiating Cells: Challenges and Opportunities for Anticancer Drug Discovery. *Nat Rev Drug Discovery* (2009) 8:806–23. doi: 10.1038/nrd2137
105. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective Identification of Tumorigenic Breast Cancer Cells. *Proc Natl Acad Sci* (2003) 100:3983–8. doi: 10.1073/pnas.0530291100
106. Maccalli C, Rasul KI, Elawad M, Ferrone S. The Role of Cancer Stem Cells in the Modulation of Anti-Tumor Immune Responses. *Semin Cancer Biol* (2018) 53:189–200. doi: 10.1016/j.semcancer.2018.09.006
107. Boyd AS, Rodrigues NP. Stem Cells Cycle Toward Immune Surveillance. *Immunity* (2018) 48:187–90. doi: 10.1016/j.immuni.2018.02.006
108. Korkaya H, Liu S, Wicha MS. Regulation of Cancer Stem Cells by Cytokine Networks: Attacking Cancer's Inflammatory Roots. *Clin Cancer Res* (2011) 17:6125–9. doi: 10.1158/1078-0432.CCR-10-2743
109. Sultan M, Coyle KM, Vidovic D, Thomas ML, Gujar S, Marcato P. Hide-and-Seek: The Interplay Between Cancer Stem Cells and the Immune System. *Carcinogenesis* (2017) 38:107–18. doi: 10.1093/carcin/bgw115
110. Nagler C, Zänker KS, Dittmar T. Cell Fusion, Drug Resistance and Recurrence Cscs. *Adv Exp Med Biol* (2011) 714:173–82. doi: 10.1007/978-94-007-0782-5_9
111. Jiang E, Yan T, Xu Z, Shang Z. Tumor Microenvironment and Cell Fusion. *BioMed Res Int* (2019) 2019:5013592. doi: 10.1155/2019/5013592
112. Pires BRB, Amorim ÍSS DE, Souza LDE, Rodrigues JA, Mencalha AL. Targeting Cellular Signaling Pathways in Breast Cancer Stem Cells and Its Implication for Cancer Treatment. *Anticancer Res* (2016) 36:5681–91. doi: 10.21873/anticanres.11151
113. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell* (2017) 168:707–23. doi: 10.1016/j.cell.2017.01.017
114. Shibue T, Weinberg RA. Emt, CSCs, and Drug Resistance: The Mechanistic Link and Clinical Implications. *Nat Rev Clin Oncol* (2017) 14:611–29. doi: 10.1038/nrclinonc.2017.44
115. Verbeke H, Struyf S, Laureys G, Van Damme J. The Expression and Role of CXC Chemokines in Colorectal Cancer. *Cytokine Growth Factor Rev* (2011) 22:345–58. doi: 10.1016/j.cytogfr.2011.09.002
116. Noy R, Pollard JW. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity* (2014) 41:49–61. doi: 10.1016/j.immuni.2014.06.010
117. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-Associated Macrophages (TAM) as Major Players of the Cancer-Related Inflammation. *J Leukoc Biol* (2009) 86:1065–73. doi: 10.1189/jlb.0609385
118. Daurkin I, Eruslanov E, Stoffs T, Perrin GQ, Algood C, Gilbert SM, et al. Tumor-Associated Macrophages Mediate Immunosuppression in the Renal Cancer Microenvironment by Activating the 15-Lipoxygenase-2 Pathway. *Cancer Res* (2011) 71:6400–9. doi: 10.1158/0008-5472.CAN-11-1261
119. Liu J, Zhang N, Li Q, Zhang W, Ke F, Leng Q, et al. Tumor-Associated Macrophages Recruit CCR6+ Regulatory T Cells and Promote the Development of Colorectal Cancer Via Enhancing CCL20 Production in Mice. *PLoS One* (2011) 6:e19495. doi: 10.1371/journal.pone.0019495
120. Yang M, Chen J, Su F, Yu B, Su F, Lin L, et al. Microvesicles Secreted by Macrophages Shuttle Invasion-Potentiating microRNAs Into Breast Cancer Cells. *Mol Cancer* (2011) 10:117. doi: 10.1186/1476-4598-10-117
121. Hao N-B, Lü M-H, Fan Y-H, Cao Y-L, Zhang Z-R, Yang S-M. Macrophages in Tumor Microenvironments and the Progression of Tumors. *Clin Dev Immunol* (2012) 2012:948098. doi: 10.1155/2012/948098
122. Ruffell B, Affara NI, Coussens LM. Differential Macrophage Programming in the Tumor Microenvironment. *Trends Immunol* (2012) 33:119–26. doi: 10.1016/j.it.2011.12.001
123. Costa NL, Valadares MC, Souza PPC, Mendonça EF, Oliveira JC, Silva TA, et al. Tumor-Associated Macrophages and the Profile of Inflammatory Cytokines in Oral Squamous Cell Carcinoma. *Oral Oncol* (2013) 49:216–23. doi: 10.1016/j.oraloncology.2012.09.012
124. Yeo E-J, Cassetta L, Qian B-Z, Lewkowich I, Li J, Stefater JA, et al. Myeloid WNT7b Mediates the Angiogenic Switch and Metastasis in Breast Cancer. *Cancer Res* (2014) 74:2962–73. doi: 10.1158/0008-5472.CAN-13-2421
125. Deryugina EI, Zajac E, Juncker-Jensen A, Kupriyanova TA, Welter L, Quigley JP. Tissue-Infiltrating Neutrophils Constitute the Major In Vivo Source of Angiogenesis-Inducing MMP-9 in the Tumor Microenvironment. *Neoplasia N Y N* (2014) 16:771–88. doi: 10.1016/j.neo.2014.08.013
126. Korbecki J, Olbromski M, Dzięgiel P. CCL18 in the Progression of Cancer. *Int J Mol Sci* (2020) 21:7955. doi: 10.3390/ijms21217955
127. Wang H, Wang X, Li X, Fan Y, Li G, Guo C, et al. Cd68(+)Hla-Dr(+) M1-like Macrophages Promote Motility of HCC Cells Via NF-κb/FAK Pathway. *Cancer Lett* (2014) 345:91–9. doi: 10.1016/j.canlet.2013.11.013
128. Candido J, Hagemann T. Cancer-Related Inflammation. *J Clin Immunol* (2013) 33(Suppl 1):S79–84. doi: 10.1007/s10875-012-9847-0
129. Yu H, Kortylewski M, Pardoll D. Crosstalk Between Cancer and Immune Cells: Role of STAT3 in the Tumour Microenvironment. *Nat Rev Immunol* (2007) 7:41–51. doi: 10.1038/nri1995
130. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of Tumor-Associated Neutrophil Phenotype by TGF-beta: "N1" Versus "N2" Tan. *Cancer Cell* (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017
131. Haqqani AS, Sandhu JK, Birnboim HC. Expression of Interleukin-8 Promotes Neutrophil Infiltration and Genetic Instability in Mutatect Tumors. *Neoplasia N Y N* (2000) 2:561–8. doi: 10.1038/sj.neo.7900110
132. Michaeli J, Shaul ME, Mishalian I, Hovav A-H, Levy L, Zolotriov L, et al. Tumor-Associated Neutrophils Induce Apoptosis of Non-Activated CD8 T-

Cells in a Tnfα and NO-dependent Mechanism, Promoting a Tumor-Supportive Environment. *Oncoimmunology* (2017) 6:e1356965. doi: 10.1080/2162402X.2017.1356965
133. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, Sierra R, et al. Arginase I-producing Myeloid-Derived Suppressor Cells in Renal Cell Carcinoma Are a Subpopulation of Activated Granulocytes. *Cancer Res* (2009) 69:1553–60. doi: 10.1158/0008-5472.CAN-08-1921
134. Shaul ME, Fridlender ZG. Neutrophils as Active Regulators of the Immune System in the Tumor Microenvironment. *J Leukoc Biol* (2017) 102:343–9. doi: 10.1189/jlb.5MR1216-508R
135. Matsushita H, Hosoi A, Ueha S, Abe J, Fujieda N, Tomura M, et al. Cytotoxic T Lymphocytes Block Tumor Growth Both by Lytic Activity and Ifnγ-Dependent Cell-Cycle Arrest. *Cancer Immunol Res* (2015) 3:26–36. doi: 10.1158/2326-6066.CIR-14-0098
136. Chiang CL-L, Balint K, Coukos G, Kandalaft LE. Potential Approaches for More Successful Dendritic Cell-Based Immunotherapy. *Expert Opin Biol Ther* (2015) 15:569–82. doi: 10.1517/14712598.2015.1000298
137. Gao Q, Qiu S-J, Fan J, Zhou J, Wang X-Y, Xiao Y-S, et al. Intratumoral Balance of Regulatory and Cytotoxic T Cells Is Associated With Prognosis of Hepatocellular Carcinoma After Resection. *J Clin Oncol* (2007) 25:2586–93. doi: 10.1200/JCO.2006.09.4565
138. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, et al. Quantification of Regulatory T Cells Enables the Identification of High-Risk Breast Cancer Patients and Those At Risk of Late Relapse. *J Clin Oncol* (2006) 24:5373–80. doi: 10.1200/JCO.2006.05.9584
139. Protti MP, De Monte L. Cross-Talk Within the Tumor Microenvironment Mediates Th2-Type Inflammation in Pancreatic Cancer. *Oncoimmunology* (2012) 1:89–91. doi: 10.4161/onci.1.1.17939
140. Bharadwaj U, Li M, Zhang R, Chen C, Yao Q. Elevated Interleukin-6 and G-CSF in Human Pancreatic Cancer Cell Conditioned Medium Suppress Dendritic Cell Differentiation and Activation. *Cancer Res* (2007) 67:5479–88. doi: 10.1158/0008-5472.CAN-06-3963
141. Cortez-Retamozo V, Etzrodt M, Newton A, Rauch PJ, Chudnovskiy A, Berger C, et al. Origins of Tumor-Associated Macrophages and Neutrophils. *Proc Natl Acad Sci USA* (2012) 109:2491–6. doi: 10.1073/pnas.1113744109
142. Moretta A. Natural Killer Cells and Dendritic Cells: Rendezvous in Abused Tissues. *Nat Rev Immunol* (2002) 2:957–64. doi: 10.1038/nri956
143. Wilson EB, El-Jawhari JJ, Neilson AL, Hall GD, Melcher AA, Meade JL, et al. Human Tumour Immune Evasion Via TGF-β Blocks Nk Cell Activation But Not Survival Allowing Therapeutic Restoration of Anti-Tumour Activity. *PLoS One* (2011) 6:e22842. doi: 10.1371/journal.pone.0022842
144. Gillard-Bocquet M, Caer C, Cagnard N, Crozet L, Perez M, Fridman WH, et al. Lung Tumor Microenvironment Induces Specific Gene Expression Signature in Intratumoral NK Cells. *Front Immunol* (2013) 4:19. doi: 10.3389/fimmu.2013.00019
145. Coronella JA, Telleman P, Kingsbury GA, Truong TD, Hays S, Junghans RP. Evidence for an Antigen-Driven Humoral Immune Response in Medullary Ductal Breast Cancer. *Cancer Res* (2001) 61:7889–99.
146. Milne K, Köbel M, Kalloger SE, Barnes RO, Gao D, Gilks CB, et al. Systematic Analysis of Immune Infiltrates in High-Grade Serous Ovarian Cancer Reveals CD20, FoxP3 and TIA-1 as Positive Prognostic Factors. *PLoS One* (2009) 4:e6412. doi: 10.1371/journal.pone.0006412
147. Ran Z, Yue-Bei L, Qiu-Ming Z, Huan Y. Regulatory B Cells and Its Role in Central Nervous System Inflammatory Demyelinating Diseases. *Front Immunol* (2020) 11:1884. doi: 10.3389/fimmu.2020.01884
148. Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, et al. Tumor-Evoked Regulatory B Cells Promote Breast Cancer Metastasis by Converting Resting CD4+ T Cells to T Regulatory Cells. *Cancer Res* (2011) 71:3505–15. doi: 10.1158/0008-5472.CAN-10-4316
149. Schioppa T, Moore R, Thompson RG, Rosser EC, Kulbe H, Nedospasov S, et al. B Regulatory Cells and the Tumor-Promoting Actions of TNF-α During Squamous Carcinogenesis. *Proc Natl Acad Sci USA* (2011) 108:10662–7. doi: 10.1073/pnas.1100994108
150. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated Regulation of Myeloid Cells by Tumours. *Nat Rev Immunol* (2012) 12:253–68. doi: 10.1038/nri3175
151. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor Associated CD8+ T-Cell Tolerance Induced by Bone Marrow Derived Immature Myeloid Cells. *J Immunol Baltim Md 1950* (2005) 175:4583–92. doi: 10.4049/jimmunol.175.7.4583
152. Huang B, Pan P-Y, Li Q, Sato AI, Levy DE, Bromberg J, et al. Gr-1+CD115+ Immature Myeloid Suppressor Cells Mediate the Development of Tumor-Induced T Regulatory Cells and T-Cell Anergy in Tumor-Bearing Host. *Cancer Res* (2006) 66:1123–31. doi: 10.1158/0008-5472.CAN-05-1299
153. Laoui D, Keirsse J, Morias Y, Van Overmeire E, Geeraerts X, Elkrim Y, et al. The Tumour Microenvironment Harbours Ontogenically Distinct Dendritic Cell Populations With Opposing Effects on Tumour Immunity. *Nat Commun* (2016) 7:13720. doi: 10.1038/ncomms13720
154. Broz ML, Binnewies M, Boldajipour B, Nelson AE, Pollack JL, Erle DJ, et al. Dissecting the Tumor Myeloid Compartment Reveals Rare Activating Antigen-Presenting Cells Critical for T Cell Immunity. *Cancer Cell* (2014) 26:638–52. doi: 10.1016/j.ccell.2014.09.007
155. Michea P, Noël F, Zakine E, Czerwinska U, Sirven P, Abouzid O, et al. Adjustment of Dendritic Cells to the Breast-Cancer Microenvironment Is Subset Specific. *Nat Immunol* (2018) 19:885–97. doi: 10.1038/s41590-018-0145-8
156. Barry KC, Hsu J, Broz ML, Cueto FJ, Binnewies M, Combes AJ, et al. A Natural Killer–Dendritic Cell Axis Defines Checkpoint Therapy–Responsive Tumor Microenvironments. *Nat Med* (2018) 24:1178–91. doi: 10.1038/s41591-018-0085-8
157. Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM, et al. Type I Interferon Is Selectively Required by Dendritic Cells for Immune Rejection of Tumors. *J Exp Med* (2011) 208:1989–2003. doi: 10.1084/jem.20101158
158. Mercier IL, Poujol D, Sanlaville A, Sisirak V, Gobert M, Durand I, et al. Tumor Promotion by Intratumoral Plasmacytoid Dendritic Cells Is Reversed by TLR7 Ligand Treatment. *Cancer Res* (2013) 73:4629–40. doi: 10.1158/0008-5472.CAN-12-3058
159. Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/Inos-Producing Dendritic Cells Mediate Innate Immune Defense Against Bacterial Infection. *Immunity* (2003) 19:59–70. doi: 10.1016/S1074-7613(03)00171-7
160. Fujimura T, Kambayashi Y, Aiba S. Crosstalk Between Regulatory T Cells (Tregs) and Myeloid Derived Suppressor Cells (MDSCS) During Melanoma Growth. *Oncoimmunology* (2012) 1:1433–4. doi: 10.4161/onci.21176
161. Wu Q, Li B, Sun S, Sun S. Unraveling Adipocytes and Cancer Links: Is There a Role for Senescence? *Front Cell Dev Biol* (2020) 8:282. doi: 10.3389/fcell.2020.00282
162. Cao Y. Adipocyte and Lipid Metabolism in Cancer Drug Resistance. *J Clin Invest* (2019) 129:3006–17. doi: 10.1172/JCI127201
163. Bochet L, Lehuédé C, Dauvillier S, Wang YY, Dirat B, Laurent V, et al. Adipocyte-Derived Fibroblasts Promote Tumor Progression and Contribute to the Desmoplastic Reaction in Breast Cancer. *Cancer Res* (2013) 73:5657–68. doi: 10.1158/0008-5472.CAN-13-0530
164. Deng T, Lyon CJ, Bergin S, Caligiuri MA, Hsueh WA. Obesity, Inflammation, and Cancer. *Annu Rev Pathol* (2016) 11:421–49. doi: 10.1146/annurev-pathol-012615-044359
165. Tchkonia T, Morbeck DE, Von Zglinicki T, Van Deursen J, Lustgarten J, Scrable H, et al. Fat Tissue, Aging, and Cellular Senescence. *Aging Cell* (2010) 9:667–84. doi: 10.1111/j.1474-9726.2010.00608.x
166. Pan Y, Yu Y, Wang X, Zhang T. Tumor-Associated Macrophages in Tumor Immunity. *Front Immunol* (2020) 11:583084. doi: 10.3389/fimmu.2020.583084
167. Sánchez-Jiménez F, Pérez-Pérez A, de la Cruz-Merino L, Sánchez-Margalet V. Obesity and Breast Cancer: Role of Leptin. *Front Oncol* (2019) 9:596. doi: 10.3389/fonc.2019.00596
168. Petruzzelli M, Schweiger M, Schreiber R, Campos-Olivas R, Tsoli M, Allen J, et al. A Switch From White to Brown Fat Increases Energy Expenditure in Cancer-Associated Cachexia. *Cell Metab* (2014) 20:433–47. doi: 10.1016/j.cmet.2014.06.011
169. Wu Q, Li J, Li Z, Sun S, Zhu S, Wang L, et al. Exosomes From the Tumour-Adipocyte Interplay Stimulate Beige/Brown Differentiation and Reprogram Metabolism in Stromal Adipocytes to Promote Tumour Progression. *J Exp Clin Cancer Res CR* (2019) 38:223. doi: 10.1186/s13046-019-1210-3
170. Ingram JR, Dougan M, Rashidian M, Knoll M, Keliher EJ, Garrett S, et al. Pd-L1 is an Activation-Independent Marker of Brown Adipocytes. *Nat Commun* (2017) 8:647. doi: 10.1038/s41467-017-00799-8

171. Henke E, Nandigama R, Ergün S. Extracellular Matrix in the Tumor Microenvironment and Its Impact on Cancer Therapy. *Front Mol Biosci* (2020) 6:160. doi: 10.3389/fmolb.2019.00160
172. Tan T-T, Coussens LM. Humoral Immunity, Inflammation and Cancer. *Curr Opin Immunol* (2007) 19:209–16. doi: 10.1016/j.coi.2007.01.001
173. Wever OD, Demetter P, Mareel M, Bracke M. Stromal Myofibroblasts are Drivers of Invasive Cancer Growth. *Int J Cancer* (2008) 123:2229–38. doi: 10.1002/ijc.23925
174. Desmoulière A, Guyot C, Gabbiani G. The Stroma Reaction Myofibroblast: A Key Player in the Control of Tumor Cell Behavior. *Int J Dev Biol* (2004) 48:509–17. doi: 10.1387/ijdb.041802ad
175. Paszek MJ, Weaver VM. The Tension Mounts: Mechanics Meets Morphogenesis and Malignancy. *J Mammary Gland Biol Neoplasia* (2004) 9:325–42. doi: 10.1007/s10911-004-1404-x
176. Dituri F, Mazzocca A, Giannelli G, Antonaci S. Pi3k Functions in Cancer Progression, Anticancer Immunity and Immune Evasion by Tumors. *Clin Dev Immunol* (2011) 2011:e947858. doi: 10.1155/2011/947858
177. Lu H, Clauser KR, Tam WL, Fröse J, Ye X, Eaton EN, et al. A Breast Cancer Stem Cell Niche Supported by Juxtacrine Signalling From Monocytes and Macrophages. *Nat Cell Biol* (2014) 16:1105–17. doi: 10.1038/ncb3041
178. Stahl M, Schupp J, Jäger B, Schmid M, Zissel G, Müller-Quernheim J, et al. Lung Collagens Perpetuate Pulmonary Fibrosis Via CD204 and M2 Macrophage Activation. *PLoS One* (2013) 8:e81382. doi: 10.1371/journal.pone.0081382
179. Bollyky PL, Wu RP, Falk BA, Lord JD, Long SA, Preisinger A, et al. ECM Components Guide IL-10 Producing Regulatory T-Cell (TR1) Induction From Effector Memory T-Cell Precursors. *Proc Natl Acad Sci* (2011) 108:7938–43. doi: 10.1073/pnas.1017360108
180. Tomaso TD, Mazzoleni S, Wang E, Sovena G, Clavenna D, Franzin A, et al. Immunobiological Characterization of Cancer Stem Cells Isolated From Glioblastoma Patients. *Clin Cancer Res* (2010) 16:800–13. doi: 10.1158/1078-0432.CCR-09-2730
181. Zhou W, Ke SQ, Huang Z, Flavahan W, Fang X, Paul J, et al. Periostin Secreted by Glioblastoma Stem Cells Recruits M2 Tumour-Associated Macrophages and Promotes Malignant Growth. *Nat Cell Biol* (2015) 17:170–82. doi: 10.1038/ncb3090
182. Yakubenko VP, Cui K, Ardell CL, Brown KE, West XZ, Gao D, et al. Oxidative Modifications of Extracellular Matrix Promote the Second Wave of Inflammation Via β2 Integrins. *Blood* (2018) 132:78–88. doi: 10.1182/blood-2017-10-810176
183. Martinez FO, Gordon S. The M1 and M2 Paradigm of Macrophage Activation: Time for Reassessment. *F1000Prime Rep* (2014) 6:13. doi: 10.12703/P6-13
184. Wei J, Wu A, Kong L-Y, Wang Y, Fuller G, Fokt I, et al. Hypoxia Potentiates Glioma-Mediated Immunosuppression. *PLoS One* (2011) 6:e16195. doi: 10.1371/journal.pone.0016195
185. Xue X, Shah YM. Hypoxia-Inducible Factor-2α Is Essential in Activating the COX2/mPGES-1/PGE 2 Signaling Axis in Colon Cancer. *Carcinogenesis* (2013) 34:163–9. doi: 10.1093/carcin/bgs313
186. Schaaf MB, Garg AD, Agostinis P. Defining the Role of the Tumor Vasculature in Antitumor Immunity and Immunotherapy. *Cell Death Dis* (2018) 9:1–14. doi: 10.1038/s41419-017-0061-0
187. Ostroukhova M, Qi Z, Oriss TB, Dixon-McCarthy B, Rabir R, Ray A. Treg-Mediated Immunosuppression Involves Activation of the Notch-HES1 Axis by Membrane-Bound TGF-β. *J Clin Invest* (2006) 116:996–1004. doi: 10.1172/JCI26490
188. Zhang F, Wang H, Wang X, Jiang G, Liu H, Zhang G, et al. Tgf-β Induces M2-like Macrophage Polarization Via SNAIL-Mediated Suppression of a Pro-Inflammatory Phenotype. *Oncotarget* (2016) 7:52294–306. doi: 10.18632/oncotarget.10561
189. Ruella M, Klichinsky M, Kenderian SS, Shestova O, Ziober A, Kraft DO, et al. Overcoming the Immunosuppressive Tumor Microenvironment of Hodgkin Lymphoma Using Chimeric Antigen Receptor T Cells. *Cancer Discov* (2017) 7:1154–67. doi: 10.1158/2159-8290.CD-16-0850
190. Powell J, Mota F, Steadman D, Soudy C, Miyauchi JT, Crosby S, et al. Small Molecule Neuropilin-1 Antagonists Combine Antiangiogenic and Antitumor Activity With Immune Modulation Through Reduction of Transforming Growth Factor Beta (Tgfβ) Production in Regulatory T-Cells. *J Med Chem* (2018) 61:4135–54. doi: 10.1021/acs.jmedchem.8b00210
191. Gavalas NG, Tsiatas M, Tsitsilonis O, Politi E, Ioannou K, Ziogas AC, et al. VEGF Directly Suppresses Activation of T Cells From Ascites Secondary to Ovarian Cancer Via VEGF Receptor Type 2. *Br J Cancer* (2012) 107:1869–75. doi: 10.1038/bjc.2012.468
192. Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. *Cell* (2009) 136:642–55. doi: 10.1016/j.cell.2009.01.035
193. Gangaraju VK, Lin H. MicroRNAs: Key Regulators of Stem Cells. *Nat Rev Mol Cell Biol* (2009) 10:116–25. doi: 10.1038/nrm2621
194. Kadera BE, Li L, Toste PA, Wu N, Adams C, Dawson DW, et al. MicroRNA-21 in Pancreatic Ductal Adenocarcinoma Tumor-Associated Fibroblasts Promotes Metastasis. *PLoS One* (2013) 8:e71978. doi: 10.1371/journal.pone.0071978
195. Shah SH, Miller P, Garcia-Contreras M, Ao Z, Machlin L, Issa E, et al. Hierarchical Paracrine Interaction of Breast Cancer Associated Fibroblasts With Cancer Cells Via hMAPK-microRNAs to Drive ER-negative Breast Cancer Phenotype. *Cancer Biol Ther* (2015) 16:1671–81. doi: 10.1080/15384047.2015.1071742
196. Pang W, Su J, Wang Y, Feng H, Dai X, Yuan Y, et al. Pancreatic Cancer-Secreted miR-155 Implicates in the Conversion From Normal Fibroblasts to Cancer-Associated Fibroblasts. *Cancer Sci* (2015) 106:1362–9. doi: 10.1111/cas.12747
197. Taddei ML, Cavallini L, Comito G, Giannoni E, Folini M, Marini A, et al. Senescent Stroma Promotes Prostate Cancer Progression: The Role of Mir-210. *Mol Oncol* (2014) 8:1729–46. doi: 10.1016/j.molonc.2014.07.009
198. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as Oncogenes and Tumor Suppressors. *Dev Biol* (2007) 302:1–12. doi: 10.1016/j.ydbio.2006.08.028
199. Zhang Y, Yang P, Sun T, Li D, Xu X, Rui Y, et al. miR-126 and miR-126* Repress Recruitment of Mesenchymal Stem Cells and Inflammatory Monocytes to Inhibit Breast Cancer Metastasis. *Nat Cell Biol* (2013) 15:284–94. doi: 10.1038/ncb2690
200. Toh TB, Lim JJ, Chow EK-H. Epigenetics in Cancer Stem Cells. *Mol Cancer* (2017) 16:29. doi: 10.1186/s12943-017-0596-9
201. Suzuki H, Maruyama R, Yamamoto E, Kai M. Epigenetic Alteration and microRNA Dysregulation in Cancer. *Front Genet* (2013) 4:258. doi: 10.3389/fgene.2013.00258
202. Yi M, Xu L, Jiao Y, Luo S, Li A, Wu K. The Role of Cancer-Derived microRNAs in Cancer Immune Escape. *J Hematol OncolJ Hematol Oncol* (2020) 13:25. doi: 10.1186/s13045-020-00848-8
203. Gao F, Zhao Z-L, Zhao W-T, Fan Q-R, Wang S-C, Li J, et al. miR-9 Modulates the Expression of Interferon-Regulated Genes and MHC Class I Molecules in Human Nasopharyngeal Carcinoma Cells. *Biochem Biophys Res Commun* (2013) 431:610–6. doi: 10.1016/j.bbrc.2012.12.097
204. Mari L, Hoefnagel SJM, Zito D, van de Meent M, van Endert P, Calpe S, et al. microRNA 125a Regulates MHC-I Expression on Esophageal Adenocarcinoma Cells, Associated With Suppression of Antitumor Immune Response and Poor Outcomes of Patients. *Gastroenterology* (2018) 155:784–98. doi: 10.1053/j.gastro.2018.06.030
205. Colangelo T, Polcaro G, Ziccardi P, Pucci B, Muccillo L, Galgani M, et al. Proteomic Screening Identifies Calreticulin as a Mir-27a Direct Target Repressing MHC Class I Cell Surface Exposure In Colorectal Cancer. *Cell Death Dis* (2016) 7:e2120. doi: 10.1038/cddis.2016.28
206. Chen HL, Gabrilovich D, Tampé R, Girgis KR, Nadaf S, Carbone DP. A Functionally Defective Allele of TAP1 Results in Loss of MHC Class I Antigen Presentation in a Human Lung Cancer. *Nat Genet* (1996) 13:210–3. doi: 10.1038/ng0696-210
207. Ysla RM, Wilson GM, Brewer G. Assays of Adenylate Uridylate-Rich Element-Mediated Mrna Decay in Cells. *Methods Enzymol* (2008) 449:47–71. doi: 10.1016/S0076-6879(08)02403-8
208. Raghavan M, Wijeyesakere SJ, Peters LR, Del Cid N. Calreticulin in the Immune System: Ins and Outs. *Trends Immunol* (2013) 34:13–21. doi: 10.1016/j.it.2012.08.002
209. Carosella ED, Rouas-Freiss N, Tronik-Le Roux D, Moreau P, LeMaoult J. Hla-G: An Immune Checkpoint Molecule. *Adv Immunol* (2015) 127:33–144. doi: 10.1016/bs.ai.2015.04.001
210. Lin A, Yan W-H. Heterogeneity of HLA-G Expression in Cancers: Facing the Challenges. *Front Immunol* (2018) 9:2164. doi: 10.3389/fimmu.2018.02164
211. Yan W-H. Human Leukocyte Antigen-G in Cancer: Are They Clinically Relevant? *Cancer Lett* (2011) 311:123–30. doi: 10.1016/j.canlet.2011.07.019

212. Yang J, Li B, Lu H, Chen Y, Lu C, Zhu R, et al. Serum miR-152, miR-148a, miR-148b, and miR-21 as Novel Biomarkers in Non-Small Cell Lung Cancer Screening. *Tumour Biol J Int Soc Oncodev Biol Med* (2015) 36:3035–42. doi: 10.1007/s13277-014-2938-1
213. Zhu X, Han T, Wang X, Li Y, Yang H, Luo Y, et al. Overexpression of miR-152 Leads to Reduced Expression of Human Leukocyte Antigen-G and Increased Natural Killer Cell Mediated Cytolysis in JEG-3 Cells. *Am J Obstet Gynecol* (2010) 202:592.e1–7. doi: 10.1016/j.ajog.2010.03.002
214. Tao S, He H, Chen Q, Yue W. GPER Mediated Estradiol Reduces miR-148a to Promote HLA-G Expression in Breast Cancer. *Biochem Biophys Res Commun* (2014) 451:74–8. doi: 10.1016/j.bbrc.2014.07.073
215. Ashizawa M, Okayama H, Ishigame T, Thar Min AK, Saito K, Ujiie D, et al. Mirna-148a-3p Regulates Immunosuppression in DNA Mismatch Repair-Deficient Colorectal Cancer by Targeting Pd-L1. *Mol Cancer Res MCR* (2019) 17:1403–13. doi: 10.1158/1541-7786.MCR-18-0831
216. Gao L, Guo Q, Li X, Yang X, Ni H, Wang T, et al. MiR-873/PD-L1 Axis Regulates the Stemness of Breast Cancer Cells. *EBioMedicine* (2019) 41:395–407. doi: 10.1016/j.ebiom.2019.02.034
217. Qu F, Ye J, Pan X, Wang J, Gan S, Chu C, et al. MicroRNA-497-5p Down-Regulation Increases PD-L1 Expression in Clear Cell Renal Cell Carcinoma. *J Drug Target* (2019) 27:67–74. doi: 10.1080/1061186X.2018.1479755
218. Anastasiadou E, Stroopinsky D, Alimperti S, Jiao AL, Pyzer AR, Cippitelli C, et al. Epstein–Barr Virus-Encoded EBNA2 Alters Immune Checkpoint PD-L1 Expression by Downregulating miR-34a in B-Cell Lymphomas. *Leukemia* (2019) 33:132–47. doi: 10.1038/s41375-018-0178-x
219. Wang Y, Wang L. miR-34a Attenuates Glioma Cells Progression and Chemoresistance Via Targeting PD-L1. *Biotechnol Lett* (2017) 39:1485–92. doi: 10.1007/s10529-017-2397-z
220. Zhang X, Xu L, Wang F. Hsa_Circ_0020397 Regulates Colorectal Cancer Cell Viability, Apoptosis and Invasion by Promoting the Expression of the miR-138 Targets TERT and PD-L1. *Cell Biol Int* (2017) 41:1056–64. doi: 10.1002/cbin.10826
221. Zhang T, Chen H, Zhou Y, Dong W, Cai H, Tan W-S. Cooperation of FGF/MEK/ERK and Wnt/β-Catenin Pathway Regulators to Promote the Proliferation and Pluripotency of Mouse Embryonic Stem Cells in Serum- and Feeder-Free Conditions. *Bioresour Bioprocess* (2019) 6:12. doi: 10.1186/s40643-019-0249-5
222. Baranwal AK, Mehra NK. Major Histocompatibility Complex Class I Chain-Related A (Mica) Molecules: Relevance in Solid Organ Transplantation. *Front Immunol* (2017) 8:182. doi: 10.3389/fimmu.2017.00182
223. López-Cobo S, Romera-Cárdenas G, García-Cuesta EM, Reyburn HT, Valés-Gómez M. Transfer of the Human NKG2D Ligands UL16 Binding Proteins (ULBP) 1–3 Is Related to Lytic Granule Release and Leads to Ligand Retransfer and Killing of ULBP-recipient Natural Killer Cells. *Immunology* (2015) 146:70–80. doi: 10.1111/imm.12482
224. Hayakawa Y. Targeting NKG2D in Tumor Surveillance. *Expert Opin Ther Targets* (2012) 16:587–99. doi: 10.1517/14728222.2012.681378
225. Heinemann A, Zhao F, Pechlivanis S, Eberle J, Steinle A, Diederichs S, et al. Tumor Suppressive MicroRNAs Mir-34a/C Control Cancer Cell Expression of ULBP2, A Stress-Induced Ligand of the Natural Killer Cell Receptor Nkg2d. *Cancer Res* (2012) 72:460–71. doi: 10.1158/0008-5472.CAN-11-1977
226. Nguyen VHL, Yue C, Du KY, Salem M, O'Brien J, Peng C. The Role of microRNAs in Epithelial Ovarian Cancer Metastasis. *Int J Mol Sci* (2020) 21:7093. doi: 10.3390/ijms21197093
227. Jiang Y, Luan Y, He D, Chen G. miR-125b Expression Affects Tumor Growth of Multiple Myeloma Via Targeting MKK7. *Int J Clin Exp Pathol* (2017) 10:8487–94.
228. Shen J, Pan J, Du C, Si W, Yao M, Xu L, et al. Silencing NKG2D Ligand-Targeting miRNAs Enhances Natural Killer Cell-Mediated Cytotoxicity in Breast Cancer. *Cell Death Dis* (2017) 8:e2740–0. doi: 10.1038/cddis.2017.158
229. Min D, Lv X-b, Wang X, Zhang B, Meng W, Yu F, et al. Downregulation of miR-302c and miR-520c by 1,25(OH)2D3 Treatment Enhances the Susceptibility of Tumour Cells to Natural Killer Cell-Mediated Cytotoxicity. *Br J Cancer* (2013) 109:723–30. doi: 10.1038/bjc.2013.337
230. Cheong JE, Sun L. Targeting the IDO1/TDO2-KYN-AhR Pathway for Cancer Immunotherapy - Challenges and Opportunities. *Trends Pharmacol Sci* (2018) 39:307–25. doi: 10.1016/j.tips.2017.11.007
231. Mbongue JC, Nicholas DA, Torrez TW, Kim N-S, Firek AF, Langridge WHR. The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity. *Vaccines* (2015) 3:703–29. doi: 10.3390/vaccines3030703
232. Mellor AL, Lemos H, Huang L. Indoleamine 2,3-Dioxygenase and Tolerance: Where Are We Now? *Front Immunol* (2017) 8:1360. doi: 10.3389/fimmu.2017.01360
233. Eichmüller SB, Osen W, Mandelboim O, Seliger B. Immune Modulatory Micrornas Involved in Tumor Attack and Tumor Immune Escape. *JNCI J Natl Cancer Inst* (2017) 109. doi: 10.1093/jnci/djx034
234. Wang Z, Tan Y, Yu W, Zheng S, Zhang S, Sun L, et al. Small Role With Big Impact: miRNAs as Communicators in the Cross-Talk Between Cancer-Associated Fibroblasts and Cancer Cells. *Int J Biol Sci* (2017) 13:339–48. doi: 10.7150/ijbs.17680
235. Yang F, Ning Z, Ma L, Liu W, Shao C, Shu Y, et al. Exosomal miRNAs and miRNA Dysregulation in Cancer-Associated Fibroblasts. *Mol Cancer* (2017) 16:148. doi: 10.1186/s12943-017-0718-4
236. Wang J, Guan X, Zhang Y, Ge S, Zhang L, Li H, et al. Exosomal Mir-27a Derived From Gastric Cancer Cells Regulates the Transformation of Fibroblasts Into Cancer-Associated Fibroblasts. *Cell Physiol Biochem* (2018) 49:869–83. doi: 10.1159/000493218
237. Fang T, Lv H, Lv G, Li T, Wang C, Han Q, et al. Tumor-Derived Exosomal miR-1247-3p Induces Cancer-Associated Fibroblast Activation to Foster Lung Metastasis of Liver Cancer. *Nat Commun* (2018) 9:191. doi: 10.1038/s41467-017-02583-0
238. Zhou Y, Ren H, Dai B, Li J, Shang L, Huang J, et al. Hepatocellular Carcinoma-Derived Exosomal miRNA-21 Contributes to Tumor Progression by Converting Hepatocyte Stellate Cells to Cancer-Associated Fibroblasts. *J Exp Clin Cancer Res* (2018) 37:324. doi: 10.1186/s13046-018-0965-2
239. Hossain A, Kuo MT, Saunders GF. Mir-17-5p Regulates Breast Cancer Cell Proliferation by Inhibiting Translation of AIB1 Mrna. *Mol Cell Biol* (2006) 26:8191–201. doi: 10.1128/MCB.00242-06
240. Wang T, Tao W, Zhang L, Li S. Oncogenic Role of microRNA-20a in Human Multiple Myeloma. *OncoTargets Ther* (2017) 10:4465–74. doi: 10.2147/OTT.S143612
241. Chen S, Zhang Y, Kuzel TM, Zhang B. Regulating Tumor Myeloid-Derived Suppressor Cells by Micrornas. *Cancer Cell Microenviron* (2015) 2:e637. doi: 10.1093/jnci/djx034
242. Chen C, Liu J, Luo Y. MicroRNAs in Tumor Immunity: Functional Regulation in Tumor-Associated Macrophages. *J Zhejiang Univ Sci B* (2020) 21:12–28. doi: 10.1631/jzus.B1900452
243. Mathsyaraja H, Thies K, Taffany DA, Deighan C, Liu T, Yu L, et al. Csf1-ETS2-induced microRNA in Myeloid Cells Promote Metastatic Tumor Growth. *Oncogene* (2015) 34:3651–61. doi: 10.1038/onc.2014.294
244. Xi J, Huang Q, Wang L, Ma X, Deng Q, Kumar M, et al. miR-21 Depletion in Macrophages Promotes Tumoricidal Polarization and Enhances PD-1 Immunotherapy. *Oncogene* (2018) 37:3151–65. doi: 10.1038/s41388-018-0178-3
245. Chen Y, Wang S-X, Mu R, Luo X, Liu Z-S, Liang B, et al. Dysregulation of the miR-324-5p-CUEDC2 Axis Leads to Macrophage Dysfunction and is Associated With Colon Cancer. *Cell Rep* (2014) 7:1982–93. doi: 10.1016/j.celrep.2014.05.007
246. Llorente A, Skotland T, Sylvänne T, Kauhanen D, Róg T, Orłowski A, et al. Molecular Lipidomics of Exosomes Released by PC-3 Prostate Cancer Cells. *Biochim Biophys Acta* (2013) 1831:1302–9. doi: 10.1016/j.bbalip.2013.04.011
247. Li I, Nabet BY. Exosomes in the Tumor Microenvironment as Mediators of Cancer Therapy Resistance. *Mol Cancer* (2019) 18:32. doi: 10.1186/s12943-019-0975-5
248. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, et al. Intercellular Transfer of the Oncogenic Receptor EGFRvIII by Microvesicles Derived From Tumour Cells. *Nat Cell Biol* (2008) 10:619–24. doi: 10.1038/ncb1725
249. Melo SA, Sugimoto H, O'Connell JT, Kato N, Villanueva A, Vidal A, et al. Cancer Exosomes Perform Cell-Independent microRNA Biogenesis and Promote Tumorigenesis. *Cancer Cell* (2014) 26:707–21. doi: 10.1016/j.ccell.2014.09.005
250. Ristorcelli E, Beraud E, Mathieu S, Lombardo D, Verine A. Essential Role of Notch Signaling in Apoptosis of Human Pancreatic Tumoral Cells Mediated by Exosomal Nanoparticles. *Int J Cancer* (2009) 125:1016–26. doi: 10.1002/ijc.24375

251. Quail DF, Joyce JA. Microenvironmental Regulation of Tumor Progression and Metastasis. *Nat Med* (2013) 19:1423–37. doi: 10.1038/nm.3394
252. Laplane L, Duluc D, Larmonier N, Pradeu T, Bikfalvi A. The Multiple Layers of the Tumor Environment. *Trends Cancer* (2018) 4:802–9. doi: 10.1016/j.trecan.2018.10.002
253. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and Genetic Properties of Tumors Associated With Local Immune Cytolytic Activity. *Cell* (2015) 160:48–61. doi: 10.1016/j.cell.2014.12.033
254. O'Donnell JS, Long GV, Scolyer RA, Teng MWL, Smyth MJ. Resistance to PD1/PDL1 Checkpoint Inhibition. *Cancer Treat Rev* (2017) 52:71–81. doi: 10.1016/j.ctrv.2016.11.007
255. McGranahan N, Furness AJS, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal Neoantigens Elicit T Cell Immunoreactivity and Sensitivity to Immune Checkpoint Blockade. *Science* (2016) 351:1463–9. doi: 10.1126/science.aaf1490
256. Anagnostou V, Smith KN, Forde PM, Niknafs N, Bhattacharya R, White J, et al. Evolution of Neoantigen Landscape During Immune Checkpoint Blockade in Non–Small Cell Lung Cancer. *Cancer Discov* (2017) 7:264–76. doi: 10.1158/2159-8290.CD-16-0828
257. Akalay I, Janji B, Hasmim M, Noman MZ, André F, De Cremoux P, et al. Epithelial-to-Mesenchymal Transition and Autophagy Induction in Breast Carcinoma Promote Escape From T-Cell-Mediated Lysis. *Cancer Res* (2013) 73:2418–27. doi: 10.1158/0008-5472.CAN-12-2432
258. Ferrand N, Gnanapragasam A, Dorothee G, Redeuilh G, Larsen AK, Sabbah M. Loss of WISP2/CCN5 in Estrogen-Dependent MCF7 Human Breast Cancer Cells Promotes a Stem-Like Cell Phenotype. *PLoS One* (2014) 9: e87878. doi: 10.1371/journal.pone.0087878
259. Fritah A, Saucier C, De Wever O, Bracke M, Bièche I, Lidereau R, et al. Role of WISP-2/CCN5 in the Maintenance of a Differentiated and Noninvasive Phenotype in Human Breast Cancer Cells. *Mol Cell Biol* (2008) 28:1114–23. doi: 10.1128/MCB.01335-07
260. Tojo M, Hamashima Y, Hanyu A, Kajimoto T, Saitoh M, Miyazono K, et al. The ALK-5 Inhibitor A-83-01 Inhibits Smad Signaling and Epithelial-to-Mesenchymal Transition by Transforming Growth Factor-Beta. *Cancer Sci* (2005) 96:791–800. doi: 10.1111/j.1349-7006.2005.00103.x
261. Noman MZ, Janji B, Abdou A, Hasmim M, Terry S, Tan TZ, et al. The Immune Checkpoint Ligand PD-L1 Is Upregulated in EMT-Activated Human Breast Cancer Cells by a Mechanism Involving ZEB-1 and Mir-200. *Oncoimmunology* (2017) 6:e1263412. doi: 10.1080/2162402X.2016.1263412
262. Terry S, Buart S, Tan TZ, Gros G, Noman MZ, Lorens JB, et al. Acquisition of Tumor Cell Phenotypic Diversity Along the EMT Spectrum Under Hypoxic Pressure: Consequences on Susceptibility to Cell-Mediated Cytotoxicity. *Oncoimmunology* (2017) 6:e1271858. doi: 10.1080/2162402X.2016.1271858
263. Barry M, Bleackley RC. Cytotoxic T Lymphocytes: All Roads Lead to Death. *Nat Rev Immunol* (2002) 2:401–9. doi: 10.1038/nri819
264. Palena C, Polev DE, Tsang KY, Fernando RI, Litzinger M, Krukovskaya LL, et al. The Human T-Box Mesodermal Transcription Factor Brachyury Is a Candidate Target for T-Cell-Mediated Cancer Immunotherapy. *Clin Cancer Res* (2007) 13:2471–8. doi: 10.1158/1078-0432.CCR-06-2353
265. Hamilton DH, Huang B, Fernando RI, Tsang K-Y, Palena C. WEE1 Inhibition Alleviates Resistance to Immune Attack of Tumor Cells Undergoing Epithelial-Mesenchymal Transition. *Cancer Res* (2014) 74:2510–9. doi: 10.1158/0008-5472.CAN-13-1894
266. Tripathi SC, Peters HL, Taguchi A, Katayama H, Wang H, Momin A, et al. Immunoproteasome Deficiency is a Feature of Non-Small Cell Lung Cancer With a Mesenchymal Phenotype and Is Associated With a Poor Outcome. *Proc Natl Acad Sci USA* (2016) 113:E1555–64. doi: 10.1073/pnas.1521812113
267. Dustin ML, Long EO. Cytotoxic Immunological Synapses. *Immunol Rev* (2010) 235:24–34. doi: 10.1111/j.0105-2896.2010.00904.x
268. Dustin ML. What Counts in the Immunological Synapse? *Mol Cell* (2014) 54:255–62. doi: 10.1016/j.molcel.2014.04.001
269. Dustin ML, Chakraborty AK, Shaw AS. Understanding the Structure and Function of the Immunological Synapse. *Cold Spring Harb Perspect Biol* (2010) 2:a002311. doi: 10.1101/cshperspect.a002311
270. Comrie WA, Burkhardt JK. Action and Traction: Cytoskeletal Control of Receptor Triggering At the Immunological Synapse. *Front Immunol* (2016) 7:68. doi: 10.3389/fimmu.2016.00068
271. Yu Y, Smoligovets AA, Groves JT. Modulation of T Cell Signaling by the Actin Cytoskeleton. *J Cell Sci* (2013) 126:1049–58. doi: 10.1242/jcs.098210
272. Milner JD, Holland SM. The Cup Runneth Over: Lessons From the Ever-Expanding Pool of Primary Immunodeficiency Diseases. *Nat Rev Immunol* (2013) 13:635–48. doi: 10.1038/nri3493
273. Orange JS. Formation and Function of the Lytic NK-cell Immunological Synapse. *Nat Rev Immunol* (2008) 8:713–25. doi: 10.1038/nri2381
274. Hamaï A, Meslin F, Benlalam H, Jalil A, Mehrpour M, Faure F, et al. ICAM-1 has a Critical Role in the Regulation of Metastatic Melanoma Tumor Susceptibility to CTL Lysis by Interfering With PI3K/AKT Pathway. *Cancer Res* (2008) 68:9854–64. doi: 10.1158/0008-5472.CAN-08-0719
275. Anikeeva N, Somersalo K, Sims TN, Thomas VK, Dustin ML, Sykulev Y. Distinct Role of Lymphocyte Function-Associated Antigen-1 in Mediating Effective Cytolytic Activity by Cytotoxic T Lymphocytes. *Proc Natl Acad Sci USA* (2005) 102:6437. doi: 10.1073/pnas.0502467102
276. Abouzahr S, Bismuth G, Gaudin C, Caroll O, Van Endert P, Jalil A, et al. Identification of Target Actin Content and Polymerization Status as a Mechanism of Tumor Resistance After Cytolytic T Lymphocyte Pressure. *Proc Natl Acad Sci USA* (2006) 103:1428–33. doi: 10.1073/pnas.0510454103
277. Wainwright EN, Scaffidi P. Epigenetics and Cancer Stem Cells: Unleashing, Hijacking, and Restricting Cellular Plasticity. *Trends Cancer* (2017) 3:372–86. doi: 10.1016/j.trecan.2017.04.004
278. Shang S, Hua F, Hu Z-W. The Regulation of β-Catenin Activity and Function in Cancer: Therapeutic Opportunities. *Oncotarget* (2017) 8:33972. doi: 10.18632/oncotarget.15687
279. MacDonald BT, Tamai K, He X. Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. *Dev Cell* (2009) 17:9–26. doi: 10.1016/j.devcel.2009.06.016
280. Hoffmeyer K, Raggioli A, Rudloff S, Anton R, Hierholzer A, Del Valle I, et al. Wnt/β-Catenin Signaling Regulates Telomerase in Stem Cells and Cancer Cells. *Science* (2012) 336:1549–54. doi: 10.1126/science.1218370
281. Myant KB, Cammareri P, McGhee EJ, Ridgway RA, Huels DJ, Cordero JB, et al. ROS Production and NF-κb Activation Triggered by RAC1 Facilitate WNT-Driven Intestinal Stem Cell Proliferation and Colorectal Cancer Initiation. *Cell Stem Cell* (2013) 12:761–73. doi: 10.1016/j.stem.2013.04.006
282. Barker N, Clevers H. Mining the Wnt Pathway for Cancer Therapeutics. *Nat Rev Drug Discovery* (2006) 5:997–1014. doi: 10.1038/nrd2154
283. Polakis P. Wnt Signaling and Cancer. *Genes Dev* (2000) 14:1837–51.
284. Lindvall C, Bu W, Williams BO, Li Y. Wnt Signaling, Stem Cells, and the Cellular Origin of Breast Cancer. *Stem Cell Rev* (2007) 3:157–68. doi: 10.1007/s12015-007-0025-3
285. Klarmann GJ, Decker A, Farrar WL. Epigenetic Gene Silencing in the Wnt Pathway in Breast Cancer. *Epigenetics* (2008) 3:59–63. doi: 10.4161/epi.3.2.5899
286. Suzuki H, Watkins DN, Jair K-W, Schuebel KE, Markowitz SD, Chen WD, et al. Epigenetic Inactivation of SFRP Genes Allows Constitutive WNT Signaling in Colorectal Cancer. *Nat Genet* (2004) 36:417–22. doi: 10.1038/ng1330
287. Koinuma K, Yamashita Y, Liu W, Hatanaka H, Kurashina K, Wada T, et al. Epigenetic Silencing of AXIN2 in Colorectal Carcinoma With Microsatellite Instability. *Oncogene* (2006) 25:139–46. doi: 10.1038/sj.onc.1209009
288. Hussain M, Rao M, Humphries AE, Hong JA, Liu F, Yang M, et al. Tobacco Smoke Induces Polycomb-Mediated Repression of Dickkopf-1 in Lung Cancer Cells. *Cancer Res* (2009) 69:3570–8. doi: 10.1158/0008-5472.CAN-08-2807
289. Jiang X, Tan J, Li J, Kivimäe S, Yang X, Zhuang L, et al. DACT3 is an Epigenetic Regulator of Wnt/beta-Catenin Signaling in Colorectal Cancer and Is a Therapeutic Target of Histone Modifications. *Cancer Cell* (2008) 13:529–41. doi: 10.1016/j.ccr.2008.04.019
290. Beachy PA, Karhadkar SS, Berman DM. Tissue Repair and Stem Cell Renewal in Carcinogenesis. *Nature* (2004) 432:324–31. doi: 10.1038/nature03100
291. Ingham PW, McMahon AP. Hedgehog Signaling in Animal Development: Paradigms and Principles. *Genes Dev* (2001) 15:3059–87. doi: 10.1101/gad.938601
292. Sellheyer K. Basal Cell Carcinoma: Cell of Origin, Cancer Stem Cell Hypothesis and Stem Cell Markers. *Br J Dermatol* (2011) 164:696–711. doi: 10.1111/j.1365-2133.2010.10158.x
293. Youssef KK, Van Keymeulen A, Lapouge G, Beck B, Michaux C, Achouri Y, et al. Identification of the Cell Lineage At the Origin of Basal Cell Carcinoma. *Nat Cell Biol* (2010) 12:299–305. doi: 10.1038/ncb2031

294. Teglund S, Toftgård R. Hedgehog Beyond Medulloblastoma and Basal Cell Carcinoma. *Biochim Biophys Acta* (2010) 1805:181–208. doi: 10.1016/j.bbcan.2010.01.003
295. Biegel JA, Zhou JY, Rorke LB, Stenstrom C, Wainwright LM, Fogelgren B. Germ-Line and Acquired Mutations of INI1 in Atypical Teratoid and Rhabdoid Tumors. *Cancer Res* (1999) 59:74–9.
296. Cui W, Wang L-H, Wen Y-Y, Song M, Li B-L, Chen X-L, et al. Expression and Regulation Mechanisms of Sonic Hedgehog in Breast Cancer. *Cancer Sci* (2010) 101:927–33. doi: 10.1111/j.1349-7006.2010.01495.x
297. Duan Z-H, Wang H-C, Zhao D-M, Ji X-X, Song M, Yang X-J, et al. Cooperatively Transcriptional and Epigenetic Regulation of Sonic Hedgehog Overexpression Drives Malignant Potential of Breast Cancer. *Cancer Sci* (2015) 106:1084–91. doi: 10.1111/cas.12697
298. Andersson ER, Lendahl U. Therapeutic Modulation of Notch Signalling–Are We There Yet? *Nat Rev Drug Discovery* (2014) 13:357–78. doi: 10.1038/nrd4252
299. Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, et al. Notch Signalling Regulates Stem Cell Numbers In Vitro and In Vivo. *Nature* (2006) 442:823–6. doi: 10.1038/nature04940
300. Ghoshal P, Nganga AJ, Moran-Giuati J, Szafranek A, Johnson TR, Bigelow AJ, et al. Loss of the SMRT/NCoR2 Corepressor Correlates With JAG2 Overexpression in Multiple Myeloma. *Cancer Res* (2009) 69:4380–7. doi: 10.1158/0008-5472.CAN-08-3467
301. Jin L, Vu TT, Datta PK. Abstract 1709: STRAP Mediates the Stemness of Human Colorectal Cancer Cells by Epigenetic Regulation of Notch Pathway. *Cancer Res* (2016) 76:1709–9. doi: 10.1158/1538-7445.AM2016-1709
302. Shah AN, Summy JM, Zhang J, Park SI, Parikh NU, Gallick GE. Development and Characterization of Gemcitabine-Resistant Pancreatic Tumor Cells. *Ann Surg Oncol* (2007) 14:3629–37. doi: 10.1245/s10434-007-9583-5
303. Shukla S, Meeran SM. Epigenetics of Cancer Stem Cells: Pathways and Therapeutics. *Biochim Biophys Acta BBA - Gen Subj* (2014) 1840:3494–502. doi: 10.1016/j.bbagen.2014.09.017
304. Arumugam T, Ramachandran V, Fournier KF, Wang H, Marquis L, Abbruzzese JL, et al. Epithelial to Mesenchymal Transition Contributes to Drug Resistance in Pancreatic Cancer. *Cancer Res* (2009) 69:5820–8. doi: 10.1158/0008-5472.CAN-08-2819
305. Chow EK-H, Fan L, Chen X, Bishop JM. Oncogene-Specific Formation of Chemoresistant Murine Hepatic Cancer Stem Cells. *Hepatology* (2012) 56:1331–41. doi: 10.1002/hep.25776
306. Chung-Pu W, Anna MC, Suresh VA. Reversal of ABC Drug Transporter-Mediated Multidrug Resistance in Cancer Cells: Evaluation of Current Strategies. *Curr Mol Pharmacol* (2008) 1:93–105.
307. Wang X, Low XC, Hou W, Abdullah LN, Toh TB, Mohd Abdul Rashid M, et al. Epirubicin-Adsorbed Nanodiamonds Kill Chemoresistant Hepatic Cancer Stem Cells. *ACS Nano* (2014) 8:12151–66. doi: 10.1021/nn503491e
308. Cho S, Lu M, He X, Ee P-LR, Bhat U, Schneider E, et al. Notch1 Regulates the Expression of the Multidrug Resistance Gene ABCC1/MRP1 in Cultured Cancer Cells. *Proc Natl Acad Sci* (2011) 108:20778–83. doi: 10.1073/pnas.1019452108
309. Kim B, Stephen SL, Hanby AM, Horgan K, Perry SL, Richardson J, et al. Chemotherapy Induces Notch1-Dependent MRP1 Up-Regulation, Inhibition of Which Sensitizes Breast Cancer Cells to Chemotherapy. *BMC Cancer* (2015) 15:634. doi: 10.1186/s12885-015-1625-y
310. To KKW, Polgar O, Huff LM, Morisaki K, Bates SE. Histone Modifications at the ABCG2 Promoter Following Treatment With Histone Deacetylase Inhibitor Mirror Those in Multidrug-Resistant Cells. *Mol Cancer Res* (2008) 6:151–64. doi: 10.1158/1541-7786.MCR-07-0175
311. Tang DG. Understanding Cancer Stem Cell Heterogeneity and Plasticity. *Cell Res* (2012) 22:457–72. doi: 10.1038/cr.2012.13
312. Chaffer CL, Brueckmann I, Scheel C, Kaestli AJ, Wiggins PA, Rodrigues LO, et al. Normal and Neoplastic Nonstem Cells can Spontaneously Convert to a Stem-Like State. *Proc Natl Acad Sci* (2011) 108:7950–5. doi: 10.1073/pnas.1102454108
313. Chaffer CL, Marjanovic ND, Lee T, Bell G, Kleer CG, Reinhardt F, et al. Poised Chromatin At the ZEB1 Promoter Enables Breast Cancer Cell Plasticity and Enhances Tumorigenicity. *Cell* (2013) 154:61–74. doi: 10.1016/j.cell.2013.06.005
314. Chen W, Dong J, Haiech J, Kilhoffer M-C, Zeniou M. Cancer Stem Cell Quiescence and Plasticity as Major Challenges in Cancer Therapy. *Stem Cells Int* (2016) 2016:e1740936. doi: 10.1155/2016/1740936
315. Iliopoulos D, Hirsch HA, Wang G, Struhl K. Inducible Formation of Breast Cancer Stem Cells and Their Dynamic Equilibrium With Non-Stem Cancer Cells Via IL6 Secretion. *Proc Natl Acad Sci* (2011) 108:1397–402. doi: 10.1073/pnas.1018898108
316. Jolly MK, Kulkarni P, Weninger K, Orban J, Levine H. Phenotypic Plasticity, Bet-Hedging, and Androgen Independence in Prostate Cancer: Role of Non-Genetic Heterogeneity. *Front Oncol* (2018) 8:50. doi: 10.3389/fonc.2018.00050
317. Morgan RG, Mortensson E, Williams AC. Targeting LGR5 in Colorectal Cancer: Therapeutic Gold or Too Plastic? *Br J Cancer* (2018) 118:1410–8. doi: 10.1038/s41416-018-0118-6
318. Tsai L-L, Yu C-C, Lo J-F, Sung W-W, Lee H, Chen S-L, et al. Enhanced Cisplatin Resistance in Oral-Cancer Stem-Like Cells Is Correlated With Upregulation of Excision-Repair Cross-Complementation Group 1. *J Dent Sci* (2012) 7:111–7. doi: 10.1016/j.jds.2012.03.006
319. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma Stem Cells Promote Radioresistance by Preferential Activation of the DNA Damage Response. *Nature* (2006) 444:756–60. doi: 10.1038/nature05236
320. Kim NH, Kim HS, Li X-Y, Lee I, Choi H-S, Kang SE, et al. A P53/miRNA-34 Axis Regulates Snail1-dependent Cancer Cell Epithelial-Mesenchymal Transition. *J Cell Biol* (2011) 195:417–33. doi: 10.1083/jcb.201103097
321. Apel A, Zentgraf H, Büchler MW, Herr I. Autophagy-a Double-Edged Sword in Oncology. *Int J Cancer* (2009) 125:991–5. doi: 10.1002/ijc.24500
322. Smith AG, Macleod KF. Autophagy, Cancer Stem Cells and Drug Resistance. *J Pathol* (2019) 247:708–18. doi: 10.1002/path.5222
323. Schito L, Semenza GL. Hypoxia-Inducible Factors: Master Regulators of Cancer Progression. *Trends Cancer* (2016) 2:758–70. doi: 10.1016/j.trecan.2016.10.016
324. Semenza GL. Defining the Role of Hypoxia-Inducible Factor 1 in Cancer Biology and Therapeutics. *Oncogene* (2010) 29:625–34. doi: 10.1038/onc.2009.441
325. Wilson WR, Hay MP. Targeting Hypoxia in Cancer Therapy. *Nat Rev Cancer* (2011) 11:393–410. doi: 10.1038/nrc3064
326. Minchinton AI, Tannock IF. Drug Penetration in Solid Tumours. *Nat Rev Cancer* (2006) 6:583–92. doi: 10.1038/nrc1893
327. Jing X, Yang F, Shao C, Wei K, Xie M, Shen H, et al. Role of Hypoxia in Cancer Therapy by Regulating the Tumor Microenvironment. *Mol Cancer* (2019) 18:157. doi: 10.1186/s12943-019-1089-9
328. Mitra SK, Schlaepfer DD. Integrin-Regulated FAK–Src Signaling in Normal and Cancer Cells. *Curr Opin Cell Biol* (2006) 18:516–23. doi: 10.1016/j.ceb.2006.08.011
329. Fischer KR, Durrans A, Lee S, Sheng J, Li F, Wong STC, et al. Epithelial-to-Mesenchymal Transition Is Not Required for Lung Metastasis But Contributes to Chemoresistance. *Nature* (2015) 527:472–6. doi: 10.1038/nature15748
330. Qiu G-Z, Jin M-Z, Dai J-X, Sun W, Feng J-H, Jin W-L. Reprogramming of the Tumor in the Hypoxic Niche: The Emerging Concept and Associated Therapeutic Strategies. *Trends Pharmacol Sci* (2017) 38:669–86. doi: 10.1016/j.tips.2017.05.002
331. Bhandari V, Li CH, Bristow RG, Boutros PC. Divergent Mutational Processes Distinguish Hypoxic and Normoxic Tumours. *Nat Commun* (2020) 11:737. doi: 10.1038/s41467-019-14052-x
332. Denko NC. Hypoxia, HIF1 and Glucose Metabolism in the Solid Tumour. *Nat Rev Cancer* (2008) 8:705–13. doi: 10.1038/nrc2468
333. Semenza GL. Hif-1: Upstream and Downstream of Cancer Metabolism. *Curr Opin Genet Dev* (2010) 20:51. doi: 10.1016/j.gde.2009.10.009
334. Reina-Campos M, Moscat J, Diaz-Meco M. Metabolism Shapes the Tumor Microenvironment. *Curr Opin Cell Biol* (2017) 48:47–53. doi: 10.1016/j.ceb.2017.05.006
335. Goto M, Miwa H, Shikami M, Tsunekawa-Imai N, Suganuma K, Mizuno S, et al. Importance of Glutamine Metabolism in Leukemia Cells by Energy Production Through Tca Cycle and by Redox Homeostasis. *Cancer Invest* (2014) 32:241–7. doi: 10.3109/07357907.2014.907419
336. Warburg O, Wind F, Negelein E. The Metabolism of Tumors in The Body. *J Gen Physiol* (1927) 8:519–30. doi: 10.1085/jgp.8.6.519

337. Hou X-M, Yuan S-Q, Zhao D, Liu X-J, Wu X-A. LDH-a Promotes Malignant Behavior Via Activation of Epithelial-to-Mesenchymal Transition in Lung Adenocarcinoma. *Biosci Rep* (2019) 39:BSR20181476. doi: 10.1042/BSR20181476
338. Angelin A, Gil-de-Gómez L, Dahiya S, Jiao J, Guo L, Levine MH, et al. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metab* (2017) 25:1282–93.e7. doi: 10.1016/j.cmet.2016.12.018
339. Hunt TK, Aslam RS, Beckert S, Wagner S, Ghani QP, Hussain MZ, et al. Aerobically-Derived Lactate Stimulates Revascularization and Tissue Repair Via Redox Mechanisms. *Antioxid Redox Signal* (2007) 9:1115–24. doi: 10.1089/ars.2007.1674
340. Altman BJ, Stine ZE, Dang CV. From Krebs to Clinic: Glutamine Metabolism to Cancer Therapy. *Nat Rev Cancer* (2016) 16:619–34. doi: 10.1038/nrc.2016.71
341. Elia I, Rossi M, Stegen S, Broekaert D, Doglioni G, van Gorsel M, et al. Breast Cancer Cells Rely on Environmental Pyruvate to Shape the Metastatic Niche. *Nature* (2019) 568:117–21. doi: 10.1038/s41586-019-0977-x
342. Nallanthighal S, Heiserman JP, Cheon D-J. The Role of the Extracellular Matrix in Cancer Stemness. *Front Cell Dev Biol* (2019) 7:86. doi: 10.3389/fcell.2019.00086
343. Quennet V, Yaromina A, Zips D, Rosner A, Walenta S, Baumann M, et al. Tumor Lactate Content Predicts for Response to Fractionated Irradiation of Human Squamous Cell Carcinomas in Nude Mice. *Radiother Oncol J Eur Soc Ther Radiol Oncol* (2006) 81:130–5. doi: 10.1016/j.radonc.2006.08.012
344. Apicella M, Giannoni E, Fiore S, Ferrari KJ, Fernández-Pérez D, Isella C, et al. Increased Lactate Secretion by Cancer Cells Sustains non-Cell-Autonomous Adaptive Resistance to MET and EGFR Targeted Therapies. *Cell Metab* (2018) 28:848–65. doi: 10.1016/j.cmet.2018.08.006
345. Wagner W, Ciszewski WM, Kania KD. L- and D-lactate Enhance DNA Repair and Modulate the Resistance of Cervical Carcinoma Cells to Anticancer Drugs Via Histone Deacetylase Inhibition and Hydroxycarboxylic Acid Receptor 1 Activation. *Cell Commun Signal CCS* (2015) 13:36. doi: 10.1186/s12964-015-0114-x
346. Beloribi-Djefaflia S, Vasseur S, Guillaumond F. Lipid Metabolic Reprogramming in Cancer Cells. *Oncogenesis* (2016) 5:e189–9. doi: 10.1038/oncsis.2015.49
347. Menendez JA, Lupu R. Fatty Acid Synthase and the Lipogenic Phenotype in Cancer Pathogenesis. *Nat Rev Cancer* (2007) 7:763–77. doi: 10.1038/nrc2222
348. Kuhajda FP. Fatty Acid Synthase and Cancer: New Application of an Old Pathway. *Cancer Res* (2006) 66:5977–80. doi: 10.1158/0008-5472.CAN-05-4673
349. Mashima T, Seimiya H, Tsuruo T. De Novo Fatty-Acid Synthesis and Related Pathways as Molecular Targets for Cancer Therapy. *Br J Cancer* (2009) 100:1369–72. doi: 10.1038/sj.bjc.6605007
350. Qin X-Y, Kojima S. Inhibition of Stearoyl-CoA Desaturase-1 Activity Suppressed SREBP Signaling in Colon Cancer Cells and Their Spheroid Growth. *Gastrointest Disord* (2019) 1:191–200. doi: 10.3390/gidisord1010014
351. Tracz-Gaszewska Z, Dobrzyn P. Stearoyl-Coa Desaturase 1 as a Therapeutic Target for the Treatment of Cancer. *Cancers* (2019) 11:948. doi: 10.3390/cancers11070948
352. Tadros S, Shukla SK, King RJ, Gunda V, Vernucci E, Abrego J, et al. De Novo Lipid Synthesis Facilitates Gemcitabine Resistance Through Endoplasmic Reticulum Stress in Pancreatic Cancer. *Cancer Res* (2017) 77:5503–17. doi: 10.1158/0008-5472.CAN-16-3062
353. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, et al. Adipocytes Promote Ovarian Cancer Metastasis and Provide Energy for Rapid Tumor Growth. *Nat Med* (2011) 17:1498–503. doi: 10.1038/nm.2492
354. Pascual G, Avgustinova A, Mejetta S, Martín M, Castellanos A, Attolini CS-O, et al. Targeting Metastasis-Initiating Cells Through the Fatty Acid Receptor CD36. *Nature* (2017) 541:41–5. doi: 10.1038/nature20791
355. Han S, Wei R, Zhang X, Jiang N, Fan M, Huang JH, et al. Cpt1a/2-Mediated FAO Enhancement-a Metabolic Target in Radioresistant Breast Cancer. *Front Oncol* (2019) 9:1201. doi: 10.3389/fonc.2019.01201
356. Peck B, Schulze A. Lipid Metabolism At the Nexus of Diet and Tumor Microenvironment. *Trends Cancer* (2019) 5:693–703. doi: 10.1016/j.trecan.2019.09.007
357. Long J, Zhang C-J, Zhu N, Du K, Yin Y-F, Tan X, et al. Lipid Metabolism and Carcinogenesis, Cancer Development. *Am J Cancer Res* (2018) 8:778–91.
358. Ma L, Hernandez MO, Zhao Y, Mehta M, Tran B, Kelly M, et al. Tumor Cell Biodiversity Drives Microenvironmental Reprogramming in Liver Cancer. *Cancer Cell* (2019) 36:418–30. doi: 10.1016/j.ccell.2019.08.007
359. Li H, Feng Z, He M-L. Lipid Metabolism Alteration Contributes to and Maintains the Properties of Cancer Stem Cells. *Theranostics* (2020) 10:7053–69. doi: 10.7150/thno.41388
360. Incio J, Ligibel JA, McManus DT, Suboj P, Jung K, Kawaguchi K, et al. Obesity Promotes Resistance to Anti-VEGF Therapy in Breast Cancer by Up-Regulating IL-6 and Potentially FGF-2. *Sci Transl Med* (2018) 10:eaag0945. doi: 10.1126/scitranslmed.aag0945
361. Hultsch S, Kankainen M, Paavolainen L, Kovanen R-M, Ikonen E, Kangaspeska S, et al. Association of Tamoxifen Resistance and Lipid Reprogramming in Breast Cancer. *BMC Cancer* (2018) 18:850. doi: 10.1186/s12885-018-4757-z
362. She K, Fang S, Du W, Fan X, He J, Pan H, et al. SCD1 Is Required for EGFR-Targeting Cancer Therapy Of Lung Cancer Via Re-Activation of EGFR/PI3K/AKT Signals. *Cancer Cell Int* (2019) 19:103. doi: 10.1186/s12935-019-0809-y
363. Salunkhe S, Mishra SV, Ghorai A, Hole A, Chandrani P, Dutt A, et al. Metabolic Rewiring In Drug Resistant Cells Exhibit Higher OXPHOS and Fatty Acids as Preferred Major Source to Cellular Energetics. *Biochim Biophys Acta Bioenerg* (2020) 1861:148300. doi: 10.1016/j.bbabio.2020.148300
364. Mims J, Bansal N, Bharadwaj MS, Chen X, Molina AJ, Tsang AW, et al. Energy Metabolism in a Matched Model of Radiation Resistance for Head and Neck Squamous Cell Cancer. *Radiat Res* (2015) 183:291–304. doi: 10.1667/RR13828.1
365. Farge T, Saland E, de Toni F, Aroua N, Hosseini M, Perry R, et al. Chemotherapy-Resistant Human Acute Myeloid Leukemia Cells Are Not Enriched for Leukemic Stem Cells but Require Oxidative Metabolism. *Cancer Discov* (2017) 7:716–35. doi: 10.1158/2159-8290.CD-16-0441
366. Iwamoto H, Abe M, Yang Y, Cui D, Seki T, Nakamura M, et al. Cancer Lipid Metabolism Confers Antiangiogenic Drug Resistance. *Cell Metab* (2018) 28:104–17.e5. doi: 10.1016/j.cmet.2018.05.005
367. Wang T, Fahrmann JF, Lee H, Li Y-J, Tripathi SC, Yue C, et al. JAK/STAT3-Regulated Fatty Acid β-Oxidation Is Critical for Breast Cancer Stem Cell Self-Renewal and Chemoresistance. *Cell Metab* (2018) 27:136–50.e5. doi: 10.1016/j.cmet.2017.11.001
368. Shen S, Faouzi S, Souquere S, Roy S, Routier E, Libenciuc C, André F, Pierron G, Scoazec J-Y, Robert C. Melanoma Persister Cells Are Tolerant to BRAF/MEK Inhibitors Via ACOX1-Mediated Fatty Acid Oxidation. *Cell Rep* (2020) 33:108421. doi: 10.1016/j.celrep.2020.108421
369. Weinberg F, Ramnath N, Nagrath D. Reactive Oxygen Species in the Tumor Microenvironment: An Overview. *Cancers* (2019) 11:1191. doi: 10.3390/cancers11081191
370. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial Reactive Oxygen Species Trigger Hypoxia-Induced Transcription. *Proc Natl Acad Sci* (1998) 95:11715–20. doi: 10.1073/pnas.95.20.11715
371. Zhang Y, Choksi S, Chen K, Pobezinskaya Y, Linnoila I, Liu Z-G. ROS Play a Critical Role in the Differentiation of Alternatively Activated Macrophages and the Occurrence of Tumor-Associated Macrophages. *Cell Res* (2013) 23:898–914. doi: 10.1038/cr.2013.75
372. Nakashima RA, Paggi MG, Pedersen PL. Contributions of Glycolysis and Oxidative Phosphorylation to Adenosine 5'-Triphosphate Production in AS-30D Hepatoma Cells. *Cancer Res* (1984) 44:5702–6.
373. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, et al. Growth Factor Regulation of Autophagy and Cell Survival in the Absence of Apoptosis. *Cell* (2005) 120:237–48. doi: 10.1016/j.cell.2004.11.046
374. Shukla SK, Purohit V, Mehla K, Gunda V, Chaika NV, Vernucci E, et al. MUC1 and HIF-1alpha Signaling Crosstalk Induces Anabolic Glucose Metabolism to Impart Gemcitabine Resistance to Pancreatic Cancer. *Cancer Cell* (2017) 32:71–87.e7. doi: 10.1016/j.ccell.2017.06.004
375. D'Autréaux B, Toledano MB. ROS as Signalling Molecules: Mechanisms That Generate Specificity in ROS Homeostasis. *Nat Rev Mol Cell Biol* (2007) 8:813–24. doi: 10.1038/nrm2256
376. Panieri E, Santoro MM. ROS Homeostasis and Metabolism: A Dangerous Liason in Cancer Cells. *Cell Death Dis* (2016) 7:e2253–3. doi: 10.1038/cddis.2016.105

377. Lu W, Hu Y, Chen G, Chen Z, Zhang H, Wang F, et al. Correction: Novel Role of NOX in Supporting Aerobic Glycolysis in Cancer Cells With Mitochondrial Dysfunction and as a Potential Target for Cancer Therapy. *PLoS Biol* (2017) 15:e1002616. doi: 10.1371/journal.pbio.1002616
378. Bedard K, Krause K-H. The NOX Family of ROS-generating NADPH Oxidases: Physiology and Pathophysiology. *Physiol Rev* (2007) 87:245–313. doi: 10.1152/physrev.00044.2005
379. Anastasiou D, Yu Y, Israelsen WJ, Jiang J-K, Boxer MB, Hong BS, et al. Pyruvate Kinase M2 Activators Promote Tetramer Formation and Suppress Tumorigenesis. *Nat Chem Biol* (2012) 8:839–47. doi: 10.1038/nchembio.1060
380. Bluemlein K, Grüning N-M, Feichtinger RG, Lehrach H, Kofler B, Ralser M. No Evidence for a Shift in Pyruvate Kinase PKM1 to PKM2 Expression During Tumorigenesis. *Oncotarget* (2011) 2:393–400. doi: 10.18632/oncotarget.278
381. Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang J, Shen M, et al. Inhibition of Pyruvate Kinase M2 by Reactive Oxygen Species Contributes to Cellular Antioxidant Responses. *Science* (2011) 334:1278–83. doi: 10.1126/science.1211485
382. Rubie C, Kempf K, Hans J, Su T, Tilton B, Georg T, et al. Housekeeping Gene Variability in Normal and Cancerous Colorectal, Pancreatic, Esophageal, Gastric and Hepatic Tissues. *Mol Cell Probes* (2005) 19:101–9. doi: 10.1016/j.mcp.2004.10.001
383. Seebacher NA, Richardson DR, Jansson PJ. Glucose Modulation Induces Reactive Oxygen Species and Increases P-Glycoprotein-Mediated Multidrug Resistance to Chemotherapeutics. *Br J Pharmacol* (2015) 172:2557–72. doi: 10.1111/bph.13079
384. Bhatt AN, Chauhan A, Khanna S, Rai Y, Singh S, Soni R, et al. Transient Elevation of Glycolysis Confers Radio-Resistance by Facilitating DNA Repair in Cells. *BMC Cancer* (2015) 15:335. doi: 10.1186/s12885-015-1368-9
385. Zhao J-G, Ren K-M, Tang J. Overcoming 5-Fu Resistance in Human non-Small Cell Lung Cancer Cells by the Combination of 5-Fu and Cisplatin Through the Inhibition of Glucose Metabolism. *Tumour Biol J Int Soc Oncodev Biol Med* (2014) 35:12305–15. doi: 10.1007/s13277-014-2543-3
386. Ma S, Jia R, Li D, Shen B. Targeting Cellular Metabolism Chemosensitizes the Doxorubicin-Resistant Human Breast Adenocarcinoma Cells. *BioMed Res Int* (2015) 2015:453986. doi: 10.1155/2015/453986
387. Vara-Ciruelos D, Russell FM, Hardie DG. The Strange Case of AMPK and Cancer: Dr Jekyll or Mr Hyde?†. *Open Biol* (2019) 9:190099. doi: 10.1098/rsob.190099
388. Ghanbari Movahed Z, Rastegari-Pouyani M, Mohammadi MH, Mansouri K. Cancer Cells Change Their Glucose Metabolism to Overcome Increased ROS: One Step From Cancer Cell to Cancer Stem Cell? *BioMed Pharmacother* (2019) 112:108690. doi: 10.1016/j.biopha.2019.108690
389. Maddocks ODK, Vousden KH. Metabolic Regulation by P53. *J Mol Med Berl Ger* (2011) 89:237–45. doi: 10.1007/s00109-011-0735-5
390. Wu GS. The Functional Interactions Between the MAPK and P53 Signaling Pathways. *Cancer Biol Ther* (2004) 3:156–61. doi: 10.4161/cbt.3.2.614
391. Bhardwaj V, He J. Reactive Oxygen Species, Metabolic Plasticity, and Drug Resistance in Cancer. *Int J Mol Sci* (2020) 21:3412. doi: 10.3390/ijms21103412
392. suruo T, Naito M, Tomida A, Fujita N, Mashima T, Sakamoto H, et al. Molecular Targeting Therapy of Cancer: Drug Resistance, Apoptosis and Survival Signal. *Cancer Sci* (2003) 94:15–21. doi: 10.1111/j.1349-7006.2003.tb01345.x
393. Kuo MT. Redox Regulation of Multidrug Resistance in Cancer Chemotherapy: Molecular Mechanisms and Therapeutic Opportunities. *Antioxid Redox Signal* (2009) 11:99–133. doi: 10.1089/ars.2008.2095
394. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE, Gottesman MM. Revisiting the Role of ABC Transporters in Multidrug-Resistant Cancer. *Nat Rev Cancer* (2018) 18:452–64. doi: 10.1038/s41568-018-0005-8
395. Perillo B, Di Donato M, Pezone A, Di Zazzo E, Giovannelli P, Galasso G, et al. ROS in Cancer Therapy: The Bright Side of the Moon. *Exp Mol Med* (2020) 52:192–203. doi: 10.1038/s12276-020-0384-2
396. Cort A, Ozben T, Saso L, De Luca C, Korkina L. Redox Control of Multidrug Resistance and Its Possible Modulation by Antioxidants. *Oxid Med Cell Longev* (2016) 2016:e4251912. doi: 10.1155/2016/4251912
397. Chiche J, Brahimi-Horn MC, Pouysségur J. Tumour Hypoxia Induces a Metabolic Shift Causing Acidosis: A Common Feature in Cancer. *J Cell Mol Med* (2010) 14:771–94. doi: 10.1111/j.1582-4934.2009.00994.x
398. Webb BA, Chimenti M, Jacobson MP, Barber DL. Dysregulated pH: A Perfect Storm for Cancer Progression. *Nat Rev Cancer* (2011) 11:671–7. doi: 10.1038/nrc3110
399. Hashim AI, Zhang X, Wojtkowiak JW, Martinez GV, Gillies RJ. Imaging Ph and Metastasis. *NMR BioMed* (2011) 24:582–91. doi: 10.1002/nbm.1644
400. Gillies RJ, Pilot C, Marunaka Y, Fais S. Targeting Acidity in Cancer and Diabetes. *Biochim Biophys Acta Rev Cancer* (2019) 1871:273–80. doi: 10.1016/j.bbcan.2019.01.003
401. Sonveaux P, Végran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, et al. Targeting Lactate-Fueled Respiration Selectively Kills Hypoxic Tumor Cells in Mice. *J Clin Invest* (2008) 118:3930–42. doi: 10.1172/JCI36843
402. Peppicelli S, Toti A, Giannoni E, Bianchini F, Margheri F, Del Rosso M, et al. Metformin is Also Effective on Lactic Acidosis-Exposed Melanoma Cells Switched to Oxidative Phosphorylation. *Cell Cycle Georget Tex* (2016) 15:1908–18. doi: 10.1080/15384101.2016.1191706
403. Mazzio EA, Boukli N, Rivera N, Soliman KFA. Pericellular Ph Homeostasis is a Primary Function of the Warburg Effect: Inversion of Metabolic Systems to Control Lactate Steady State in Tumor Cells. *Cancer Sci* (2012) 103:422–32. doi: 10.1111/j.1349-7006.2012.02206.x
404. Lamonte G, Tang X, Chen JL-Y, Wu J, Ding C-KC, Keenan MM, et al. Acidosis Induces Reprogramming of Cellular Metabolism to Mitigate Oxidative Stress. *Cancer Metab* (2013) 1:23. doi: 10.1186/2049-3002-1-23
405. Vukovic V, Tannock IF. Influence of Low Ph on Cytotoxicity of Paclitaxel, Mitoxantrone and Topotecan. *Br J Cancer* (1997) 75:1167–72. doi: 10.1038/bjc.1997.201
406. Gerweck LE, Vijayappa S, Kozin S. Tumor Ph Controls the In Vivo Efficacy of Weak Acid and Base Chemotherapeutics. *Mol Cancer Ther* (2006) 5:1275–9. doi: 10.1158/1535-7163.MCT-06-0024
407. Saunders DE, Lawrence WD, Christensen C, Wappler NL, Ruan H, Deppe G. Paclitaxel-Induced Apoptosis in MCF-7 Breast-Cancer Cells. *Int J Cancer* (1997) 70:214–20. doi: 10.1002/(sici)1097-0215(19970117)70:2<214::aid-ijc13>3.0.co;2-i
408. Williams AC, Collard TJ, Paraskeva C. An Acidic Environment Leads to p53 Dependent Induction of Apoptosis in Human Adenoma and Carcinoma Cell Lines: Implications for Clonal Selection During Colorectal Carcinogenesis. *Oncogene* (1999) 18:3199–204. doi: 10.1038/sj.onc.1202660
409. Lotz C, Kelleher DK, Gassner B, Gekle M, Vaupel P, Thews O. Role of the Tumor Microenvironment in the Activity and Expression of the P-Glycoprotein in Human Colon Carcinoma Cells. *Oncol Rep* (2007) 17:239–44. doi: 10.3892/or.17.1.239
410. Ohtsubo T, Igawa H, Saito T, Matsumoto H, Park HJ, Song CW, et al. Acidic Environment Modifies Heat- or Radiation-Induced Apoptosis in Human Maxillary Cancer Cells. *Int J Radiat Oncol Biol Phys* (2001) 49:1391–8. doi: 10.1016/s0360-3016(00)01590-x
411. Ingangi V, Minopoli M, Ragone C, Motti ML, Carriero MV. Role of Microenvironment on the Fate of Disseminating Cancer Stem Cells. *Front Oncol* (2019) 9:82. doi: 10.3389/fonc.2019.00082
412. Vander Linden C, Corbet C. Therapeutic Targeting of Cancer Stem Cells: Integrating and Exploiting the Acidic Niche. *Front Oncol* (2019) 9:159. doi: 10.3389/fonc.2019.00159
413. Gupta GP, Massagué J. Cancer Metastasis: Building a Framework. *Cell* (2006) 127:679–95. doi: 10.1016/j.cell.2006.11.001
414. Pan C, Schoppe O, Parra-Damas A, Cai R, Todorov MI, Gondi G, et al. Deep Learning Reveals Cancer Metastasis and Therapeutic Antibody Targeting in the Entire Body. *Cell* (2019) 179:1661–76.e19. doi: 10.1016/j.cell.2019.11.013
415. Friebel E, Kapolou K, Unger S, Núñez NG, Utz S, Rushing EJ, et al. Single-Cell Mapping of Human Brain Cancer Reveals Tumor-Specific Instruction of Tissue-Invading Leukocytes. *Cell* (2020) 181:1626–42.e20. doi: 10.1016/j.cell.2020.04.055
416. Shree T, Olson OC, Elie BT, Kester JC, Garfall AL, Simpson K, et al. Macrophages and Cathepsin Proteases Blunt Chemotherapeutic Response in Breast Cancer. *Genes Dev* (2011) 25:2465–79. doi: 10.1101/gad.180331.111
417. Santoni M, Massari F, Amantini C, Nabissi M, Maines F, Burattini L, et al. Emerging Role of Tumor-Associated Macrophages as Therapeutic Targets in Patients With Metastatic Renal Cell Carcinoma. *Cancer Immunol Immunother CII* (2013) 62:1757–68. doi: 10.1007/s00262-013-1487-6
418. Jinushi M, Komohara Y. Tumor-Associated Macrophages as an Emerging Target Against Tumors: Creating a New Path From Bench to Bedside. *Biochim Biophys Acta* (2015) 1855:123–30. doi: 10.1016/j.bbcan.2015.01.002

419. Pepper MS. Role of the Matrix Metalloproteinase and Plasminogen Activator-Plasmin Systems in Angiogenesis. *Arterioscler Thromb Vasc Biol* (2001) 21:1104–17. doi: 10.1161/hq0701.093685
420. Masuda M, Wakasaki T, Suzui M, Toh S, Joe AK, Weinstein IB. Stat3 Orchestrates Tumor Development and Progression: The Achilles' Heel of Head and Neck Cancers? *Curr Cancer Drug Targets* (2010) 10:117–26. doi: 10.2174/156800910790980197
421. Jinushi M, Chiba S, Yoshiyama H, Masutomi K, Kinoshita I, Dosaka-Akita H, et al. Tumor-Associated Macrophages Regulate Tumorigenicity and Anticancer Drug Responses of Cancer Stem/Initiating Cells. *Proc Natl Acad Sci USA* (2011) 108:12425–30. doi: 10.1073/pnas.1106645108
422. Shamamian P, Schwartz JD, Pocock BJ, Monea S, Whiting D, Marcus SG, et al. Activation of Progelatinase A (Mmp-2) by Neutrophil Elastase, Cathepsin G, and proteinase-3: A Role for Inflammatory Cells in Tumor Invasion and Angiogenesis. *J Cell Physiol* (2001) 189:197–206. doi: 10.1002/jcp.10014
423. Mm Q, Re R, Rg H, Cr K-P, Cl J. Breast Cancer Cells Stimulate Neutrophils to Produce Oncostatin M: Potential Implications for Tumor Progression. *Cancer Res* (2005) 65:8896–904. doi: 10.1158/0008-5472.can-05-1734
424. Shojaei F, Singh M, Thompson JD, Ferrara N. Role of Bv8 in Neutrophil-Dependent Angiogenesis in a Transgenic Model of Cancer Progression. *Proc Natl Acad Sci USA* (2008) 105:2640–5. doi: 10.1073/pnas.0712185105
425. Zhou S-L, Zhou Z-J, Hu Z-Q, Huang X-W, Wang Z, Chen E-B, et al. Tumor-Associated Neutrophils Recruit Macrophages and T-Regulatory Cells to Promote Progression of Hepatocellular Carcinoma and Resistance to Sorafenib. *Gastroenterology* (2016) 150:1646–58.e17. doi: 10.1053/j.gastro.2016.02.040
426. Ostrand-Rosenberg S, Sinha P, Beury DW, Clements VK. Cross-Talk Between Myeloid-Derived Suppressor Cells (MDSC), Macrophages, and Dendritic Cells Enhances Tumor-Induced Immune Suppression. *Semin Cancer Biol* (2012) 22:275–81. doi: 10.1016/j.semcancer.2012.01.011
427. Beury DW, Parker KH, Nyandjo M, Sinha P, Carter KA, Ostrand-Rosenberg S. Cross-Talk Among Myeloid-Derived Suppressor Cells, Macrophages, and Tumor Cells Impacts the Inflammatory Milieu of Solid Tumors. *J Leukoc Biol* (2014) 96:1109–18. doi: 10.1189/jlb.3A0414-210R
428. Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, Sung M, et al. The Novel Role of Tyrosine Kinase Inhibitor in the Reversal of Immune Suppression and Modulation of Tumor Microenvironment for Immune-Based Cancer Therapies. *Cancer Res* (2009) 69:2514–22. doi: 10.1158/0008-5472.CAN-08-4709
429. Kao J, Ko EC, Eisenstein S, Sikora AG, Fu S, Chen S-H. Targeting Immune Suppressing Myeloid-Derived Suppressor Cells in Oncology. *Crit Rev Oncol Hematol* (2011) 77:12–9. doi: 10.1016/j.critrevonc.2010.02.004
430. Ramachandran IR, Condamine T, Lin C, Herlihy SE, Garfall A, Vogl DT, et al. Bone Marrow PMN-MDSCs and Neutrophils are Functionally Similar in Protection of Multiple Myeloma From Chemotherapy. *Cancer Lett* (2016) 371:117–24. doi: 10.1016/j.canlet.2015.10.040
431. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. *Cancer Discovery* (2011) 1:54–67. doi: 10.1158/2159-8274.CD-10-0028
432. Wang Y-Q, Guo R-D, Guo R-M, Sheng W, Yin L-R. MicroRNA-182 Promotes Cell Growth, Invasion, and Chemoresistance by Targeting Programmed Cell Death 4 (PDCD4) in Human Ovarian Carcinomas. *J Cell Biochem* (2013) 114:1464–73. doi: 10.1002/jcb.24488
433. Tanaka K, Miyata H, Sugimura K, Fukuda S, Kanemura T, Yamashita K, et al. miR-27 Is Associated With Chemoresistance in Esophageal Cancer Through Transformation of Normal Fibroblasts to Cancer-Associated Fibroblasts. *Carcinogenesis* (2015) 36:894–903. doi: 10.1093/carcin/bgv067
434. Luga V, Zhang L, Viloria-Petit AM, Ogunjimi AA, Inanlou MR, Chiu E, et al. Exosomes Mediate Stromal Mobilization of Autocrine Wnt-PCP Signaling in Breast Cancer Cell Migration. *Cell* (2012) 151:1542–56. doi: 10.1016/j.cell.2012.11.024
435. Boelens MC, Wu TJ, Nabet BY, Xu B, Qiu Y, Yoon T, et al. Exosome Transfer From Stromal to Breast Cancer Cells Regulates Therapy Resistance Pathways. *Cell* (2014) 159:499–513. doi: 10.1016/j.cell.2014.09.051
436. Hu Y, Yan C, Mu L, Huang K, Li X, Tao D, et al. Fibroblast-Derived Exosomes Contribute to Chemoresistance Through Priming Cancer Stem Cells in Colorectal Cancer. *PLoS One* (2015) 10:e0125625. doi: 10.1371/journal.pone.0125625
437. Bielecka ZF, Czarnecka AM, Solarek W, Kornakiewicz A, Szczylik C. Mechanisms of Acquired Resistance to Tyrosine Kinase Inhibitors in Clear - Cell Renal Cell Carcinoma (Ccrcc). *Curr Signal Transduct Ther* (2014) 8:218–28. doi: 10.2174/1574362409666140206223014
438. Zhang H, Zhang X, Wu X, Li W, Su P, Cheng H, et al. Interference of Frizzled 1 (FZD1) Reverses Multidrug Resistance in Breast Cancer Cells Through the Wnt/β-Catenin Pathway. *Cancer Lett* (2012) 323:106–13. doi: 10.1016/j.canlet.2012.03.039
439. Chang HW, Roh J-L, Jeong E-J, Lee S, Kim S-W, Choi S-H, et al. Wnt Signaling Controls Radiosensitivity Via Cyclooxygenase-2-Mediated Ku Expression in Head and Neck Cancer. *Int J Cancer* (2008) 122:100–7. doi: 10.1002/ijc.23069
440. Cojoc M, Peitzsch C, Kurth I, Trautmann F, Kunz-Schughart LA, Telegeev GD, et al. Aldehyde Dehydrogenase is Regulated by β-Catenin/TCF and Promotes Radioresistance in Prostate Cancer Progenitor Cells. *Cancer Res* (2015) 75:1482–94. doi: 10.1158/0008-5472.CAN-14-1924
441. Park JS, Burckhardt CJ, Lazcano R, Solis LM, Isogai T, Li L, et al. Mechanical Regulation of Glycolysis Via Cytoskeleton Architecture. *Nature* (2020) 578:621–6. doi: 10.1038/s41586-020-1998-1
442. Ulrich TA, Pardo EM de J, Kumar S. The Mechanical Rigidity of the Extracellular Matrix Regulates the Structure, Motility, and Proliferation of Glioma Cells. *Cancer Res* (2009) 69:4167–74. doi: 10.1158/0008-5472.CAN-08-4859
443. Jahanban-Esfahlan R, Seidi K, Manjili MH, Jahanban-Esfahlan A, Javaheri T, Zare P. Tumor Cell Dormancy: Threat or Opportunity in the Fight Against Cancer. *Cancers* (2019) 11:1207. doi: 10.3390/cancers11081207
444. Liu Y, Lv J, Liang X, Yin X, Zhang L, Chen D, et al. Fibrin Stiffness Mediates Dormancy of Tumor-Repopulating Cells Via a Cdc42-Driven Tet2 Epigenetic Program. *Cancer Res* (2018) 78:3926–37. doi: 10.1158/0008-5472.CAN-17-3719
445. Yu H, Mouw JK, Weaver VM. Forcing Form and Function: Biomechanical Regulation of Tumor Evolution. *Trends Cell Biol* (2011) 21:47–56. doi: 10.1016/j.tcb.2010.08.015
446. Pratt SJP, Lee RM, Martin SS. The Mechanical Microenvironment in Breast Cancer. *Cancers* (2020) 12:1452. doi: 10.3390/cancers12061452
447. Jain RK, Martin JD, Stylianopoulos T. The Role of Mechanical Forces in Tumor Growth and Therapy. *Annu Rev BioMed Eng* (2014) 16:321–46. doi: 10.1146/annurev-bioeng-071813-105259
448. Kular JK, Basu S, Sharma RI. The Extracellular Matrix: Structure, Composition, Age-Related Differences, Tools for Analysis and Applications for Tissue Engineering. *J Tissue Eng* (2014) 5:2041731414557112. doi: 10.1177/2041731414557112
449. McLeod CM, Mauck RL. High Fidelity Visualization of Cell-to-Cell Variation and Temporal Dynamics in Nascent Extracellular Matrix Formation. *Sci Rep* (2016) 6:38852. doi: 10.1038/srep38852
450. Cox TR, Erler JT. Remodeling and Homeostasis of the Extracellular Matrix: Implications for Fibrotic Diseases and Cancer. *Dis Model Mech* (2011) 4:165–78. doi: 10.1242/dmm.004077
451. Pfeifer CR, Alvey CM, Irianto J, Discher DE. Genome Variation Across Cancers Scales With Tissue Stiffness - an Invasion-Mutation Mechanism and Implications for Immune Cell Infiltration. *Curr Opin Syst Biol* (2017) 2:103–14. doi: 10.1016/j.coisb.2017.04.005
452. Deville SS, Cordes N. The Extracellular, Cellular, and Nuclear Stiffness, a Trinity in the Cancer Resistome—a Review. *Front Oncol* (2019) 9:1376. doi: 10.3389/fonc.2019.01376
453. Dhillon AS, Hagan S, Rath O, Kolch W. MAP Kinase Signalling Pathways in Cancer. *Oncogene* (2007) 26:3279–90. doi: 10.1038/sj.onc.1210421
454. Cobbaut M, Karagil S, Bruno L, Diaz de la Loza MDC, Mackenzie FE, Stolinski M, et al. Dysfunctional Mechanotransduction Through the YAP/TAZ/Hippo Pathway as a Feature of Chronic Disease. *Cells* (2020) 9:151. doi: 10.3390/cells9010151
455. Wei L, Surma M, Shi S, Lambert-Cheatham N, Shi J. Novel Insights Into the Roles of Rho Kinase in Cancer. *Arch Immunol Ther Exp (Warsz)* (2016) 64:259–78. doi: 10.1007/s00005-015-0382-6
456. Humphrey JD, Dufresne ER, Schwartz MA. Mechanotransduction and Extracellular Matrix Homeostasis. *Nat Rev Mol Cell Biol* (2014) 15:802–12. doi: 10.1038/nrm3896

457. Northcott JM, Dean IS, Mouw JK, Weaver VM. Feeling Stress: The Mechanics of Cancer Progression and Aggression. *Front Cell Dev Biol* (2018) 6:17. doi: 10.3389/fcell.2018.00017
458. Chow K-H, Factor RE, Ullman KS. The Nuclear Envelope Environment and its Cancer Connections. *Nat Rev Cancer* (2012) 12:196–209. doi: 10.1038/nrc3219
459. Dahl KN, Kahn SM, Wilson KL, Discher DE. The Nuclear Envelope Lamina Network has Elasticity and a Compressibility Limit Suggestive of a Molecular Shock Absorber. *J Cell Sci* (2004) 117:4779–86. doi: 10.1242/jcs.01357
460. Denais C, Lammerding J. Nuclear Mechanics in Cancer. *Adv Exp Med Biol* (2014) 773:435–70. doi: 10.1007/978-1-4899-8032-8_20
461. Isermann P, Lammerding J. Nuclear Mechanics and Mechanotransduction in Health and Disease. *Curr Biol* (2013) 23:R1113–21. doi: 10.1016/j.cub.2013.11.009
462. Nguyen TV, Sleiman M, Moriarty T, Herrick WG, Peyton SR. Sorafenib Resistance and JNK Signaling in Carcinoma During Extracellular Matrix Stiffening. *Biomaterials* (2014) 35:5749–59. doi: 10.1016/j.biomaterials.2014.03.058
463. Joyce MH, Lu C, James ER, Hegab R, Allen SC, Suggs LJ, et al. Phenotypic Basis for Matrix Stiffness-Dependent Chemoresistance of Breast Cancer Cells to Doxorubicin. *Front Oncol* (2018) 8:337. doi: 10.3389/fonc.2018.00337
464. Liu C, Liu Y, Xie H-G, Zhao S, Xu X-X, Fan L-X, et al. Role of Three-Dimensional Matrix Stiffness in Regulating the Chemoresistance of Hepatocellular Carcinoma Cells. *Biotechnol Appl Biochem* (2015) 62:556–62. doi: 10.1002/bab.1302
465. Schrader J, Gordon-Walker TT, Aucott RL, van Deemter M, Quaas A, Walsh S, et al. Matrix Stiffness Modulates Proliferation, Chemotherapeutic Response, and Dormancy in Hepatocellular Carcinoma Cells. *Hepatol Baltim Md* (2011) 53:1192–205. doi: 10.1002/hep.24108
466. You Y, Zheng Q, Dong Y, Xie X, Wang Y, Wu S, et al. Matrix Stiffness-Mediated Effects on Stemness Characteristics Occurring in HCC Cells. *Oncotarget* (2016) 7:32221–31. doi: 10.18632/oncotarget.8515
467. Helmlinger G, Netti PA, Lichtenbeld HC, Melder RJ, Jain RK. Solid Stress Inhibits the Growth of Multicellular Tumor Spheroids. *Nat Biotechnol* (1997) 15:778–83. doi: 10.1038/nbt0897-778
468. Padera TP, Stoll BR, Tooredman JB, Capen D, di Tomaso E, Jain RK. Cancer Cells Compress Intratumour Vessels. *Nature* (2004) 427:695–5. doi: 10.1038/427695a
469. Talukdar S, Bhoopathi P, Emdad L, Das S, Sarkar D, Fisher PB. Dormancy and Cancer Stem Cells: An Enigma for Cancer Therapeutic Targeting. *Adv Cancer Res* (2019) 141:43–84. doi: 10.1016/bs.acr.2018.12.002
470. Jain RK. Antiangiogenesis Strategies Revisited: From Starving Tumors to Alleviating Hypoxia. *Cancer Cell* (2014) 26:605–22. doi: 10.1016/j.ccell.2014.10.006
471. Tse JM, Cheng G, Tyrrell JA, Wilcox-Adelman SA, Boucher Y, Jain RK, et al. Mechanical Compression Drives Cancer Cells Toward Invasive Phenotype. *Proc Natl Acad Sci USA* (2012) 109:911–6. doi: 10.1073/pnas.1118910109
472. Moffitt L, Karimnia N, Stephens A, Bilandzic M. Therapeutic Targeting of Collective Invasion in Ovarian Cancer. *Int J Mol Sci* (2019) 20:1466. doi: 10.3390/ijms20061466
473. Cheung KJ, Gabrielson E, Werb Z, Ewald AJ. Collective Invasion in Breast Cancer Requires a Conserved Basal Epithelial Program. *Cell* (2013) 155:1639–51. doi: 10.1016/j.cell.2013.11.029
474. Karakashev SV, Reginato MJ. Progress Toward Overcoming Hypoxia-Induced Resistance to Solid Tumor Therapy. *Cancer Manag Res* (2015) 7:253–64. doi: 10.2147/CMAR.S58285
475. Broders-Bondon F, Nguyen Ho-Bouldoires TH, Fernandez-Sanchez M-E, Farge E. Mechanotransduction in Tumor Progression: The Dark Side of the Force. *J Cell Biol* (2018) 217:1571–87. doi: 10.1083/jcb.201701039
476. Heldin C-H, Rubin K, Pietras K, Östman A. High Interstitial Fluid Pressure — An Obstacle in Cancer Therapy. *Nat Rev Cancer* (2004) 4:806–13. doi: 10.1038/nrc1456
477. Provenzano PP, Cuevas C, Chang AE, Goel VK, Von Hoff DD, Hingorani SR. Enzymatic Targeting of the Stroma Ablates Physical Barriers to Treatment of Pancreatic Ductal Adenocarcinoma. *Cancer Cell* (2012) 21:418–29. doi: 10.1016/j.ccr.2012.01.007
478. Marchesi F, Piemonti L, Mantovani A, Allavena P. Molecular Mechanisms of Perineural Invasion, a Forgotten Pathway of Dissemination and Metastasis. *Cytokine Growth Factor Rev* (2010) 21:77–82. doi: 10.1016/j.cytogfr.2009.11.001
479. Coarfa C, Florentin D, Putluri N, Ding Y, Au J, He D, et al. Influence of the Neural Microenvironment on Prostate Cancer. *Prostate* (2018) 78:128–39. doi: 10.1002/pros.23454
480. Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ, et al. Autonomic Nerve Development Contributes to Prostate Cancer Progression. *Science* (2013) 341:1236361. doi: 10.1126/science.1236361
481. Melamed R, Rosenne E, Shakhar K, Schwartz Y, Abudarham N, Ben-Eliyahu S. Marginating Pulmonary-NK Activity and Resistance to Experimental Tumor Metastasis: Suppression by Surgery and the Prophylactic Use of a Beta-Adrenergic Antagonist and a Prostaglandin Synthesis Inhibitor. *Brain Behav Immun* (2005) 19:114–26. doi: 10.1016/j.bbi.2004.07.004
482. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, et al. Chronic Stress Promotes Tumor Growth and Angiogenesis in a Mouse Model of Ovarian Carcinoma. *Nat Med* (2006) 12:939–44. doi: 10.1038/nm1447
483. Amit M, Takahashi H, Dragomir MP, Lindemann A, Gleber-Netto FO, Pickering CR, et al. Loss of p53 Drives Neuron Reprogramming in Head and Neck Cancer. *Nature* (2020) 578:449–54. doi: 10.1038/s41586-020-1996-3
484. Kim-Fuchs C, Le CP, Pimentel MA, Shackleford D, Ferrari D, Angst E, et al. Chronic Stress Accelerates Pancreatic Cancer Growth and Invasion: A Critical Role for Beta-Adrenergic Signaling in the Pancreatic Microenvironment. *Brain Behav Immun* (2014) 40:40–7. doi: 10.1016/j.bbi.2014.02.019
485. Pundavela J, Demont Y, Jobling P, Lincz LF, Roselli S, Thorne RF, et al. ProNGF Correlates With Gleason Score and Is a Potential Driver of Nerve Infiltration in Prostate Cancer. *Am J Pathol* (2014) 184:3156–62. doi: 10.1016/j.ajpath.2014.08.009
486. Zhao C-M, Hayakawa Y, Kodama Y, Muthupalani S, Westphalen CB, Andersen GT, et al. Denervation Suppresses Gastric Tumorigenesis. *Sci Transl Med* (2014) 6:250ra115. doi: 10.1126/scitranslmed.3009569
487. Osswald M, Jung E, Sahm F, Solecki G, Venkataramani V, Blaes J, et al. Brain Tumour Cells Interconnect to a Functional and Resistant Network. *Nature* (2015) 528:93–8. doi: 10.1038/nature16071
488. Xi G, Li YD, Grahovac G, Rajaram V, Wadhwani N, Pundy T, et al. Targeting CD133 Improves Chemotherapeutic Efficacy of Recurrent Pediatric Pilocytic Astrocytoma Following Prolonged Chemotherapy. *Mol Cancer* (2017) 16:21. doi: 10.1186/s12943-017-0593-z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 17 September 2021
doi: 10.3389/fonc.2021.723888

Immunotherapeutic Potential of T Memory Stem Cells

Yujie Li[1], *Dengqiang Wu*[2], *Xuejia Yang*[2] *and Sufang Zhou*[1,2*]

[1] Department of Biochemistry and Molecular Biology, School of Pre-Clinical Science, Guangxi Medical University, Nanning, China, [2] National Center for International Research of Bio-targeting Theranostics, Guangxi Key Laboratory of Bio-targeting Theranostics, Collaborative Innovation Center for Targeting Tumor Diagnosis and Therapy, Guangxi Medical University, Nanning, China

Memory T cells include T memory stem cells (T_{SCM}) and central memory T cells (T_{CM}). Compared with effector memory T cells (T_{EM}) and effector T cells (T_{EFF}), they have better durability and anti-tumor immunity. Recent studies have shown that although T_{SCM} has excellent self-renewal ability and versatility, if it is often exposed to antigens and inflammatory signals, T_{SCM} will behave as a variety of inhibitory receptors such as PD-1, TIM-3 and LAG-3 expression, and metabolic changes from oxidative phosphorylation to glycolysis. These changes can lead to the exhaustion of T cells. Cumulative evidence in animal experiments shows that it is the least differentiated cell in the memory T lymphocyte system and is a central participant in many physiological and pathological processes in humans. It has a good clinical application prospect, so it is more and more important to study the factors affecting the formation of T_{SCM}. This article summarizes and prospects the phenotypic and functional characteristics of T_{SCM}, the regulation mechanism of formation, and its application in treatment of clinical diseases.

Keywords: T memory stem cells, stemness, tumor immunotherapy, HIV, autoimmune diseases

OPEN ACCESS

Edited by:
Ira Ida Skvortsova,
Innsbruck Medical University, Austria

Reviewed by:
Xia Liu,
Baylor College of Medicine, United States
Sukhbir Kaur,
National Institutes of Health (NIH), United States

****Correspondence:***
Sufang Zhou
zsf200000@163.com

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *11 June 2021*
Accepted: *23 August 2021*
Published: *17 September 2021*

Citation:
Li Y, Wu D, Yang X and Zhou S (2021) Immunotherapeutic Potential of T Memory Stem Cells. Front. Oncol. 11:723888. doi: 10.3389/fonc.2021.723888

INTRODUCTION

Immunotherapy has become one of the most promising strategies in cancer treatment, and has shown good efficacy in clinical trials (1). In particular, chimeric antigen receptor-engineered T cells (CAR-T) can specifically and effectively kill tumor cells, bringing new hopes for the treatment of patients with malignant tumors (2–7). However, whether it is traditional immune cell therapy or new CAR-T cells and T-cell receptor T cells (TCR-T) therapy, all are based on terminally differentiated effector T (T_{TE}) cells, making it difficult to exert long-lasting anti-tumor effects in the body (8). Adoptive T cell therapy (ACT) is the *in vitro* expansion and reinfusion of tumor-reactive T cells, and is a potential treatment method for the treatment of advanced cancer (9–14). In infections and cancers, T lymphocytes expand and differentiate into effector cells and memory cells that clear pathogens. These cells can survive for a long time and ensure that they have a protective effect against pathogens when they are re-attacked by antigens (15). Human T lymphocytes are generally divided into naive T cell (T_N), central memory T cell (T_{CM}), effector memory T cell (T_{EM}) and effector T cell (T_{EFF}). In 2005, in the study of graft *versus* host disease (GVHD) in mice, a group of special memory T cell subsets with super proliferation and differentiation ability was observed for the first time. It produces persistent graft-*versus*-host disease, which the researchers named "stem like memory T cells" (T_{SCM}) (16). Studies have shown that adoptively infused young T cells can

self-renew and differentiate in mice, having the ability to survive for a long time, and exhibit significantly better anti-tumor capabilities than T_{TE} cells. The progressive differentiation of T lymphocytes leads to a gradual loss of function and therapeutic potential. These studies suggest that poorly differentiated immune cells may have more application potential in clinical treatment (17–21).

T_{SCM} cells have great potential in overcoming the limitations of current T cell-based immunotherapy (22–24). In mouse tumor models and human hematopoietic stem cell transplantation (HSCT) patients, T_{SCM} cells have higher antitumor activity and survival rate. However, the proportion of T_{SCM} cells in peripheral blood is low, which limits its application in immunotherapy. In this review, we summarize the latest findings, and discuss in depth the phenotype, function, differentiation mechanism and clinical application of memory T cells. It is hoped that using the therapeutic potential of T_{SCM} cells for adoptive immunotherapy provides new ideas. The conceptual work and key discoveries that formed this field of investigation are shown in **Figure 1** (25–38), which mainly summarizes the main discoveries in the process of T_{SCM} cell research in recent years and the new research on the occurrence and development of diseases, some of which are introduced in articles.

PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF MEMORY T CELLS

T_{SCM} is a T cell subset with self-renewal ability and pluripotency potential. This group of memory T cells can play the role of acquired immune function in the process of the body's fight against viruses or tumors (36, 39). T cell populations are classified by some surface markers, and distinguished according to their functions and sources, and the production of their effector cytokines. Memory T cells can be divided into T_{CM} and T_{EM}. T_{EM} cells and T_{CM} cells circulate in the blood and target the secondary lymphoid tissues. The degree of differentiation of T_{CM} cells is lower than that of T_{EM} and effector cells, and its telomeres are found to be longer, and the expression of perforin, granzyme and other effector molecules is lower (40). In addition, the T_{SCM} pool should be limited to lymph nodes and secondary lymphoid organs, which are T cells that have antigen experience. The current research results also show that T_{CM} has the function of T memory stem cells. T_{CM} has stronger immune replacement ability and stronger survival ability *in vivo* than T_{EM} cells. T_{SCM} is developed from naive T cells in a resting state. It is a group of cells between T_N and T_{CM}. It also has the characteristics of T_N cells and memory T cells (T_M), and then differentiates into T_{CM} and T_{EM}. Good et al. (41) used single-cell mass cytometry to track the proliferation history of T cells. By analyzing the changes in phenotype and protein expression of T cells at different times and in different division states, it assisted in confirming the T cell differentiation theory: $T_N \rightarrow T_{SCM} \rightarrow T_{CM} \rightarrow T_{EM}$. It is worth noting that only naive T cells and T_{SCM} cells can reconstruct the heterogeneity of the entire memory T cell subset. At present, malignant tumors are one of the important diseases threatening human health, and there is no effective method to treat them. Due to their own characteristics, T_{SCM} cells have shown their strong potential for tumor therapy.

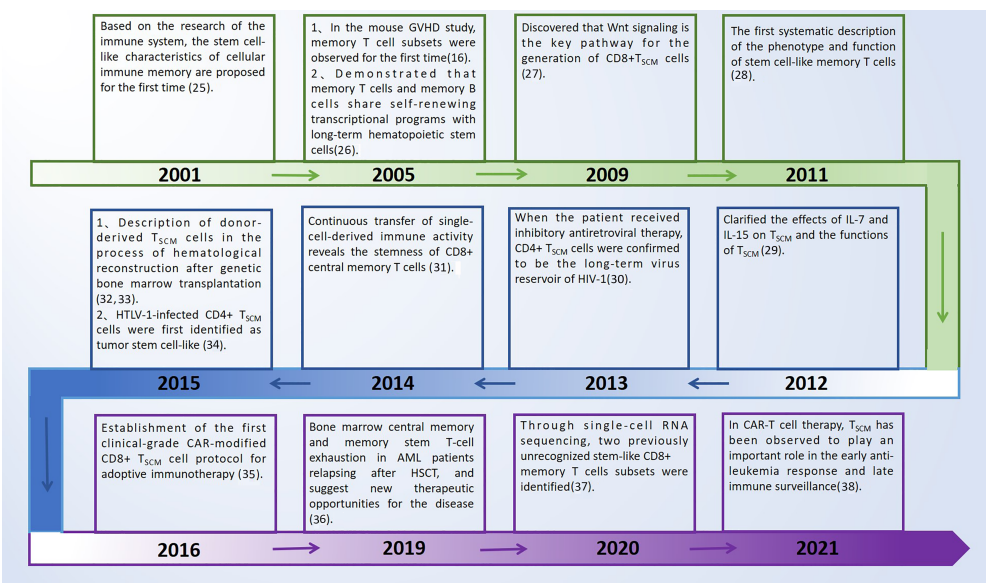


FIGURE 1 | Key discoveries on T_{SCM} cells. GVHD, graft *versus* host disease; T_{SCM} cells, T memory stem cells; HTLV-1, human T cell lymphotropic virus type 1; HIV-1, human immunodeficiency virus type 1; CAR-T, chimeric antigen receptor-engineered T cells; AML, acute myeloid leukemia; HSCT, hematopoietic stem cell transplantation.

According to the different expressions of cell surface chemokine receptor (CCR7) and lymph node homing molecules (CD62L), memory T cells are divided into T_{CM} and T_{EM}. T_{CM} highly expresses CCR7 and CD62L, homing to secondary lymphoid organs, but low expression in T_{EM}, which preferentially transports to peripheral tissues and mediates rapid effector functions. T_{SCM} cells express naive cell phenotypes (CD45RA, CD62L, CCR7, CD95, CD27, CD122), and are characterized by rapid response to antigens, expression of a variety of effector molecules, and generation of memory effector cells. $CD45RA^+$ is closely related to its memory ability. Naive cells express two molecules CD27 and CD45RA at the same time. Memory and effector cells only express CD27 or CD45RA respectively. T_{SCM} cells highly express IL-2, IFN-γ, TNF-α, Bcl- 2. IL-7 and other molecules related to early differentiation of T cells, low expression of CD57 and other molecules related to T cell senescence, showing stronger degranulation ability and the ability to produce inflammatory cytokines. Recent studies have found that by detecting the expression of CD122 or CXCR3 in healthy people by flow cytometry, the T_{SCM} $CD122^{hi}$-expressing subset demonstrate greater proliferation, greater multipotency and enhanced polyfunctionality with higher frequencies of triple positive (TNF-α, IL-2, IFN-γ) cytokine-producing cells upon exposure to recall antigen. The cell proliferation and multifunctional cytokine production of the T_{SCM} $CXCR3^{lo}$ population are also significantly increased (42). Loss of CXCR3 promotes stem-like memory precursor differentiation (43). According to these surface markers, T_{SCM} cells can be accurately distinguished. T_{SCM} cells represent a subset of minimally differentiated T cells, which are characterized by phenotypic and functional characteristics that connect naive and conventional memory cells.

The above mainly describes the surface markers of human T cell subsets. In addition to the specific T cell receptor (TCR), both human and murine T_{SCM} express common markers of memory T cells (mouse CD62L, human CCR7, human CD45RO), and anti-apoptotic marker molecules (Bcl- 2), the cytokine receptor markers related to survival and proliferation CD122 (co-receptor of IL-2, IL-7 and IL-15) and CD127 (IL-7 receptor), stem cell marker (Sca-1). Human and murine T cell subsets are defined by different phenotypes (16, 27) (**Table 1**).

DEVELOPMENT OF T_{SCM} CELLS

Manipulation to Produce T_{SCM} Cells in *(Ex) Vivo*

The relative scarcity of circulating T_{SCM} cells limits their use in tumor therapy, which has led to manufacturing protocols that expand this cell type *in vitro*. As an important participant in the function of T cells, cytokines play an important role in the maintenance and expansion of T_{SCM} subset. Recently reported related cytokines that can promote T_{SCM} expansion are shown in **Figure 2**. A large number of studies have shown that adding different cytokines to the immune cell culture system can make it differentiate into memory or effector T cells (44–47). γc-cytokine IL-2, as a T cell growth factor, is still the most common cytokine used to expand therapeutic T cell products for patients (29, 48–50). γc-cytokine IL-2, as a T cell growth factor, is still the most common cytokine used to expand therapeutic T cell products for patients. However, high IL-2 levels reduced the overall production of early memory T cells by reducing central memory T cells and augmenting effectors. The number of early memory T cells in the T cell subset could be increased by simply reducing the amount of IL-2 (51). In the *in vitro* expansion process, repeated use of IL-2 to stimulate T cells would also cause T cell depletion and reduced T cell persistence (52). IL-7 could also promote the proliferation of T_{SCM} cells by down-regulating the expression of programmed cell death protein 1 (PD-1) and Foxp3, and promoted the ability of $CD4^+$ T cells to produce IFN-γ, IL-2, TNF-α and granzyme B. The involvement of STAT5 in IL-7-induced polyfunctionality, this the polyfunctional phenotype driven by IL-7 is associated with increased histone acetylation effector gene promoters and reveals previously unknown characteristics of IL-7 (53–56). The current study, CAR-T cells expanded in IL-15 (CAR-T/IL-15) preserved a less-differentiated T_{SCM} phenotype, defined by expression of $CD62L^+CD45RA^+CCR7^+$, as compared to cells cultured in IL-2 (CAR-T/IL-2). What's more, CAR-T/IL-15 cells exhibited reduced expression of exhaustion markers, higher anti-apoptotic properties, and increased proliferative capacity when it was attacked by antigens (57). The combined use of IL-7 and IL-15 can preserve the T_{SCM} phenotype and enhance the effectiveness of CAR-T cells (11, 29, 58–61). IL-21 was critical for the long-term maintenance and functionality of

TABLE 1 | Phenotypic markers of memory T cells.

Subset	Phenotype (Human)	Phenotype (Mice)	Characteristics
T_N	$CD45RA^+$,$CD45RO^-$,$CCR7^+$,$CD62L^+$,$CD127^+$,$CD122^+$,$CD27^+$,$CD44^-$,$CD28^+$,$CD43^-$,$CD95^-$, $CD57^-$,$CD58^-$,$CD11\alpha^-$,$(IL\text{-}7R\alpha)^+$,$CXCR3^-$,$(IL\text{-}2R\beta)^-$	$CD44^-$, $CD62L^+$, $CCR7^+$, $CXCR5^-$, $CXCR3^-$	Multidirectional differentiation ability
T_{SCM}	$CD45RA^+$,$CD45RO^-$,$CCR7^+$,$CD62L^+$,$CD127^+$,$CD122^+$,$CD27^+$,$CD44^{+/-}$,$CD28^+$, $CD43^-$, $CD95^+$,$CD57^-$,$CD58^+$,$CD11\alpha^+$,$(IL\text{-}7R\alpha)^+$,$CXCR3^+$,$(IL\text{-}2R\beta)^+$	$CD44^-$, $CD62L^-$,$(Sca\text{-}1)^+$,$CD122^+$, $(Bcl\text{-}2)^+$,$CCR5^+$,$CXCR3^+$	Self-renewal capacity and multipotency
T_{CM}	$CD45RA^-$,$CD45RO^+$,$CCR7^+$,$CD62L^+$,$CD127^+$,$CD122^+$,$CD27^+$,$CD44^+$,$CD28^+$, $CD43^-$,$CD95^+$, $CD57^-$,$CD58^+$,$CD11\alpha^+$,$(IL\text{-}7R\alpha)^+$,$CXCR3^+$,$(IL\text{-}2R\beta)^+$	$CD44^+$, $CD62L^+$, $CCR7^+$	Long-lasting immune memory
T_{EM}	$CD45RA^-$,$CD45RO^+$,$CCR7^-$,$CD62L^-$,$CD127^+$,$CD122^+$,$CD27^{+/-}$,$CD44^+$,$CD28^{+/-}$,$CD43^{+/-}$, $CD95^+$,$CD57^{+/-}$,$CD58^+$,$CD11\alpha^+$,$(IL\text{-}7R\alpha)^{+/-}$, $CXCR3^+$,$(IL\text{-}2R\beta)^+$	$CD44^+$, $CD62L^-$, $CCR7^-$	Immediate effector function
T_{TE}	$CD45RA^-$,$CD45RO^-$,$CCR7^-$,$CD62L^-$,$CD127^+$,$CD122^-$,$CD27^-$,$CD44^-$,$CD28^-$,$CD43^+$,$CD95^+$, $CD57^+$,$CD58^+$,$CD11\alpha^+$,$(IL\text{-}7R\alpha)^-$,$CXCR3^-$,$(IL\text{-}2R\beta)^+$	$CD44^-$, $CD62L^+$	Terminally differentiated effector T cells

"+" positive expression; "–" negative expression; T_N, naive T cell; T_{SCM}, stem cell memory T cell; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{TE}, terminal effector T cell.

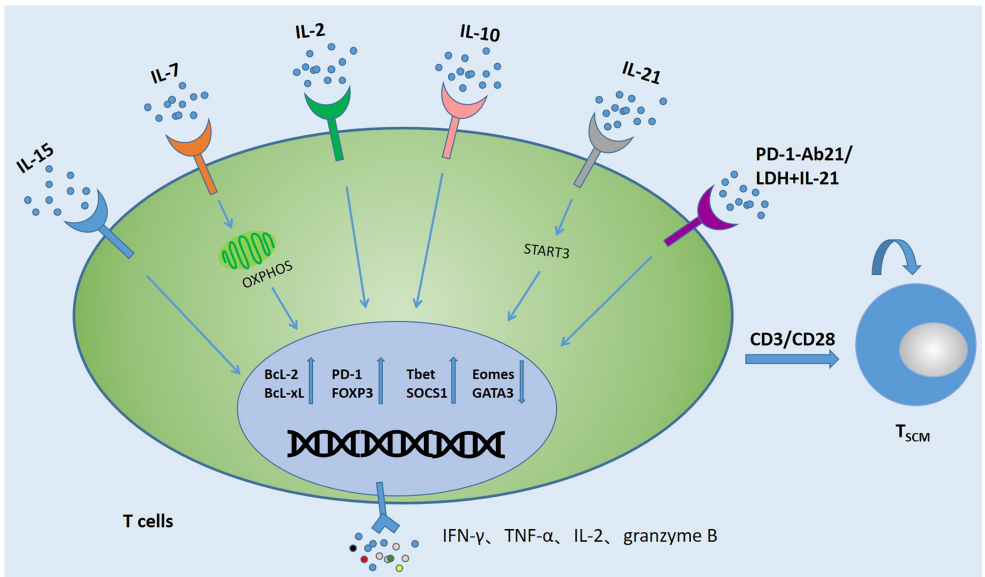


FIGURE 2 | Several strategies to induce the generation of T_{SCM}. Activating T cells (CAR-T cells, TCR-T cells, TILs, VSTs) with anti-CD3/CD28 antibodies and co-cultivating them with cytokines or combined with PD-1 and LDH can promote the production of T_{SCM} cells and change the expression levels of related anti-apoptotic proteins and metabolic molecules. In addition, the expression of TNF-α, IFN-γ, IL-2 and Granzyme B also increased significantly.

$CD8^+$T cells and the control of chronic lymphocytic choriomeningitis virus (LCWV) infection in mice. In the process of chronic infection, cell-autonomous IL-21 receptor (IL-21R)–dependent signaling by $CD8^+$ T cells was required for sustained cell proliferation and cytokine production (62, 63). IL-21 also can promote the generation of T_{SCM} cells. It activates the Janus kinase signal transducer and activator of transcription 3 pathway by upregulating signal transducer and activator of transcription 3 phosphorylation and thereby promoting the expression of T-bet and suppressor of cytokine signaling 1, while decreasing the expression of eomesodermin (Eomes) and GATA binding protein 3 (64). In the absence of IL-10, IL-21 or STAT3, virus-specific $CD8^+$ T cells (VSTs) maintain the terminal effect (T_E) differentiation state and couldn't mature into self-renewing T_{CM} cells. The maturation of protective memory T cells and memory $CD8^+$ T cell precursors was an active process that depended on the IL-10-IL-21-STAT3 signal (64, 65). Whether the formation of T_{SCM} depends on this pathway still needs further research, but it provides new ideas for subsequent research. Lactate dehydrogenase (LDH) inhibition combined with IL-21 could increase the formation of T_{SCM} cells, thereby producing more profound antitumor responses and prolonging the survival time of the host (66). In addition, a new study found that by fusion of IL-21 to anti-PD-1 antibody, IL-21 can target tumor-reactive T cells to promote T_{SCM} production. PD-1Ab21 therapy has shown greater antitumor effects in established tumor-bearing mice (67). At present, a large number of experiments have confirmed that these cytokines can promote the production of T_{SCM} and have potential antitumor effects. However, the mechanism of using cytokines, drugs and checkpoint blockade to promote the differentiation of memory T cells remains to be studied.

Oxidative Metabolic Pathway of T_{SCM} Cells

The naive T cells in the circulation are quiescent and have low metabolic requirements. They mainly use oxidative phosphorylation (OXPHOS) to produce ATP (53, 68). Generally speaking, differentiated T cells use glycolysis to proliferate, while memory T cells tend to use fatty acid oxidation (FAO)-dependent oxidative phosphorylation (OXPHOS) to produce ATP, which helps to perform long-lasting antitumor response in the tumor microenvironment (69–74). In the tumor microenvironment, tumor cells inhibit the metabolic reprogramming of T cells by competitively using glycolysis, so that the formation of memory T cells is inhibited (75, 76). It is reported that important transcription factors and cytokines, as well as MEKi and other inhibitors in the process of T cell differentiation, induce the generation of T_{SCM} by regulating T cell-related metabolic enzymes (77–79) (**Figure 3**).

Signals from TCR, costimulatory molecules, and growth factors lead to the activation of signaling pathways that promote transcriptional programs that are critical to effector function (80–82). In memory T cells, cellular stress, such as growth factor deprivation or a low ratio of ATP/AMP, will activate AMP-activated protein kinase (AMPK) and inhibit mTOR signaling (83). IL-15 also showed a similar function (57, 83).

Good et al. (41, 84) have proved through a large number of experiments that blocking the mTOR pathway by adding inhibitors can allow T cells to differentiate towards T_{SCM}-like cells, such as ITK

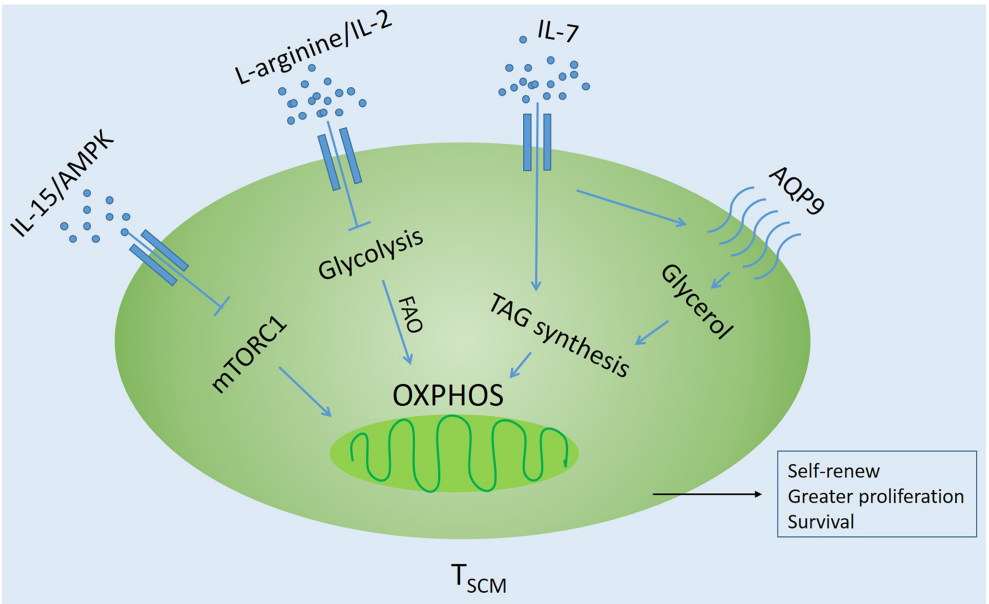


FIGURE 3 | Influencing factors regulating oxidative metabolism of T_{SCM} Cells. Inhibit glycolysis through different pathways and promote fatty acid oxidation (FAO)-dependent oxidative phosphorylation (OXPHOS). AQP9, Glycerol channel aquaporin 9; AMPK, AMP-activated protein kinase; TAG, triglyceride; mTORC1, Rapamycin Complex 1.

(IL-2-inducible T-cell kinase), TWS119 and BTK (Bruton's tyrosine kinase) inhibitors. In addition, the glycolytic function of T_{SCM} cells is reduced, and different inhibitors promote the *in vitro* generation of T_{SCM}-like cells with unique metabolic characteristics and retained polyfunctionality. It is worth noting that the drug-induced T_{SCM} cells have superior functional characteristics and self-renewing capacity after adoptive transfer. The research compound Akt inhibitor VIII inhibits AKT *in vitro*, which can preserve the differentiation and function of minor histocompatibility antigen (MIHA)-specific $CD8^+$ T cells. Moreover, transcriptome profiling revealed that AKT-inhibited $CD8^+$T cells clustered closely to naturally occurring stem cell-memory $CD8^+$ T cells. Moreover, AKT-inhibited MiHA-specific $CD8^+$ T cells showed increased polyfunctionality with co-secretion of IFN-γ and IL-2 upon antigen recall (79). Glycerol channel aquaporin 9 (AQP9) deficiency could impair the entry of glycerol into memory $CD8^+$ T cells for fatty acid esterification and triglyceride (TAG) synthesis and storage. While IL-7 could induce expression of the AQP9 in virus-specific memory $CD8^+$ T cells, but not naive cells. AQP9 is essential for their long-term survival. TAG synthase could restore the survival of lipid storage and memory T cells through ectopic expression, and it was found that TAG synthase is the central component of IL-7-mediated survival of human and mouse memory $CD8^+$ T cells (75). Three transcription factors, BAZ1B, PSIP1 and TSN, could regulate the level of L-arginine and promoted the survival of T cells. Activated T cells transform from glycolysis to oxidative phosphorylation, which promotes the production of T_{SCM} with higher survival ability and has antitumor activity in mouse models (85). Recent new studies have found that T_{SCM} induced by Meki/2 inhibition (Meki) has a natural phenotype, self-renewal ability, and enhanced pluripotency and proliferation. It is also achieved by regulating metabolism without affecting T cell receptor-mediated activation. DNA methylation analysis showed that Meki-induced T_{SCM} cells exhibited plasticity and loci-specific profiles, similar to those of T_{SCM} truly isolated from healthy donors, and had similar characteristics to naive and T_{CM} cells. Meki treatment of tumor-bearing mice also showed strong immune-mediated antitumor effects (86). These studies indicate that the regulation of glycolysis and metabolism is the key factor in inducing the formation of T_{SCM}. Therefore, targeted metabolic checkpoints can make T cells differentiate into memory and provide more young T cells for immunotherapy (74, 81, 82, 86).

The Molecules of Exhausted T Cells

T cell exhaustion is a phenomenon widely observed in humans. T_{SCM} or CAR-modified T_{SCM} expresses high levels of PD-1, TIM-3 or CTLA-4 after infiltrating the tumor, indicating that they have become exhausted T cells. Mostly due to T cell exhaustion and dysfunction by continuous TCR and cytokine stimulation. In addition, the effect of immune checkpoint inhibitors is very dependent on endogenous T cell function. However, they cannot reverse the exhaustion of T cells in cells that have undergone epigenetic changes. Therefore, this limits the long-term efficacy and wide application of cancer immunotherapy. Therefore, an in-depth understanding of the mechanism of T cell exhaustion is necessary for the study of T_{SCM} and its better clinical application. The term " exhausted T cells" was originally derived from a mouse model of LCMV. It is

now widely used to define the dysfunction state of T cells under chronic infection or tumor-induced long-term high antigen load stimulation (87). Enhanced and sustained T cell receptor stimulation is a key driver of T cell exhaustion. In recent years, the definition and identification of exhausted T cells have been divided from phenotype to transcriptional and epigenetic levels (88–90). Exhausted T cells are characterized by increased expression levels of inhibitory receptors such as PD-1, LAG3, 2B4, TIM-3 and CD28, and the gradual loss of effector functions, including impaired ability to secrete IFN-γ and tumor necrosis factor (91–95). PD-1 is mainly expressed on the surface of activated T cells and can inhibit T cell activation and proliferation. It is an important immunosuppressive molecule that plays an important role in suppressing immune responses and promoting self-tolerance (96–98). Programmed cell death ligand 1 (PD-L1) is a transmembrane protein, which is mainly expressed on the surface of antigen-presenting cells (APCs) such as dendritic cells (DCs), and can also be expressed on the surface of cancer cells and tumor infiltrating lymphocytes (TIL) (99–102). TOX is a nuclear DNA binding protein. TOX plays an important role in the development of thymus $CD4^+$ T cells, NK cells and intrinsic lymphocytes, and is critical in the differentiation of tumor-specific T cells. Recent studies have described the important role of TOX in the differentiation of exhaustive $CD8^+$ T cells and its molecular mechanism. It is unanimously found that the high expression of TOX is related to the high expression of a variety of inhibitory receptors (PD-1, TIM-3, TIGIT, CTLA-4, etc.) and the low expression of TCF1 (103). So inhibiting TOX expression may hinder the exhaustion of T cells (104–109). Many laboratories have identified a kind of exhausted T cell precursors (TPEX), which highly express molecules related to memory T cells, such as TCF1. TCF1 is a transcription factor and histone deacetylase (HDAC), which is related to the formation of T cell memory. Through single-cell RNA sequencing (scRNA-seq) and lineage tracing, the $TCF1^+Ly108^+PD\text{-}1^+CD8^+$ T cell population was identified. It was found that PD-1 stabilized the $TCF1^+$TeX precursor cell pool and confirmed that PD-1 was this early stage protector of the TCF1 population (91, 110). Exhaustion first appeared in $TCF1^+$ precursor T cells and then spread to the antigen-specific T cell pool. These findings will be important in the future to further investigate the developmental relationships in the later stages of exhaustion (111, 112).

At present, the specific mechanism of T cell exhaustion has not been fully elucidated. T cell exhaustion may be a parallel process with T cell differentiation. T cells at any stage of differentiation can be induced into exhausted T cells, which involves changes in different phenotypes and molecules. Excessive stimulation of precursor cells may be the origin of T cell failure. Under chronic infection or long-term tumor antigen stimulation, memory T cells and exhausted T cell precursors show different differentiation characteristics. Whether there is a link between the differentiation between these two subgroups should be a priority research area in the future. The possible potential developmental trajectories of exhausted T cells are shown in **Figure 4**.

At present, drugs for T cell exhaustion are still in the laboratory research or clinical trial stage. By reducing T cell exhaustion to promote the self-renewal ability and polyfunctionality of T_{SCM} cells (**Table 2**). Therefore, we do not know how to regulate the exhaustion process of T cells and reverse the exhausted state. Is it feasible to reach a certain effector state, and will there be side effects? Whether T_{SCM} can be designed to be exhaustion resistant? In general, the molecular mechanism of T_{SCM} cell formation is very complicated, and we describe them as clearly as possible in the review. More and more evidence supports the therapeutic potential of targeting exhausted T cells (115–118). We have already begun to understand the molecular mechanism of T cell exhaustion and early memory formation. Transforming exhausted T cells into rejuvenated T_{SCM} cells is the goal of our research.

CLINICAL APPLICATION

The Antitumor Effect of T_{SCM}

T_{SCM} cells are the least differentiated cells located at the top of the memory T lymphocyte hierarchy system. Compared with other T cells, they have stronger self-renewal ability and anti-tumor ability (84, 119, 120). As early as in previous studies, it has been found that T_{SCM} is considered a key determinant of immune memory and is involved in diversification of immune memory after allogeneic HSCT (32, 33). Play an important role in adult T-cell leukemia (34). With the FDA approval of CAR-T cell therapy for hematological malignancies, ACT has become a hot spot of continuous attention (63, 121–128). The clinical application of T_{SCM} cells is hindered because they are relatively rare in the circulation. According to reports, the CAR-T cell-modified T_{SCM} was cocultured with IL-2, IL-7 or IL-15 and then injected intravenously into tumor-bearing mice. It was found that the CAR-T/IL-15 group have the best anti-tumor effect (57). Guan et al. (129) prepared allogeneic antigen-specific $CD8^+$ T_{SCM}. It showed a proliferation history and rapidly differentiated into effector cells upon the E007 [the EB virus (EBV) transformed B lymphoblastoid cell lines (LCLs)] re-stimulation. Importantly, the prepared T_{SCM} cells could survive for a long time and reconstituted other T cell subsets *in vivo*, and could effectively eliminate E007 cells after being transferred to LCL burden mice. KUN et al. (120) presented a novel tumor therapeutic modality of the cryo-thermal therapy. After 90 days of cryo-thermal therapy, it can enhance the cytolytic function of $CD8^+$ T cells, induce $CD8^+$ T cells to differentiate into T_{SCM}, and $CD4^+$ T cells to differentiate into dominant $CD4^-$ CTL, Th1 and TFH subets. Cryo-thermal therapy not only inhibits lung metastasis, but also promotes the regression of implanted melanoma and prolongs survival time (35, 130, 131). It was found that after antigen chimeric modification of T_{SCM}, CD19-specific CAR T cell adoptive transfer has a significant antitumor effect on leukemia and lymphoma, and the therapeutic potential seems to be related to persistence *in vivo* (128, 129, 132, 133).

In SIP (an ex-vivo culture system modeled after the temporal changes of essential cytokines in an acute infection), TIL in the

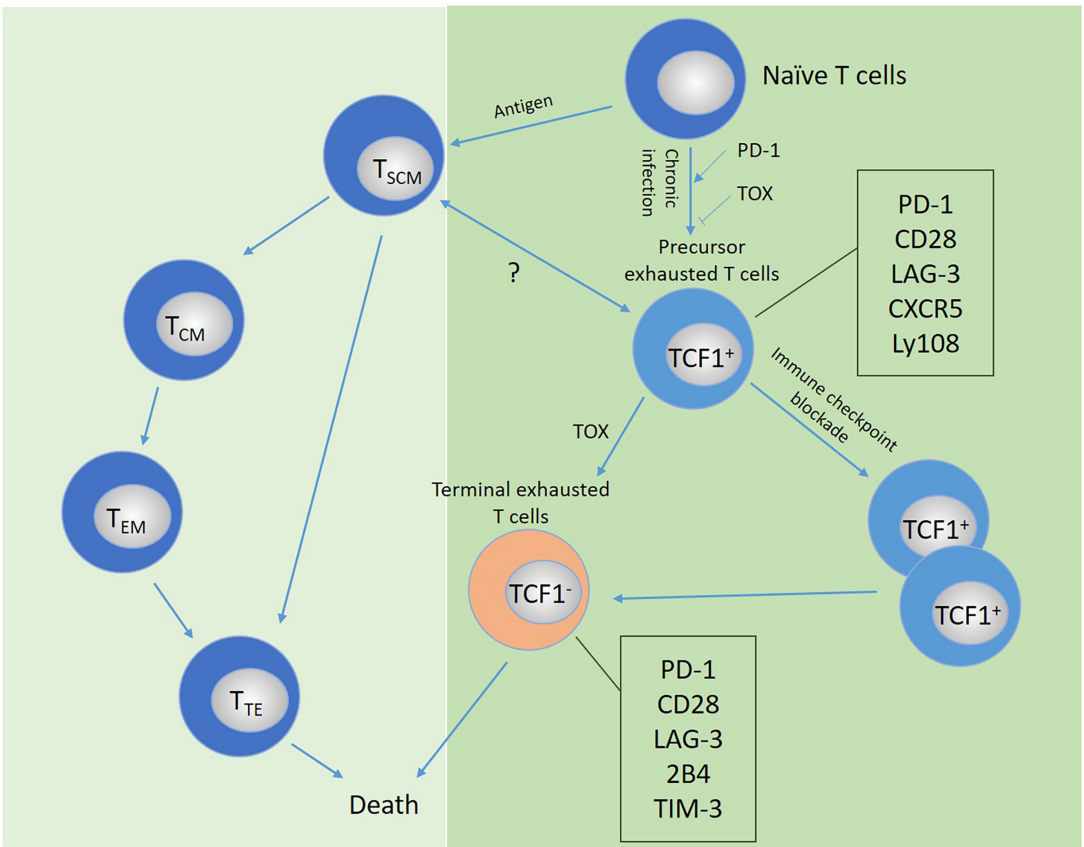


FIGURE 4 | Possible developmental trajectory of exhausted T cells and the comparison and relationship with memory or effector T cells. Under continuous antigen stimulation, T cells transform from precursor exhausted cells into terminally exhausted T cell populations, which mainly depends on the expression of the transcription factor TCF-1, accompanied by the high expression of a variety of inhibitory receptors. The relationship between the differentiation of T cell subsets and exhausted T cells remains to be explored. PD-1, PD ligand 1; TCF1, T cell factor-1; TIM-3, T-cell immunoglobulin domain and mucin domain protein 3; LAG-3, lymphocyte activation gene 3; TOX, thymocyte selection-associated high-motility group (HMG) box protein.

TABLE 2 | Key discoveries in the formation of induced culture T_{SCM}.

Year	Authors	Discovery
2013	Nicoletta Cieri et al.	IL-7 and IL-15 instructed the generation of human memory stem T cells from naïve precursors (29).
2016	Lenka V. Hurton et al.	IL-15 could maintain the long-term persistence of CAR-T modified T_{SCM} (48, 57).
2016	Godehard Scholz et al.	Promote the generation of T_{SCM} by inhibiting the mTORC1 pathway (39, 84).
2016	Alvarez-Fernandez, C et al.	IL-21, IL-7 and IL-15 could effectively promote the generation of T_{SCM} under short anti-CD3/CD28 costimulation (113).
2017	T aisuke Kondo et al.	Coculture of activated T cells and stromal cells expressing Notch ligand could produce T_{SCM} cells with low expression of inhibitory receptors (89).
2017	TANJA KAARTINEN et al.	Simply reducing the amount of IL-2 could promote the generation of T_{SCM} (51).
2018	Charlotte M. Mousset et al.	AKT inhibitors promoted the *in vitro* generation of T_{SCM}-like $CD8^+$ T cells with a unique metabolic profile and retained polyfunctionality (79).
2018	Taisuke Kondo et al.	The coculture of activated T cells with IL-7, IL-15 and op9-hdll1 cells could effectively generate T_{SCM} cells (58).
2018	Yingshi Chen et al.	IL-21 promoted the generation of T_{SCM} cells more effectively than other common γ-chain cytokines (64).
2020	Taisuke Kondo et al.	The Notch-foxm1 axis played a key role in the metabolism of CAR-T modified T_{SCM} (74).
2020	Dalton Hermans et al.	LDH inhibition combined with IL-21 increase the formation of T_{SCM} cells (66).
2020	Pilipow, K et al.	Promote the formation of T_{SCM} by adding antioxidants (114).
2021	Ying Li et al.	IL-21 fusion anti-PD-1 antibody promoted the generation of T_{SCM} (67).
2021	Vivek Verma et al.	Meki was confirmed to induce reprogramming of $CD8^+$ T cells into T_{SCM} (86).

Op9-hdll1, op9 cells expressing notch ligand, delta-like 1; Foxm1, forkhead box m1.

bone marrow of patients diagnosed with acute myeloid leukemia (AML) was treated with similar SIP, and it was found that these lymphocytes can be re-transformed into mutant $CD45RA^+$ central memory T lymphocytes (T_{CMRA}) with similar characteristics of T_{SCM}. The expression of pro-inflammatory cytokines, TNF-α, IFN-γ and IL-2 increased, and T_{CMRA} also exhibited cytotoxicity against autologous AML blast cells (134). In addition, similar effects have been shown in the treatment of Hodgkin's lymphoma. It showed a survival advantage, had higher tumor invasion and enhanced antitumor effect (133). Tumor immunotherapy is a promising treatment method. Transfect antigen-specific TCR gene or CAR vector to T_{SCM} to obtain CAR-T cells with poor differentiation and greater proliferation ability (135–139). A clinical trial study found that the genetically modified T_{SCM} can survive in the body for up to 12 years and has good safety and function (140). Recent studies have found that through integration site analysis, it is possible to study the fate of different types of CAR-T cells in patients, and it has been observed that T_{SCM} plays a central role in the early anti-leukemia response and late immune surveillance (38). This shows that this small portion of T cells is critical to the long-term success of CAR-T cell therapy. This new insight may help us improve CAR-T cell therapy and find out which patients are at higher risk of recurrence, and may benefit from stem cell transplantation after CAR-T cell therapy.

To date, CAR-T cells have achieved remarkable results in the treatment of hematological malignancies. However, despite extensive research, CAR-T cells have not been so successful in the treatment of solid tumors (141). Therefore, how to increase the trafficking and extravasation of T cells to the tumor sites and encourage the proliferation of T cells in the tumor is a problem that needs to be solved urgently. T_{SCM} have been shown to eradicate large tumors even when limited numbers of cells were transferred (28). Studies have found that chimeric T cells with multiple antigens may be a new direction for the treatment of solid tumors (71, 141, 142). At present, there are relatively few reports on the treatment of solid tumors with CAR-T-modified T_{SCM}, so it is more challenging for CAR-T-modified T_{SCM} to target solid tumors. The future should be a priority research area. In summary, memory T cell subsets have good clinical application prospects in clinical antitumor immunotherapy, and can provide personalized treatment plans for improving the prognosis of patients (134, 143). In short, these studies provide a strong scientific basis and practical methods for the rapid advancement of T_{SCM} cells in clinical trials of human adoptive immunotherapy.

The Importance of T_{SCM} in HIV-1 Immunotherapy and Vaccine Research

T_{SCM} cells play a key role in the pathogenesis of human immunodeficiency virus (HIV) infection (30, 144–146). The exhaustion of these cells will lead to the deterioration of the immune system and the development of AIDS. HIV-1 is an important part of the virus reservoirs. During HIV-1 infection, $CD4^+$ T_{SCM} cells are confirmed to be the longest-lived HIV-1. Virus storage is one of the factors that cause persistent HIV-1 infection (147, 148). Therefore, $CD4^+$ T_{SCM} cells can be used as a new target to clear the HIV-1 virus reservoir. The virus-latent cells are mainly concentrated in $CD4^+$ T_{SCM}. $CD4^+$ T_{SCM} expresses lower levels of CCR5, but can still support the production and latent infection of R5-tropic HIV-1 (149, 150). In addition, $CD4^+$ T_{SCM} is highly permissible for VSV-G-HIV-1 virus infection *in vitro*, and expresses relatively low levels of intracellular viral restriction factors, such as SAMHD1, Trim5alpha, and APOBEC3G. Moreover, these restriction factors can prevent HIV-1 from replicating in myeloid and dendritic cells (151–153). It was found that the $CD4^+$ T_{SCM} of untreated HIV-1 infected persons contained high levels of HIV-1 RNA, which all indicated the sensitivity of $CD4^+$ T_{SCM} cells to HIV-1. The study also found that in patients undergoing antiretroviral therapy (ART), $CD4^+$ T_{SCM} cells also have viral DNA that can be activated. Moreover, among the subsets of $CD4^+$ T memory cells, the number of HIV-1 DNA in T_{SCM} cells is the highest. During HIV infection, T cells play an important role in controlling virus replication. In patients receiving inhibitory antiretroviral therapy, $CD8^+$ T_{SCM} with stem cell characteristics was found to be more abundant than untreated patients (154). In addition, prolonging the treatment time can increase the ratio of $CD8^+$ T_{SCM}, and preferentially secrete IL-2 under viral stimulation, indicating that $CD8^+$ T_{SCM} is an important part of the cellular immune response to HIV-1. Able to maintain long-term, non-antigen-dependent cellular immune memory for HIV-1, which plays a key role in HIV control, but it seems unable to survive and proliferate during untreated infections (149). It is worth noting that HIV-1 specific $CD8^+$ T_{SCM} cells may not directly participate in the antiviral process, but play a role by secreting IL-2 to maintain their own proliferation and differentiation (155–157). Recent studies have found that vaccination of the subtype C prophylactic HIV-1 vaccine candidate can induce more T_{SCM} and antiviral. Compared with MVA alone and placebo, it induces more peripheral $CD8^+$ T_{SCM} cells and a higher level of $CD8^+$ T cell-mediated inhibition of the replication of different HIV-1 branches can respond to acute HIV infection or effectively control the chronic replication of HIV (152). Recently, a cross-sectional study of 20 cases of HIV-infected patients on treatment alone and 20 cases of ART has revealed a new subset of $CD4^+$ T cells: follicular regulatory T cells (TFR). The TFR of HIV^+ patients had anti-apoptotic properties, high proliferation rate and T_{SCM}-like properties, which leaded to the expansion of TFR, which in turn leaded to the dysfunction of TFH. Therefore, TFR cells may also become a new and potential therapeutic target for the treatment of HIV infection (158). How to target T_{SCM} therapy to provide new ideas for the development of new strategies for HIV-1 vaccines and immunotherapy still needs to continue to be explored and studied.

T_{SCM} and Autoimmune Diseases

T_{SCM} cells provide long-term protective immunity for anti-tumor immunity, which is probably based on reactivity to self-antigens. Therefore, as a by-product of antitumor, T_{SCM}-

mediated autoimmunity is inevitable (18, 159). Recent related studies have reported that T_{SCM} cells are associated with a variety of autoimmune diseases. Systemic lupus erythematosus (SLE) is a chronic connective tissue disease involving multiple organs that occurs in young women. Compared with healthy controls, the percentage of $CD4^+$ and $CD8^+$ T_{SCM} cells in SLE patients increased significantly. Differentiated TFH cells increase the antibodies produced by their own B cells. T_{SCM} cells play a role in the pathogenesis of SLE by maintaining TFH cells (132). Moreover, compared with healthy controls, the $CD4^+$ T_{SCM} of rheumatoid arthritis (RA) patients increased significantly (160). In the presence of IL-6, TCRs are easily activated to produce inflammatory cytokines. T_{SCM} cells may be a continuous source of the pathogenicity of RA (161). In patients with immune thrombocytopenia (ITP), the ratio of $CD4^+$ and $CD8^+$ T cells in the peripheral blood is unbalanced. The percentage of $CD8^+$ T_{SCM} in peripheral blood of ITP patients was significantly reduced after glucocorticoid treatment, indicating that the imbalance of the ratio of $CD8^+$ T_{SCM} may be involved in the occurrence and development of ITP (162). In addition, the frequency of acquired aplastic anemia (AA) $CD8^+$ T_{SCM} after immunosuppressive treatment was significantly higher than that of healthy controls. The frequency of $CD8^+$ T_{SCM} is also elevated in patients with autoimmune uveitis or sickle cell disease (130). B-cell-specific $CD8^+$ T_{SCM} cells with high expression of glucose transporter 1 (GLUT1) can be detected in T1D patients. WZB117, a selective inhibitor of Gult-1, effectively inhibits T_{SCM} cells in type 1 diabetes (T1D) patients by inhibiting glucose metabolism (53). Long-term autoreactive or abnormally activated T_{SCM} cells may induce self-renewing inflammatory cell responses. Studies have found that rapamycin (mTORC1 inhibitor) is outstanding in the treatment of autoimmune diseases (163). The above studies indicate that T_{SCM} may be a potential therapeutic target for these autoimmune diseases. The possible role of T_{SCM} cells in other diseases with severe cellular immune response, such as autoimmune hepatitis, thyroiditis, and certain types of glomerulonephritis, is currently unclear, but represents a priority research area in the future.

CONCLUSION

T_{SCM} is a long-lived memory cell with self-renewal ability and multi-differentiation potential. Different subsets of memory T cells can be identified based on their surface markers, gene expression profiles, and metabolic methods. At the same time, clinical-grade memory T cells can be obtained through *in vitro* induction and culture for cell transfer. The formation of memory T cells in the body has been confirmed in pre-clinical trials. The genetically modified T_{SCM} can survive in the body for up to 12 years and has good safety and function (140, 164). Convincing evidence in mice and humans shows that T_{SCM} cells are an important tool for adoptive immunity in tumor immunotherapy (143, 162). On the contrary, it is precisely because of their powerful immune reconstruction ability that they play a double-edged role in human diseases, and they are also potential therapeutic targets for autoimmune diseases and HIV (**Figure 5**). However, there are still many problems that need to be solved, elucidating the molecular mechanism of maintaining the phenotype of T_{SCM} cells and the influence of epigenetic modification, how to obtain a sufficient number of clinical grade T_{SCM} for induction culture. The infused T_{SCM} cells are easily affected by the immune microenvironment and are difficult to exert antitumor effects, and how the T_{SCM} cells target the tumor site to kill tumor cells is a

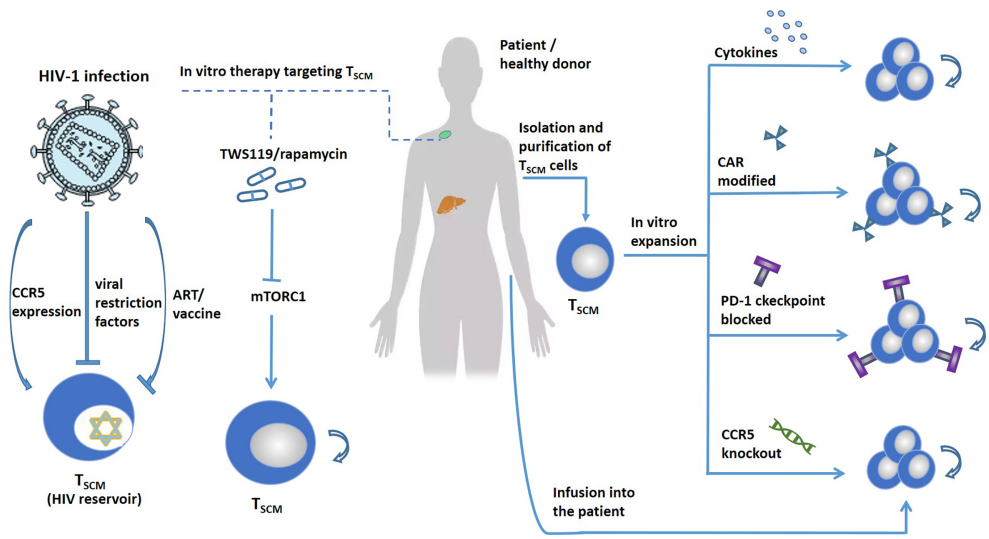


FIGURE 5 | Target T_{SCM} cells to treat human diseases. T_{SCM} cells can exacerbate human disease. Left, treat T_{SCM}-driven diseases, such as autoimmune diseases, HIV, etc., by blocking the production of T_{SCM}. The expression of CCR5 promotes the infection of T_{SCM} cells with HIV. Viral restriction factors and vaccines can target T_{SCM} cells to treat HIV. Blocking the mTORC1 pathway promotes the self-renewal and differentiation of T_{SCM}. Right, T_{SCM} cells are expanded *in vitro* by adding cytokines, CAR modification, immune checkpoint blocking, and gene editing. Stars represent cells latently infected with HIV.

problem worthy of attention at present. CAR-modified T_{SCM} cells, although there is good preclinical evidence that they have anti-tumor activity, when they are intravenously infused into solid tumor patients, they still lack persistence and efficacy (71, 133, 142). At the same time, it is worth noting that a single treatment method cannot effectively eliminate tumor cells. Immune cell therapy should be combined with PD-1 monoclonal antibody, CTLA-4 monoclonal antibody or radiotherapy, chemotherapy and other treatment methods, so that patients can get better efficacy (165). T_{SCM} has long existed in the HIV-1 virus reservoir, so future research is necessary to determine whether the low virus accumulation in T_{SCM} cells represents a significant feature of HIV-1 infection. More effort is needed to clarify the changes between the different states of T_{SCM} cells in health and disease. Although significant progress has been made in tumor therapy, there is still a gap in our understanding of the role of T_{SCM} cells in autoimmunity and viral infections.

AUTHOR CONTRIBUTIONS

YL designed the study and wrote the manuscript. DW and XY collected the literature. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Guangxi Science and Technology Research Base and Talent-specific Project (AD18126021); National Science and Technology Major Project for New Drug Innovation (2018ZX09733001-004-002); National Natural Science Foundation of China (NSFC) project (No. 81872491); Key Laboratory of the Ministry of Education Project for Early Prevention and Treatment of Regional High-risk Tumors (GKE2018-03, GKE2019-09, GKE-ZZ202007).

REFERENCES

1. Miller JD, van der Most RG, Akondy RS, Glidewell JT, Albott S, Masopust D, et al. Human Effector and Memory CD8+ T Cell Responses to Smallpox and Yellow Fever Vaccines. *Immunity* (2008) 28(5):710–22. doi: 10.1016/j.immuni.2008.02.020
2. Figueroa JA, Reidy A, Mirandola L, Trotter K, Suvorava N, Figueroa A, et al. Chimeric Antigen Receptor Engineering: A Right Step in the Evolution of Adoptive Cellular Immunotherapy. *Int Rev Immunol* (2015) 34(2):154–87. doi: 10.3109/08830185.2015.1018419
3. Khouri R, Silva-Santos G, Dierckx T, Menezes SM, Decanine D, Theys K, et al. A Genetic IFN/STAT1/FAS Axis Determines CD4 T Stem Cell Memory Levels and Apoptosis in Healthy Controls and Adult T-Cell Leukemia Patients. *Oncoimmunology* (2018) 7(5):e1426423. doi: 10.1080/2162402X.2018.1426423
4. Li X, Chen W. Mechanisms of Failure of Chimeric Antigen Receptor T-Cell Therapy. *Curr Opin Hematol* (2019) 26(6):427–33. doi: 10.1097/MOH.0000000000000548
5. Martinez M, Moon EK. CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment. *Front Immunol* (2019) 10:128. doi: 10.3389/fimmu.2019.00128
6. Zhao J, Song Y, Liu D. Clinical Trials of Dual-Target CAR T Cells, Donor-Derived CAR T Cells, and Universal CAR T Cells for Acute Lymphoid Leukemia. *J Hematol Oncol* (2019) 12(1):17. doi: 10.1186/s13045-019-0705-x
7. Xu L, Yao D, Tan J, He Z, Yu Z, Chen J, et al. Memory T Cells Skew Toward Terminal Differentiation in the CD8+ T Cell Population in Patients With Acute Myeloid Leukemia. *J Hematol Oncol* (2018) 11(1):93. doi: 10.1186/s13045-018-0636-y
8. Thommen DS, Schumacher TN. T Cell Dysfunction in Cancer. *Cancer Cell* (2018) 33(4):547–62. doi: 10.1016/j.ccell.2018.03.012
9. Sheng SY, Gu Y, Chuan Gang L, Tang YY, Yong Zou J, Qing Zhang Y, et al. The Characteristics of Naive-Like T Cells in Tumor-Infiltrating Lymphocytes From Human Lung Cancer. *J Immunother* (2017) 40(1):1–10. doi: 10.1097/CJI.0000000000000147
10. Zheng C, Zheng L, Yoo JK, Guo H, Zhang Y, Guo X, et al. Landscape of Infiltrating T Cells in Liver Cancer Revealed by Single-Cell Sequencing. *Cell* (2017) 169(7):1342–56.e1316. doi: 10.1016/j.cell.2017.05.035
11. Arcangeli S, Falcone L, Camisa B, De Girardi F, Biondi M, Giglio F, et al. Next-Generation Manufacturing Protocols Enriching TSCM CAR T Cells Can Overcome Disease-Specific T Cell Defects in Cancer Patients. *Front Immunol* (2020) 11:1217. doi: 10.3389/fimmu.2020.01217
12. Krishna S, Lowery FJ, Copeland AR, Bahadiroglu E, Mukherjee R, Jia L, et al. Stem-Like CD8 T Cells Mediate Response of Adoptive Cell Immunotherapy Against Human Cancer. *Science* (2020) 370(6522):1328–34. doi: 10.1126/science.abb9847
13. Vahidi Y, Bagheri M, Ghaderi A, Faghih Z. CD8-Positive Memory T Cells in Tumor-Draining Lymph Nodes of Patients With Breast Cancer. *BMC Cancer* (2020) 20(1):257. doi: 10.1186/s12885-020-6714-x
14. Wu TD, Madireddi S, de Almeida PE, Banchereau R, Chen YJ, Chitre AS, et al. Peripheral T Cell Expansion Predicts Tumour Infiltration and Clinical Response. *Nature* (2020) 579(7798):274–8. doi: 10.1038/s41586-020-2056-8
15. Liu Q, Sun Z, Chen L. Memory T Cells: Strategies for Optimizing Tumor Immunotherapy. *Protein Cell* (2020) 11(8):549–64. doi: 10.1007/s13238-020-00707-9
16. Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Host-Reactive CD8+ Memory Stem Cells in Graft-*Versus*-Host Disease. *Nat Med* (2005) 11(12):1299–305. doi: 10.1038/nm1326
17. Qian C, W Y, Cai H, Laroye C, De Carvalho Bittencourt M, Clement L, et al. Adenovirus-Specific T-Cell Subsets in Human Peripheral Blood and After IFN-γ Immunomagnetic Selection. *J Immunother* (Qian2016) 39(1):27–35. doi: 10.1097/CJI.0000000000000105
18. Gao S, Liang X, Wang H, Bao B, Zhang K, Zhu Y, et al. Stem Cell-Like Memory T Cells: A Perspective From the Dark Side. *Cell Immunol* (2021) 361:104273. doi: 10.1016/j.cellimm.2020.104273
19. Xu L, Zhang Y, Luo G, Li Y. The Roles of Stem Cell Memory T Cells in Hematological Malignancies. *J Hematol Oncol* (2015) 8:113. doi: 10.1186/s13045-015-0214-5
20. Lugli E, Gattinoni L, Roberto A, Mavilio D, Price DA, Restifo NP, et al. Identification, Isolation and *In Vitro* Expansion of Human and Nonhuman Primate T Stem Cell Memory Cells. *Nat Protoc* (2013) 8(1):33–42. doi: 10.1038/nprot.2012.143
21. Ando M, Ito M, Srirat T, Kondo T, Yoshimura A. Memory T Cell, Exhaustion, and Tumor Immunity. *Immunol Med* (2020) 43(1):1–9. doi: 10.1080/25785826.2019.1698261
22. Morrot A. Human Stem Memory T Cells (TSCM) as Critical Players in the Long-Term Persistence of Immune Responses. *Ann Transl Med* (2017) 5(5):120. doi: 10.21037/atm.2017.02.28
23. Guo X, Zhang Y, Zheng L, Zheng C, Song J, Zhang Q, et al. Global Characterization of T Cells in Non-Small-Cell Lung Cancer by Single-Cell Sequencing. *Nat Med* (2018) 24(7):978–85. doi: 10.1038/s41591-018-0045-3
24. Chebloune Y, Moussa M, Arrode-Brusés G, Ronfort C, Bose D, Gagnon J, et al. A Single Lentivector DNA Based Immunization Contains a Late Heterologous SIVmac251 Mucosal Challenge Infection. *Vaccine* (2020) 38(21):3729–39. doi: 10.1016/j.vaccine.2020.03.053
25. Fearon DT, Manders P, Wagner SD. Arrested Differentiation, The Self-Renewing Memory Lymphocyte, and Vaccination. *Science* (2001) 293(5528):248–50. doi: 10.1126/science.1062589

26. Luckey CJ, B D, Goldrath AW, Weissman IL, Benoist C, Mathis D. Memory T and Memory B Cells Share a Transcriptional Program of Self-Renewal With Long-Term Hematopoietic Stem Cells. *Proc Natl Acad Sci USA* (2006) 103(9):3304–9. doi: 10.1073/pnas.0511137103
27. Gattinoni L, Zhong XS, Palmer DC, Ji Y, Hinrichs CS, Yu Z, et al. Wnt Signaling Arrests Effector T Cell Differentiation and Generates CD8+ Memory Stem Cells. *Nat Med* (2009) 15(7):808–13. doi: 10.1038/nm.1982
28. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A Human Memory T Cell Subset With Stem Cell-Like Properties. *Nat Med* (2011) 17 (10):1290–7. doi: 10.1038/nm.2446
29. Cieri N, Camisa B, Cocchiarella F, Forcato M, Oliveira G, Provasi E, et al. IL-7 and IL-15 Instruct the Generation of Human Memory Stem T Cells From Naive Precursors. *Blood* (2013) 121(4):573–84. doi: 10.1182/blood-2012-05-431718
30. Buzon MJ, Sun H, Li C, Shaw A, Seiss K, Ouyang Z, et al. HIV-1 Persistence in CD4+ T Cells With Stem Cell-Like Properties. *Nat Med* (2014) 20(2):139–42. doi: 10.1038/nm.3445
31. Graef P, Buchholz VR, Stemberger C, Flossdorf M, Henkel L, Schiemann M, et al. Serial Transfer of Single-Cell-Derived Immunocompetence Reveals Stemness of CD8(+) Central Memory T Cells. *Immunity* (2014) 41(1):116–26. doi: 10.1016/j.immuni.2014.05.018
32. Roberto A, Castagna L, Zanon V, Bramanti S, Crocchiolo R, McLaren JE, et al. Role of Naive-Derived T Memory Stem Cells in T-Cell Reconstitution Following Allogeneic Transplantation. *Blood* (2015) 125(18):2855–64. doi: 10.1182/blood-2014-11-608406
33. Cieri N, Oliveira G, Greco R, Forcato M, Taccioli C, Cianciotti B, et al. Generation of Human Memory Stem T Cells After Haploidentical T-Replete Hematopoietic Stem Cell Transplantation. *Blood* (2015) 125(18):2865–74. doi: 10.1182/blood-2014-11-608539
34. Nagai Y, Kawahara M, Hishizawa M, Shimazu Y, Sugino N, Fujii S, et al. T Memory Stem Cells are the Hierarchical Apex of Adult T-Cell Leukemia. *Blood* (2015) 125(23):3527–35. doi: 10.1182/blood-2014-10-607465
35. Sabatino M, Hu J, Sommariva M, Gautam S, Fellowes V, Hocker JD, et al. Generation of Clinical-Grade CD19-Specific CAR-Modified CD8+ Memory Stem Cells for the Treatment of Human B-Cell Malignancies. *Blood* (2016) 128(4):519–28. doi: 10.1182/blood-2015-11-683847
36. Noviello M, Manfredi F, Ruggiero E, Perini T, Oliveira G, Cortesi F, et al. Bone Marrow Central Memory and Memory Stem T-Cell Exhaustion in AML Patients Relapsing After HSCT. *Nat Commun* (2019) 10(1):1065. doi: 10.1038/s41467-019-08871-1
37. Galletti G, De Simone G, Mazza EMC, Puccio S, Mezzanotte C, Bi TM, et al. Two Subsets of Stem-Like CD8(+) Memory T Cell Progenitors With Distinct Fate Commitments in Humans. *Nat Immunol* (2020) 21(12):1552–62. doi: 10.1038/s41590-020-0791-5
38. Biasco L, Izotova N, Rivat C, Ghorashian S, Richardson R, Guvenel A, et al. Clonal Expansion of T Memory Stem Cells Determines Early Anti-Leukemic Responses and Long-Term CAR T Cell Persistence in Patients. *Nat Cancer* (2021) 2(6):629–42. doi: 10.1038/s43018-021-00207-7
39. Yan C, Chang J, Song X, Yan F, Yu W, An Y, et al. Memory Stem T Cells Generated by Wnt Signaling From Blood of Human Renal Clear Cell Carcinoma Patients. *Cancer Biol Med* (2019) 16(1):109–24. doi: 10.20892/j.issn.2095-3941.2018.0118
40. Costa Del Amo P, Lahoz-Beneytez J, Boelen L, Ahmed R, Miners KL, Zhang Y, et al. Human TSCM Cell Dynamics *In Vivo* Are Compatible With Long-Lived Immunological Memory and Stemness. *PloS Biol* (2018) 16(6): e2005523. doi: 10.1371/journal.pbio.2005523
41. Good Z, Borges L, Vivanco Gonzalez N, Sahaf B, Samusik N, Tibshirani R, et al. Proliferation Tracing With Single-Cell Mass Cytometry Optimizes Generation of Stem Cell Memory-Like T Cells. *Nat Biotechnol* (2019) 37 (3):259–66. doi: 10.1038/s41587-019-0033-2
42. Zhao Y, Cai C, Samir J, Palgen JL, Keoshkerian E, Li H, et al. Human CD8 T-Stem Cell Memory Subsets Phenotypic and Functional Characterization are Defined by Expression of CD122 or CXCR3. *Eur J Immunol* (2021) 51 (7):1732–47. doi: 10.1002/eji.202049057
43. Duckworth BC, Lafouresse F, Wimmer VC, Broomfield BJ, Dalit L, Alexandre YO, et al. Effector and Stem-Like Memory Cell Fates are Imprinted in Distinct Lymph Node Niches Directed by CXCR3 Ligands. *Nat Immunol* (2021) 22(4):434–48. doi: 10.1038/s41590-021-00878-5
44. Santegoets SJAM, Turksma AW, Suhoski MM, Stam AGM, Albelda SM, Hooijberg E, et al. IL-21 Promotes the Expansion of CD27+ CD28+ Tumor Infiltrating Lymphocytes With High Cytotoxic Potential and Low Collateral Expansion of Regulatory T Cells. *Transl Med* (2013) 11(1):37. doi: 10.1186/1479-5876-11-37
45. Becker TC, Wherry EJ, Boone D, Murali-Krishna K, Antia R, Ma A, et al. Interleukin 15 Is Required for Proliferative Renewal of Virus-Specific Memory CD8 T Cells. *J Exp Med* (2002) 195(12):1541–8. doi: 10.1084/jem.20020369
46. Yi JS, Du M, Zajac AJ. A Vital Role for Interleukin-21 in the Control of a Chronic Viral Infection. *Science* (2009) 324(5934):1572–6. doi: 10.1126/science.1175194
47. Hashimoto M, Im SJ, Araki K, Ahmed R. Cytokine-Mediated Regulation of CD8 T-Cell Responses During Acute and Chronic Viral Infection. *Cold Spring Harb Perspect Biol* (2019) 11(1):a028464. doi: 10.1101/cshperspect.a028464
48. Hurton LV, Singh H, Najjar AM, Switzer KC, Mi T, Maiti S, et al. Tethered IL-15 Augments Antitumor Activity and Promotes a Stem-Cell Memory Subset in Tumor-Specific T Cells. *Proc Natl Acad Sci USA* (2016) 113(48): E7788–97. doi: 10.1073/pnas.1610544113
49. Martkamchan S, Onlamoon N, Wang S, Pattanapanyasat K, Ammaranond P. The Effects of Anti-CD3/CD28 Coated Beads and IL-2 on Expanded T Cell for Immunotherapy. *Adv Clin Exp Med* (2016) 25(5):821–8. doi: 10.17219/acem/35771
50. Perdomo-Celis F, Medina-Moreno S, Davis H, Bryant J, Taborda NA, Rugeles MT, et al. Characterization of CXCR5(+) CD8(+) T-Cells in Humanized NSG Mice. *Immunobiology* (2020) 225(2):151885. doi: 10.1016/j.imbio.2019.11.020
51. Kaartinen T, Luostarinen A, Maliniemi P, Keto J, Arvas M, Belt H, et al. Low Interleukin-2 Concentration Favors Generation of Early Memory T Cells Over Effector Phenotypes During Chimeric Antigen Receptor T-Cell Expansion. *Cytotherapy* (2017) 19(6):689–702. doi: 10.1016/j.jcyt.2017.03.067
52. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, et al. Acquisition of Full Effector Function *In Vitro* Paradoxically Impairs the *In Vivo* Antitumor Efficacy of Adoptively Transferred CD8+ T Cells. *J Clin Invest* (2005) 115(6):1616–26. doi: 10.1172/JCI24480
53. Vignali D, Cantarelli E, Bordignon C, Canu A, Citro A, Annoni A, et al. Detection and Characterization of CD8 + Autoreactive Memory Stem T Cells in Patients With Type 1 Diabetes. *Diabetes* (2018) 67(5):936–45. doi: 10.2337/db17-1390
54. Abdelsamed HA, Moustaki A, Fan Y, Dogra P, Ghoneim HE, Zebley CC, et al. Human Memory CD8 T Cell Effector Potential Is Epigenetically Preserved During *In Vivo* Homeostasis. *J Exp Med* (2017) 214(6):1593–606. doi: 10.1084/jem.20161760
55. Adachi K, Kano Y, Nagai T, Okuyama N, Sakoda Y, Tamada K. IL-7 and CCL19 Expression in CAR-T Cells Improves Immune Cell Infiltration and CAR-T Cell Survival in the Tumor. *Nat Biotechnol* (2018) 36(4):346–51. doi: 10.1038/nbt.4086
56. Zanon V, Lugli E. Differentiation of Diverse Progenies of Memory T Cells From Naive CD8(+) T Cell Precursors. *Methods Mol Biol* (2017) 1514:103–10. doi: 10.1007/978-1-4939-6548-9_8
57. Alizadeh D, Wong RA, Yang X, Wang D, Pecoraro JR, Kuo CF, et al. IL15 Enhances CAR-T Cell Antitumor Activity by Reducing Mtorc1 Activity and Preserving Their Stem Cell Memory Phenotype. *Cancer Immunol Res* (2019) 7(5):759–72. doi: 10.1158/2326-6066.CIR-18-0466
58. Kondo T, Imura Y, Chikuma S, Hibino S, Omata-Mise S, Ando M, et al. Generation and Application of Human Induced-Stem Cell Memory T Cells for Adoptive Immunotherapy. *Cancer Sci* (2018) 109(7):2130–40. doi: 10.1111/cas.13648
59. Herda S, Heimann A, Obermayer B, Ciraolo E, Althoff S, Russ J, et al. Long-Term *In Vitro* Expansion Ensures Increased Yield of Central Memory T Cells as Perspective for Manufacturing Challenges. *Int J Cancer* (2021) 148 (12):3097–110. doi: 10.1002/ijc.33523
60. Manfredi F, Abbati D, Cianciotti BC, Stasi L, Potenza A, Ruggiero E, et al. Flow Cytometry Data Mining by Cytochain Identifies Determinants of Exhaustion and Stemness in TCR-Engineered T Cells. *Eur J Immunol* (2021) 51(8):1992–2005. doi: 10.1002/eji.202049103

61. Castella M, Caballero-Banos M, Ortiz-Maldonado V, Gonzalez-Navarro EA, Sune G, Antonana-Vidosola A, et al. Point-Of-Care CAR T-Cell Production (ARI-0001) Using a Closed Semi-Automatic Bioreactor: Experience From an Academic Phase I Clinical Trial. *Front Immunol* (2020) 11:482. doi: 10.3389/fimmu.2020.00482
62. Fröhlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, et al. IL-21R on T Cells Is Critical for Sustained Functionality and Control of Chronic Viral Infection. *Science* (2009) 324(5934):1576–80. doi: 10.1126/science.1172815
63. Wu S, Zhu W, Peng Y, Wang L, Hong Y, Huang L, et al. The Antitumor Effects of Vaccine-Activated CD8(+) T Cells Associate With Weak TCR Signaling and Induction of Stem-Like Memory T Cells. *Cancer Immunol Res* (2017) 5(10):908–19. doi: 10.1158/2326-6066.CIR-17-0016
64. Chen Y, Y F, Jiang Y, Chen J, Wu K, Chen X, et al. Adoptive Transfer of Interleukin-21-Stimulated Human CD8+ T Memory Stem Cells Efficiently Inhibits Tumor Growth. *Immunother* (2018) 41(6):274–83. doi: 10.1097/CJI.0000000000000229
65. Cui W, Liu Y, Weinstein JS, Craft J, Kaech SM. An Interleukin-21-Interleukin-10-STAT3 Pathway Is Critical for Functional Maturation of Memory CD8+ T Cells. *Immunity* (2011) 35(5):792–805. doi: 10.1016/j.immuni.2011.09.017
66. Hermans D, Gautam S, Garcia-Canaveras JC, Gromer D, Mitra S, Spolski R, et al. Lactate Dehydrogenase Inhibition Synergizes With IL-21 to Promote CD8(+) T Cell Stemness and Antitumor Immunity. *Proc Natl Acad Sci USA* (2020) 117(11):6047–55. doi: 10.1073/pnas.1920413117
67. Li Y, Cong Y, Jia M, He Q, Zhong H, Zhao Y, et al. Targeting IL-21 to Tumor-Reactive T Cells Enhances Memory T Cell Responses and Anti-PD-1 Antibody Therapy. *Nat Commun* (2021) 12(1):951. doi: 10.1038/s41467-021-21241-0
68. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, et al. Enhancing CD8 T-Cell Memory by Modulating Fatty Acid Metabolism. *Nature* (2009) 460(7251):103–7. doi: 10.1038/nature08097
69. Johnson MO, Wolf MM, Madden MZ, Andrejeva G, Sugiura A, Contreras DC, et al. Distinct Regulation of Th17 and Th1 Cell Differentiation by Glutaminase-Dependent Metabolism. *Cell* (2018) 175(7):1780–95.e1719. doi: 10.1016/j.cell.2018.10.001
70. Zhang JY, Zhao YL, Lv YP, Cheng P, Chen W, Duan M, et al. Modulation of CD8(+) Memory Stem T Cell Activity and Glycogen Synthase Kinase 3beta Inhibition Enhances Anti-Tumoral Immunity in Gastric Cancer. *Oncoimmunology* (2018) 7(4):e1412900. doi: 10.1080/2162402X.2017.1412900
71. Klampatsa A, Dimou V, Albelda SM. Mesothelin-Targeted CAR-T Cell Therapy for Solid Tumors. *Expert Opin Biol Ther* (2021) 21(4):473–86. doi: 10.1080/14712598.2021.1843628
72. Sinclair LV, Rolf J, Emslie E, Shi Y-B, Taylor PM, Cantrell DA. Control of Amino-Acid Transport by Antigen Receptors Coordinates the Metabolic Reprogramming Essential for T Cell Differentiation. *Nat Immunol* (2013) 14(5):500–8. doi: 10.1038/ni.2556
73. O'Sullivan D, van der Windt GJ, Huang SC, Curtis JD, Chang CH, Buck MD, et al. Memory CD8(+) T Cells Use Cell-Intrinsic Lipolysis to Support the Metabolic Programming Necessary for Development. *Immunity* (2014) 41(1):75–88. doi: 10.1016/j.immuni.2014.06.005
74. Kondo T, Ando M, Nagai N, Tomisato W, Srirat T, Liu B, et al. The NOTCH-FOXM1 Axis Plays a Key Role in Mitochondrial Biogenesis in the Induction of Human Stem Cell Memory-Like CAR-T Cells. *Cancer Res* (2020) 80(3):471–83. doi: 10.1158/0008-5472.CAN-19-1196
75. Cui G, Staron MM, Gray SM, Ho PC, Amezquita RA, Wu J, et al. IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8+ T Cell Longevity. *Cell* (2015) 161(4):750–61. doi: 10.1016/j.cell.2015.03.021
76. Chang CH, Pearce EL. Emerging Concepts of T Cell Metabolism as a Target of Immunotherapy. *Nat Immunol* (2016) 17(4):364–8. doi: 10.1038/ni.3415
77. Hu Z, Zou Q, Su B. Regulation of T Cell Immunity by Cellular Metabolism. *Front Med* (2018) 12(4):463–72. doi: 10.1007/s11684-018-0668-2
78. Ma R, Ji T, Zhang H, Dong W, Chen X, Xu P, et al. A Pck1-Directed Glycogen Metabolic Program Regulates Formation and Maintenance of Memory CD8(+) T Cells. *Nat Cell Biol* (2018) 20(1):21–7. doi: 10.1038/s41556-017-0002-2
79. Mousset CM, Hobo W, Ji Y, Fredrix H, De Giorgi V, Allison RD, et al. Ex Vivo AKT-Inhibition Facilitates Generation of Polyfunctional Stem Cell Memory-Like CD8(+) T Cells for Adoptive Immunotherapy. *Oncoimmunology* (2018) 7(10):e1488565. doi: 10.1080/2162402X.2018.1488565
80. Giuliani C. The Flavonoid Quercetin Induces AP-1 Activation in FRTL-5 Thyroid Cells. *Antioxidants (Basel)* (2019) 8(5):112. doi: 10.3390/antiox8050112
81. Kared H, Tan SW, Lau MC, Chevrier M, Tan C, How W, et al. Immunological History Governs Human Stem Cell Memory CD4 Heterogeneity via the Wnt Signaling Pathway. *Nat Commun* (2020) 11(1):821. doi: 10.1038/s41467-020-14442-6
82. Kolan SS, Li G, Wik JA, Malachin G, Guo S, Kolan P, et al. Cellular Metabolism Dictates T Cell Effector Function in Health and Disease. *Scand J Immunol* (2020) 92(5):e12956. doi: 10.1111/sji.12956
83. Ma EH, Poffenberger MC, Wong AH, Jones RG. The Role of AMPK in T Cell Metabolism and Function. *Curr Opin Immunol* (2017) 46:45–52. doi: 10.1016/j.coi.2017.04.004
84. Scholz G, Jandus C, Zhang L, Grandclement C, Lopez-Mejia IC, Soneson C, et al. Modulation of mTOR Signalling Triggers the Formation of Stem Cell-Like Memory T Cells. *EBioMedicine* (2016) 4:50–61. doi: 10.1016/j.ebiom.2016.01.019
85. Geiger R, Rieckmann JC, Wolf T, Basso C, Feng Y, Fuhrer T, et al. L-Arginine Modulates T Cell Metabolism and Enhances Survival and Anti-Tumor Activity. *Cell* (2016) 167(3):829–42.e813. doi: 10.1016/j.cell.2016.09.031
86. Verma V, Jafarzadeh N, Boi S, Kundu S, Jiang Z, Fan Y, et al. MEK Inhibition Reprograms CD8(+) T Lymphocytes Into Memory Stem Cells With Potent Antitumor Effects. *Nat Immunol* (2021) 22(1):53–66. doi: 10.1038/s41590-020-00818-9
87. Utzschneider DT, Charmoy M, Chennupati V, Pousse L, Ferreira DP, Calderon-Copete S, et al. T Cell Factor 1-Expressing Memory-Like CD8(+) T Cells Sustain the Immune Response to Chronic Viral Infections. *Immunity* (2016) 45(2):415–27. doi: 10.1016/j.immuni.2016.07.021
88. Wu T, Ji Y, Moseman EA, Xu HC, Manglani M, Kirby M, et al. The TCF1-Bcl6 Axis Counteracts Type I Interferon to Repress Exhaustion and Maintain T Cell Stemness. *Sci Immunol* (2016) 1(6):eaai8593. doi: 10.1126/sciimmunol.aai8593
89. Kondo T, Morita R, Okuzono Y, Nakatsukasa H, Sekiya T, Chikuma S, et al. Notch-Mediated Conversion of Activated T Cells Into Stem Cell Memory-Like T Cells for Adoptive Immunotherapy. *Nat Commun* (2017) 8:15338. doi: 10.1038/ncomms15338
90. Huang X, Yang Y. Driving an Improved CAR for Cancer Immunotherapy. *J Clin Invest* (2016) 126(8):2795–8. doi: 10.1172/JCI88959
91. Chen Z, Ji Z, Ngiow SF, Manne S, Cai Z, Huang AC, et al. TCF-1-Centered Transcriptional Network Drives an Effector *Versus* Exhausted CD8 T Cell-Fate Decision. *Immunity* (2019) 51(5):840–55.e845. doi: 10.1016/j.immuni.2019.09.013
92. Raghu D, Xue HH, Mielke LA. Control of Lymphocyte Fate, Infection, and Tumor Immunity by TCF-1. *Trends Immunol* (2019) 40(12):1149–62. doi: 10.1016/j.it.2019.10.006
93. He QF, Xu Y, Li J, Huang ZM, Li XH, Wang X. CD8+ T-Cell Exhaustion in Cancer: Mechanisms and New Area for Cancer Immunotherapy. *Brief Funct Genomics* (2019) 18(2):99–106. doi: 10.1093/bfgp/ely006
94. Kurachi M. CD8(+) T Cell Exhaustion. *Semin Immunopathol* (2019) 41(3):327–37. doi: 10.1007/s00281-019-00744-5
95. Sawant DV, Yano H, Chikina M, Zhang Q, Liao M, Liu C, et al. Adaptive Plasticity of IL-10(+) and IL-35(+) Treg Cells Cooperatively Promotes Tumor T Cell Exhaustion. *Nat Immunol* (2019) 20(6):724–35. doi: 10.1038/s41590-019-0346-9
96. Siddiqui I, Schaeuble K, Chennupati V, Fuertes Marraco SA, Calderon-Copete S, Pais Ferreira D, et al. Intratumoral Tcf1(+)PD-1(+)CD8(+) T Cells With Stem-Like Properties Promote Tumor Control in Response to Vaccination and Checkpoint Blockade Immunotherapy. *Immunity* (2019) 50(1):195–211.e110. doi: 10.1016/j.immuni.2018.12.021
97. Dosset M, Joseph EL, Rivera Vargas T, Apetoh L. Modulation of Determinant Factors to Improve Therapeutic Combinations With Immune Checkpoint Inhibitors. *Cells* (2020) 9(7):1727. doi: 10.3390/cells9071727
98. Utzschneider DT, Gabriel SS, Chisanga D, Gloury R, Gubser PM, Vasanthakumar A, et al. Early Precursor T Cells Establish and Propagate

T Cell Exhaustion in Chronic Infection. *Nat Immunol* (2020) 21(10):1256–66. doi: 10.1038/s41590-020-0760-z
99. Saeidi A, Zandi K, Cheok YY, Saeidi H, Wong WF, Lee CYQ, et al. T-Cell Exhaustion in Chronic Infections: Reversing the State of Exhaustion and Reinvigorating Optimal Protective Immune Responses. *Front Immunol* (2018) 9:2569. doi: 10.3389/fimmu.2018.02569
100. Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, et al. T-Cell Exhaustion Signatures Vary With Tumor Type and Are Severe in Glioblastoma. *Clin Cancer Res* (2018) 24(17):4175–86. doi: 10.1158/1078-0432.CCR-17-1846
101. Blank CU, Haining WN, Held W, Hogan PG, Kallies A, Lugli E, et al. Defining 'T Cell Exhaustion'. *Nat Rev Immunol* (2019) 19(11):665–74. doi: 10.1038/s41577-019-0221-9
102. Fribourg M, Anderson L, Fischman C, Cantarelli C, Perin L, La Manna G, et al. T-Cell Exhaustion Correlates With Improved Outcomes in Kidney Transplant Recipients. *Kidney Int* (2019) 96(2):436–49. doi: 10.1016/j.kint.2019.01.040
103. Kim K, Park S, Park SY, Kim G, Park SM, Cho JW, et al. Single-Cell Transcriptome Analysis Reveals TOX as a Promoting Factor for T Cell Exhaustion and a Predictor for Anti-PD-1 Responses in Human Cancer. *Genome Med* (2020) 12(1):22. doi: 10.1186/s13073-020-00722-9
104. Heim K, Binder B, Sagar, Wieland D, Hensel N, Llewellyn-Lacey S, et al. TOX Defines the Degree of CD8+ T Cell Dysfunction in Distinct Phases of Chronic HBV Infection. *Gut* (2020) 70(8):1550–60. doi: 10.1136/gutjnl-2020-322404
105. Seo H, Chen J, Gonzalez-Avalos E, Samaniego-Castruita D, Das A, Wang YH, et al. TOX and TOX2 Transcription Factors Cooperate With NR4A Transcription Factors to Impose CD8(+) T Cell Exhaustion. *Proc Natl Acad Sci USA* (2019) 116(25):12410–5. doi: 10.1073/pnas.1905675116
106. Wang X, He Q, Shen H, Xia A, Tian W, Yu W, et al. TOX Promotes the Exhaustion of Antitumor CD8(+) T Cells by Preventing PD1 Degradation in Hepatocellular Carcinoma. *J Hepatol* (2019) 71(4):731–41. doi: 10.1016/j.jhep.2019.05.015
107. Yao C, Sun HW, Lacey NE, Ji Y, Moseman EA, Shih HY, et al. Single-Cell RNA-Seq Reveals TOX as a Key Regulator of CD8(+) T Cell Persistence in Chronic Infection. *Nat Immunol* (2019) 20(7):890–901. doi: 10.1038/s41590-019-0403-4
108. Khan O, Giles JR, McDonald S, Manne S, Ngiow SF, Patel KP, et al. TOX Transcriptionally and Epigenetically Programs CD8(+) T Cell Exhaustion. *Nature* (2019) 571(7764):211–8. doi: 10.1038/s41586-019-1325-x
109. Xu-Monette ZY, Zhou J, Young KH. PD-1 Expression and Clinical PD-1 Blockade in B-Cell Lymphomas. *Blood* (2018) 131(1):68–83. doi: 10.1182/blood-2017-07-740993
110. Zeng Z, Wei F, Ren X. Exhausted T Cells and Epigenetic Status. *Cancer Biol Med* (2020) 17(4):923–36. doi: 10.20892/j.issn.2095-3941.2020.0338
111. Beltra JC, Manne S, Abdel-Hakeem MS, Kurachi M, Giles JR, Chen Z, et al. Developmental Relationships of Four Exhausted CD8(+) T Cell Subsets Reveals Underlying Transcriptional and Epigenetic Landscape Control Mechanisms. *Immunity* (2020) 52(5):825–41.e828. doi: 10.1016/j.immuni.2020.04.014
112. Cai MC, Zhao X, Cao M, Ma P, Chen M, Wu J, et al. T-Cell Exhaustion Interrelates With Immune Cytolytic Activity to Shape the Inflamed Tumor Microenvironment. *J Pathol* (2020) 251(2):147–59. doi: 10.1002/path.5435
113. Alvarez-Fernandez C, Escriba-Garcia L, Vidal S, Sierra J, Briones J. A Short CD3/CD28 Costimulation Combined With IL-21 Enhance the Generation of Human Memory Stem T Cells for Adoptive Immunotherapy. *J Transl Med* (2016) 14(1):214. doi: 10.1186/s12967-016-0973-y
114. Pilipow K, Scamardella E, Lugli E. Generating Stem-Like Memory T Cells With Antioxidants for Adoptive Cell Transfer Immunotherapy of Cancer. *Methods Enzymol* (2020) 631:137–58. doi: 10.1016/bs.mie.2019.08.016
115. Tuluc F, Spitsin S, Tustin NB, Murray JB, Tustin R3rd, Schankel LA, et al. Decreased PD-1 Expression on CD8 Lymphocyte Subsets and Increase in CD8 Tscm Cells in Children With HIV Receiving Raltegravir. *AIDS Res Hum Retroviruses* (2017) 33(2):133–42. doi: 10.1089/AID.2016.0108
116. Kallies A, Zehn D, Utzschneider DT. Precursor Exhausted T Cells: Key to Successful Immunotherapy? *Nat Rev Immunol* (2020) 20(2):128–36. doi: 10.1038/s41577-019-0223-7
117. Terranova-Barberio M, Pawlowska N, Dhawan M, Moasser M, Chien AJ, Melisko ME, et al. Exhausted T Cell Signature Predicts Immunotherapy Response in ER-Positive Breast Cancer. *Nat Commun* (2020) 11(1):3584. doi: 10.1038/s41467-020-17414-y
118. Verdon DJ, Mulazzani M, Jenkins MR. Cellular and Molecular Mechanisms of CD8(+) T Cell Differentiation, Dysfunction and Exhaustion. *Int J Mol Sci* (2020) 21(19):7357. doi: 10.3390/ijms21197357
119. Kagoya Y, Nakatsugawa M, Yamashita Y, Ochi T, Guo T, Anczurowski M, et al. BET Bromodomain Inhibition Enhances T Cell Persistence and Function in Adoptive Immunotherapy Models. *J Clin Invest* (2016) 126 (9):3479–94. doi: 10.1172/JCI86437
120. He K, Liu P, Xu LX. The Cryo-Thermal Therapy Eradicated Melanoma in Mice by Eliciting CD4(+) T-Cell-Mediated Antitumor Memory Immune Response. *Cell Death Dis* (2017) 8(3):e2703. doi: 10.1038/cddis.2017.125
121. Anderson KG, Stromnes IM, Greenberg PD. Obstacles Posed by the Tumor Microenvironment to T Cell Activity: A Case for Synergistic Therapies. *Cancer Cell* (2017) 31(3):311–25. doi: 10.1016/j.ccell.2017.02.008
122. He W, Zhang H, Han F, Chen X, Lin R, Wang W, et al. CD155T/TIGIT Signaling Regulates CD8(+) T-Cell Metabolism and Promotes Tumor Progression in Human Gastric Cancer. *Cancer Res* (2017) 77(22):6375–88. doi: 10.1158/0008-5472.CAN-17-0381
123. Hashimoto M, Kamphorst AO, Im SJ, Kissick HT, Pillai RN, Ramalingam SS, et al. CD8 T Cell Exhaustion in Chronic Infection and Cancer: Opportunities for Interventions. *Annu Rev Med* (2018) 69:301–18. doi: 10.1146/annurev-med-012017-043208
124. Choi BD, Maus MV, June CH, Sampson JH. Immunotherapy for Glioblastoma: Adoptive T-Cell Strategies. *Clin Cancer Res* (2019) 25 (7):2042–8. doi: 10.1158/1078-0432.CCR-18-1625
125. Atrash S, Bano K, Harrison B, Abdallah AO. CAR-T Treatment for Hematological Malignancies. *J Investig Med* (2020) 68(5):956–64. doi: 10.1136/jim-2020-001290
126. Abramson JS. Anti-CD19 CAR T-Cell Therapy for B-Cell Non-Hodgkin Lymphoma. *Transfus Med Rev* (2020) 34(1):29–33. doi: 10.1016/j.tmrv.2019.08.003
127. Kasakovski D, Xu L, Li Y. T Cell Senescence and CAR-T Cell Exhaustion in Hematological Malignancies. *J Hematol Oncol* (2018) 11(1):91. doi: 10.1186/s13045-018-0629-x
128. Blaeschke F, Stenger D, Kaeuferle T, Willier S, Lotfi R, Kaiser AD, et al. Induction of a Central Memory and Stem Cell Memory Phenotype in Functionally Active CD4(+) and CD8(+) CAR T Cells Produced in an Automated Good Manufacturing Practice System for the Treatment of CD19 (+) Acute Lymphoblastic Leukemia. *Cancer Immunol Immunother* (2018) 67 (7):1053–66. doi: 10.1007/s00262-018-2155-7
129. Guan L, Li X, Wei J, Liang Z, Yang J, Weng X, et al. Antigen-Specific CD8+ Memory Stem T Cells Generated From Human Peripheral Blood Effectively Eradicate Allogeneic Targets in Mice. *Stem Cell Res Ther* (2018) 9(1):337. doi: 10.1186/s13287-018-1080-1
130. Hosokawa K, Muranski P, Feng X, Townsley DM, Liu B, Knickelbein J, et al. Memory Stem T Cells in Autoimmune Disease: High Frequency of Circulating CD8+ Memory Stem Cells in Acquired Aplastic Anemia. *J Immunol* (2016) 196(4):1568–78. doi: 10.4049/jimmunol.1501739
131. Wang Y, Whittall T, Neil S, Britton G, Mistry M, Rerks-Ngarm S, et al. A Novel Mechanism Linking Memory Stem Cells With Innate Immunity in Protection Against HIV-1 Infection. *Sci Rep* (2017) 7(1):1057. doi: 10.1038/s41598-017-01188-3
132. Lee YJ, Park JA, Kwon H, Choi YS, Jung KC, Park SH, et al. Role of Stem Cell-Like Memory T Cells in Systemic Lupus Erythematosus. *Arthritis Rheumatol* (2018) 70(9):1459–69. doi: 10.1002/art.40524
133. Alvarez-Fernandez C, Escriba-Garcia L, Caballero AC, Escudero-Lopez E, Ujaldon-Miro C, Montserrat-Torres R, et al. Memory Stem T Cells Modified With a Redesigned CD30-Chimeric Antigen Receptor Show an Enhanced Antitumor Effect in Hodgkin Lymphoma. *Clin Transl Immunol* (2021) 10(4): e1268. doi: 10.1002/cti2.1268
134. Teo YWB, Linn YC, Goh YT, Li S, Ho LP. Tumor Infiltrating Lymphocytes From Acute Myeloid Leukemia Marrow can be Reverted to CD45RA+ Central Memory State by Reactivation in SIP (Simulated Infective Protocol). *Immunobiology* (2019) 224(4):526–31. doi: 10.1016/j.imbio.2019.05.001

135. Hoffmann JM, Schubert ML, Wang L, Huckelhoven A, Sellner L, Stock S, et al. Differences in Expansion Potential of Naive Chimeric Antigen Receptor T Cells From Healthy Donors and Untreated Chronic Lymphocytic Leukemia Patients. *Front Immunol* (2017) 8:1956. doi: 10.3389/fimmu.2017.01956
136. Liu J, Zhong JF, Zhang X, Zhang C. Allogeneic CD19-CAR-T Cell Infusion After Allogeneic Hematopoietic Stem Cell Transplantation in B Cell Malignancies. *J Hematol Oncol* (2017) 10(1):35. doi: 10.1186/s13045-017-0405-3
137. Mollanoori H, Shahraki H, Rahmati Y, Teimourian S. CRISPR/Cas9 and CAR-T Cell, Collaboration of Two Revolutionary Technologies in Cancer Immunotherapy, an Instruction for Successful Cancer Treatment. *Hum Immunol* (2018) 79(12):876–82. doi: 10.1016/j.humimm.2018.09.007
138. Agarwal S, Weidner T, Thalheimer FB, Buchholz CJ. *In Vivo* Generated Human CAR T Cells Eradicate Tumor Cells. *Oncoimmunology* (2019) 8(12):e1671761. doi: 10.1080/2162402X.2019.1671761
139. Brudno JN, Kochenderfer JN. Recent Advances in CAR T-Cell Toxicity: Mechanisms, Manifestations and Management. *Blood Rev* (2019) 34:45–55. doi: 10.1016/j.blre.2018.11.002
140. Biasco L, Scala S, Ricci LB, Dionisio F, Baricordi C, Calabria A, et al. *In Vivo* Tracking of T Cells in Humans Unveils Decade-Long Survival and Activity of Genetically Modified T Memory Stem Cells. *Transl Med* (2015) 7(273):273ra213. doi: 10.1126/sciimmunol.aay0555
141. Jin L, Tao H, Karachi A, Long Y, Hou AY, Na M, et al. CXCR1- or CXCR2-Modified CAR T Cells Co-Opt IL-8 for Maximal Antitumor Efficacy in Solid Tumors. *Nat Commun* (2019) 10(1):4016. doi: 10.1038/s41467-019-11869-4
142. Choe JH, W PB, Simic MS, Gilbert RD, Li AW, Krasnow NA, et al. SynNotch-CAR T Cells Overcome Challenges of Specificity, Heterogeneity, and Persistence in Treating Glioblastoma. *Sci Transl Med* (2021) 13(591):eabe7378. doi: 10.1126/scitranslmed.abe7378
143. Padgett LE, Dinh HQ, Wu R, Gaddis DE, Araujo DJ, Winkels H, et al. Naive CD8(+) T Cells Expressing CD95 Increase Human Cardiovascular Disease Severity. *Arterioscler Thromb Vasc Biol* (2020) 40(12):2845–59. doi: 10.1161/ATVBAHA.120.315106
144. Flynn JK, Gorry PR. Stem Memory T Cells (TSCM)-Their Role in Cancer and HIV Immunotherapies. *Clin Transl Immunol* (2014) 3(7):e20. doi: 10.1038/cti.2014.16
145. Flynn JK, Paukovics G, Cashin K, Borm K, Ellett A, Roche M, et al. Quantifying Susceptibility of CD4+ Stem Memory T-Cells to Infection by Laboratory Adapted and Clinical HIV-1 Strains. *Viruses* (2014) 6(2):709–26. doi: 10.3390/v6020709
146. Chahroudi A, Silvestri G, Lichterfeld M. T Memory Stem Cells and HIV: A Long-Term Relationship. *Curr HIV/AIDS Rep* (2015) 12(1):33–40. doi: 10.1007/s11904-014-0246-4
147. Calascibetta F, Micci L, Carnathan D, Lawson B, Vanderford TH, Bosinger SE, et al. Antiretroviral Therapy in Simian Immunodeficiency Virus-Infected Sooty Mangabeys: Implications for AIDS Pathogenesis. *J Virol* (2016) 90(16):7541–51. doi: 10.1128/JVI.00598-16
148. Cartwright EK, Palesch D, Mavigner M, Paiardini M, Chahroudi A, Silvestri G. Initiation of Antiretroviral Therapy Restores CD4+ T Memory Stem Cell Homeostasis in Simian Immunodeficiency Virus-Infected Macaques. *J Virol* (2016) 90(15):6699–708. doi: 10.1128/JVI.00492-16
149. Vigano S, Negron J, Ouyang Z, Rosenberg ES, Walker BD, Lichterfeld M, et al. Prolonged Antiretroviral Therapy Preserves HIV-1-Specific CD8 T Cells With Stem Cell-Like Properties. *J Virol* (2015) 89(15):7829–40. doi: 10.1128/JVI.00789-15
150. Lu X, Song B, Weng W, Xia H, Su B, Wu H, et al. CD4(+) T Memory Stem Cells Correlate With Disease Progression in Chronically HIV-1-Infected Patients. *Viral Immunol* (2017) 30(9):642–8. doi: 10.1089/vim.2017.0017
151. Guardo AC, Zarama A, Gonzalez T, Bargallo ME, Rojas J, Martinez E, et al. Effects on Immune System and Viral Reservoir of a Short-Cycle Antiretroviral Therapy in Virologically Suppressed HIV-Positive Patients. *AIDS* (2019) 33(6):965–72. doi: 10.1097/QAD.0000000000002169
152. Munusamy Ponnan S, Hayes P, Fernandez N, Thiruvengadam K, Pattabiram S, Nesakumar M, et al. Evaluation of Antiviral T Cell Responses and TSCM Cells in Volunteers Enrolled in a Phase I HIV-1 Subtype C Prophylactic Vaccine Trial in India. *PloS One* (2020) 15(2):e0229461. doi: 10.1371/journal.pone.0229461
153. Manganaro L, Hong P, Hernandez MM, Argyle D, Mulder LCF, Potla U, et al. IL-15 Regulates Susceptibility of CD4(+) T Cells to HIV Infection. *Proc Natl Acad Sci USA* (2018) 115(41):E9659–67. doi: 10.1073/pnas.1806695115
154. Utay NS, Vigil KJ, Somasunderam A, Aulicino PC, Smulevitz B, Chiadika S, et al. Timing of Antiretroviral Therapy Initiation Determines Rectal Natural Killer Cell Populations. *AIDS Res Hum Retroviruses* (2020) 36(4):314–23. doi: 10.1089/AID.2019.0225
155. Finley J. Elimination of Cancer Stem Cells and Reactivation of Latent HIV-1 via AMPK Activation: Common Mechanism of Action Linking Inhibition of Tumorigenesis and the Potential Eradication of HIV-1. *Med Hypotheses* (2017) 104:133–46. doi: 10.1016/j.mehy.2017.05.032
156. Fenwick C, Joo V, Jacquier P, Noto A, Banga R, Perreau M, et al. T-Cell Exhaustion in HIV Infection. *Immunol Rev* (2019) 292(1):149–63. doi: 10.1111/imr.12823
157. Sharov KS. HIV/SARS-CoV-2 Co-Infection: T Cell Profile, Cytokine Dynamics and Role of Exhausted Lymphocytes. *Int J Infect Dis* (2021) 102:163–9. doi: 10.1016/j.ijid.2020.10.049
158. Zhao S, Xu W, Tu B, Hong WG, Zhang Z, Chen WW, et al. Alterations of the Frequency and Functions of Follicular Regulatory T Cells and Related Mechanisms in HIV Infection. *J Infect* (2020) 81(5):776–84. doi: 10.1016/j.jinf.2020.09.014
159. Roederer M, Quaye L, Mangino M, Beddall MH, Mahnke Y, Chattopadhyay P, et al. The Genetic Architecture of the Human Immune System: A Bioresource for Autoimmunity and Disease Pathogenesis. *Cell* (2015) 161(2):387–403. doi: 10.1016/j.cell.2015.02.046
160. Cianciotti BC, Ruggiero E, Campochiaro C, Oliveira G, Magnani ZI, Baldini M, et al. CD4+ Memory Stem T Cells Recognizing Citrullinated Epitopes Are Expanded in Patients With Rheumatoid Arthritis and Sensitive to Tumor Necrosis Factor Blockade. *Arthritis Rheumatol* (2020) 72(4):565–75. doi: 10.1002/art.41157
161. Lee YJ, Park EH, Park JW, Jung KC, Lee EB. Proinflammatory Features of Stem Cell-Like Memory T Cells From Human Patients With Rheumatoid Arthritis. *J Immunol* (2021) 207(2):381–8. doi: 10.4049/jimmunol.2000814
162. Cao J, Zhang C, Han X, Cheng H, Chen W, Qi K, et al. Emerging Role of Stem Cell Memory-Like T Cell in Immune Thrombocytopenia. *Scand J Immunol* (2019) 89(3):e12739. doi: 10.1111/sji.12739
163. Bride KL, Vincent T, Smith-Whitley K, Lambert MP, Bleesing JJ, Seif AE, et al. Sirolimus Is Effective in Relapsed/Refractory Autoimmune Cytopenias: Results of a Prospective Multi-Institutional Trial. *Blood* (2016) 127(1):17–28. doi: 10.1182/blood-2015-07-657981
164. Flynn JK, Gorry PR. T Cell Therapies-are T Memory Stem Cells the Answer? *Ann Transl Med* (2015) 3(17):251. doi: 10.3978/j.issn.2305-5839.2015.08.13
165. Morrot A. Lifelong Protection Mediated by Stem Cell-Like CD8(+) T Memory Subset Cells (Tscm) Induced by Vaccination. *Ann Transl Med* (2016) 4(11):221. doi: 10.21037/atm.2016.05.38

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 03 January 2022
doi: 10.3389/fimmu.2021.791551

O-Acetyl-GD2 as a Therapeutic Target for Breast Cancer Stem Cells

Jing-Yan Cheng[1†], Jung-Tung Hung[1†], Juway Lin[1], Fei-Yun Lo[1], Jing-Rong Huang[1], Shih-Pin Chiou[1], Ya-Hui Wang[1], Ruey-Jen Lin[1], Jen-Chine Wu[1], John Yu[1] and Alice L. Yu[1,2,3,4]*

[1] Institute of Stem Cell and Translational Cancer Research, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan, [2] Chang Gung Univeristy, Taoyuan, Taiwan, [3] Department of Pediatrics, University of California in San Diego, CA, United States, [4] Genomics Research Center, Academia Sinica, Taipei, Taiwan

Synopsis: A sugar-lipid molecule called OAcGD2 is a novel marker for breast cancer stem cells. Treatment with anti-OAcGD2 mAb8B6 may have superior anticancer efficacy by targeting cancer stem cells, thereby reducing metastasis and recurrence of cancer.

Background: Cancer stem cells (CSCs) that drive tumor progression and disease recurrence are rare subsets of tumor cells. CSCs are relatively resistant to conventional chemotherapy and radiotherapy. Eradication of CSCs is thus essential to achieve durable responses. GD2 was reported to be a CSC marker in human triple-negative breast cancer, and anti-GD2 immunotherapy showed reduced tumor growth in cell lines. Using a specific anti-OAcGD2 antibody, mAb8D6, we set out to determine whether OAcGD2$^+$ cells exhibit stem cell properties and mAb8D6 can inhibit tumor growth by targeting OAcGD2$^+$CSCs.

Method: OAcGD2 expression in patient-derived xenografts (PDXs) of breast cancer was determined by flow cytometric analyses using mAb8D6. The stemness of OAcGD2$^+$ cells isolated by sorting and the effects of mAb8B6 were assessed by CSC growth and mammosphere formation *in vitro* and tumor growth *in vivo* using PDX models.

Result: We found that the OAcGD2 expression levels in six PDXs of various molecular subtypes of breast cancer highly correlated with their previously defined CSC markers in these PDXs. The sorted OAcGD2$^+$ cells displayed a greater capacity for mammosphere formation *in vitro* and tumor initiation *in vivo* than OAcGD2$^-$ cells. In addition, the majority of OAcGD2$^+$ cells were aldehyde dehydrogenase (ALDH$^+$) or CD44^{hi}CD24^{lo}, the known CSC markers in breast cancer. Treatment of PDXs-bearing mice with mAb8B6, but not doxorubicin, suppressed the tumor growth, along with reduced CSCs as assessed by CSC markers and *in vivo* tumorigenicity. *In vitro*, mAb8B6 suppressed proliferation and mammosphere formation and induced apoptosis of OAcGD2$^+$ breast cancer cells harvested from PDXs, in a dose-dependent manner. Finally, administration of mAb8B6 *in vivo* dramatically suppressed tumor growth of OAcGD2$^+$ breast CSCs (BCSCs) with complete tumor abrogation in 3/6 mice.

OPEN ACCESS

Edited by:
Cristina Maccalli, Sidra Medicine, Qatar

Reviewed by:
Enrique J. Arenas, Vall d'Hebron Institute of Oncology (VHIO), Spain
Dinorah Friedmann-Morvinski, Tel Aviv University, Israel

****Correspondence:***
Alice L. Yu
a1yu@ucsd.edu

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *08 October 2021*
Accepted: *06 December 2021*
Published: *03 January 2022*

Citation:
Cheng J-Y, Hung J-T, Lin J, Lo F-Y, Huang J-R, Chiou S-P, Wang Y-H, Lin R-J, Wu J-C, Yu J and Yu AL (2022) O-Acetyl-GD2 as a Therapeutic Target for Breast Cancer Stem Cells. Front. Immunol. 12:791551. doi: 10.3389/fimmu.2021.791551

Conclusion: OAcGD2 is a novel marker for CSC in various subtypes of breast cancer. Anti-OAcGD2 mAb8B6 directly eradicated OAcGD2$^+$ cells and reduced tumor growth in PDX model. Our data demonstrate the potential of mAb8B6 as a promising immunotherapeutic agent to target BCSCs.

Keywords: glycosphingolipid (GSL) glycans, breast cancer stem cells markers, immunotherapy, antibody, PDX (patient-derived xenografts)

INTRODUCTION

Tumors are complex tissues comprising phenotypically and functionally heterogeneous cancer cells (1, 2). One of the pivotal subpopulations in a tumor is cancer stem cells (CSCs), which are highly tumorigenic and chemoresistant (3, 4). CSCs harbor the capacity for self-renewal and differentiation and display resistance to chemotherapy and radiation (5). After treatment with doxorubicin, tumor cells showed increased expression of CSC-like cell surface markers and cytokines, along with increased tumorigenicity *in vitro* and *in vivo* (6, 7). Increased production of cytokines, such as IL-6, IL-8, and MCP-1, and upregulation of transcription factors, including HIF-1α and Stat3, have been observed after treatment with chemotherapeutic agents (8–11). Thus, a great deal of effort has been devoted to the search of clinically relevant biomarkers for better identification and targeting of CSCs.

Ganglioside GD2 has been reported to be a surface marker on CD44^{hi}/CD24^{lo} BCSCs in triple-negative human breast cancer cell lines and patient samples (12). Reduction of GD2 expression by *ST8SIA1* (GD3 synthase) knockdown inhibited mammosphere formation and cell motility *in vitro*, completely blocked tumor formation *in vivo*, and changed the CSC phenotype to a non-CSC phenotype (12). In addition, Liang et al. showed that GD2, GD3, and their corresponding biosynthetic enzyme GD2/GM2 synthase maintained a stem cell phenotype in BCSCs (13). Furthermore, GD2 may be associated with cMET to activate the cMET signaling pathway, which in turn induces stem cell characteristics of glioblastoma (14). These findings suggest that GD2 might serve as a marker of BCSCs. However, the anti-GD2 antibody mAb14G2a used in these studies to identify the GD2$^+$ cells are known to cross-react with OAcGD2 (15, 16). Thus, it remains unclear whether BCSCs delineated by mAb14G2a is GD2 or OAcGD2.

OAcGD2 is the O-acetyl derivative of GD2 ganglioside. Tumors that express GD2 often concomitantly express OAcGD2 (16). Biological functions of OAcGD2 remain unclear, but O-acetylation is frequently associated with cancer aggressiveness. O-acetylation of GD3 protected glioma cells from apoptosis (17), enhanced their survival, and conferred chemoresistance of leukemia cells (18). In addition, O-acetylation plays an important role in modulating the plasticity of chromatin structure in CSCs by changing the electrical property of acetylated sites of histone and covering up the ubiquitination sites to stabilize many non-histone proteins (19). The presence of OAcGD2 in breast cancer cell lines has been reported (20), but its role in breast cancer and CSCs remains unknown.

In this report, we demonstrated that OAcGD2-positive breast cancer cells displayed characteristic hallmarks of BCSCs. Targeting OAcGD2$^+$ BCSCs by a specific antibody triggered apoptosis and hampered mammosphere formation *in vitro* and suppressed the tumor growth *via* reducing BCSCs *in vivo*. These findings suggest that OAcGD2 is not only a new biomarker for BCSCs but also an ideal target for immunotherapy targeting BCSCs.

Abbreviations: ALDH, aldehyde dehydrogenase; BCSC, breast cancer stem cell; CSC, cancer stem cell; DOX, doxorubicin, OAcGD2, O-acetly-GD2; PDX, patient-derived xenograft; TNBC, triple-negative breast cancer.

RESULT

Expression of OAcGD2 in Breast Cancer PDXs

According to the previous report for OAcGD2 detection by IHC (16), frozen section of the tumor must be used for IHC of OAcGD2 as the deparaffination process may leach out hydrophobic glycolipid molecules such as OAcGD2. Since our original breast cancer specimens are available only as paraffin-embedded tissues, it is difficult to assess OAcGD2 expression in primary tumors. Fortunately, in recent years, the focus of the CSC field has shifted to the use of freshly isolated tumor specimens and early-passage patient-derived xenografts (PDXs), instead of using cultured tumor cell lines (21). Xenotransplantation assays have become an important strategy to assess CSC subpopulations and their activities. We have established five breast cancer PDXs with various molecular subtypes including luminal A and B, triple-negative breast cancer (**Supplemental Table 1**), and identified ALDH as a BCSC marker for BC0244, BC0634, BC0350, VBC108, and CD44$^+$CD24$^-$ as BCSC marker for BC0145 PDXs (**Table 1**) (22–25). In addition, ALDH is identified as a BCSC marker for PDX AS-B244, which was a subclone of BC0244, designated as AS-B244 (25). Flow cytometry analysis showed that 13–30% of the PDX cells expressed the indicated BCSC markers (**Table 1**). Examination of their expression of OAcGD2 by flow cytometry with anti-OAcGD2, mAb8B6, showed that 30–100% of BCSCs in these six PDXs expressed OAcGD2. Furthermore, Pearson correlation analysis of BCSCs and OAcGD2 expression in these PDXs showed positive correlation of BCSC percentage with OAcGD2 MFI (r=0.8115, p=0.05), and percentage of OAcGD2$^+$ in BCSCs (r=0.85, p=0.03), but not with percentage of OAcGD2$^+$ cells (r=0.42, p=0.41) (**Figure 1A**). Thus, the

TABLE 1 | Expression of OAcGD2 in PDXs of breast cancer.

PDXs	CSC Markers	% of CSCs	% of OAcGD2$^+$ Cellsand Expression Level (MFI)	% of OAcGD2$^+$ in CSCs
BC0244	ALDH$^+$	28–30%	70–73% (2,846)	100%
BC0145	CD44$^+$CD24$^-$	25–27%	35–40% (2,488)	100%
BC0634	ALDH$^+$	15–16%	28–30% (380)	75–78%
BC0350R1	ALDH$^+$	20–25%	58–60% (936)	70–72%
BCV108	ALDH$^+$	15–18%	40–43% (1,371)	40–43%
AS-B244	ALDH$^+$	13–15%	56–59% (1,132)	30–32%

Breast cancer PDXs were stained with anti-CD44-APC, anti-CD24-FITC, or ALDEFLUOR™ kit for detection of BCSCs-enriched subpopulation, as well as with anti-OAcGD2, and analyzed on an EC800 flow cytometer. MFI (mean fluorescence intensity) denotes the intensity of the OAcGD2 expression.

amount (MFI) of OAcGD2 is much more pertinent to stemness than its percentage. These findings suggest that OAcGD2 may serve as a marker for further enrichment of BCSCs.

Expression of OAcGD2 in Breast Cancer Stem Cells

The hallmark of CSCs is their ability to initiate tumors better than their bulk tumor counterparts (26). To determine whether OAcGD2-expressing ALDH$^+$ or CD44$^+$CD24$^-$ BCSCs are more tumorigenic than OAcGD2 negative ALDH$^+$ or CD44$^+$CD24$^-$ BCSCs, we sorted the highest and lowest OAcGD2-expressing ALDH$^+$ BC0244 (**Figure 1B**) or CD44$^+$CD24$^-$ BC0145 cells (**Figure 1C**) to assess their mammosphere-forming ability. The OAcGD2$^+$ALDH$^+$ BC0244 and OAcGD2$^+$CD44$^+$CD24$^-$ BC0145 cells formed more mammospheres when compared with OAcGD2$^-$ALDH$^+$ BC0244 (40.2 ± 4.4 vs. 21.5 ± 2.1) and OAcGD2$^-$CD44$^+$CD24$^-$ BC0145 cells (30.0 ± 3.3 *vs.* 15.1 ± 1.7) ($p < 0.05$ for both) (**Figures 1D, E**). To determine the tumor-initiating potentials of OAcGD2$^+$ BCSCs, we sorted OAcGD2^{low} and OAcGD2^{high} BCSCs from BC0244 and BC0145 cells and injected these cells into the mammary fat pad of NOD/SCID mice (n = 3/group) at the cell doses of 10^2, 10^3, and 10^4. As shown in **Table 2**, OAcGD2^{low} subpopulation of BC0244 and BC0145 failed to show any tumor engraftment at all three cell

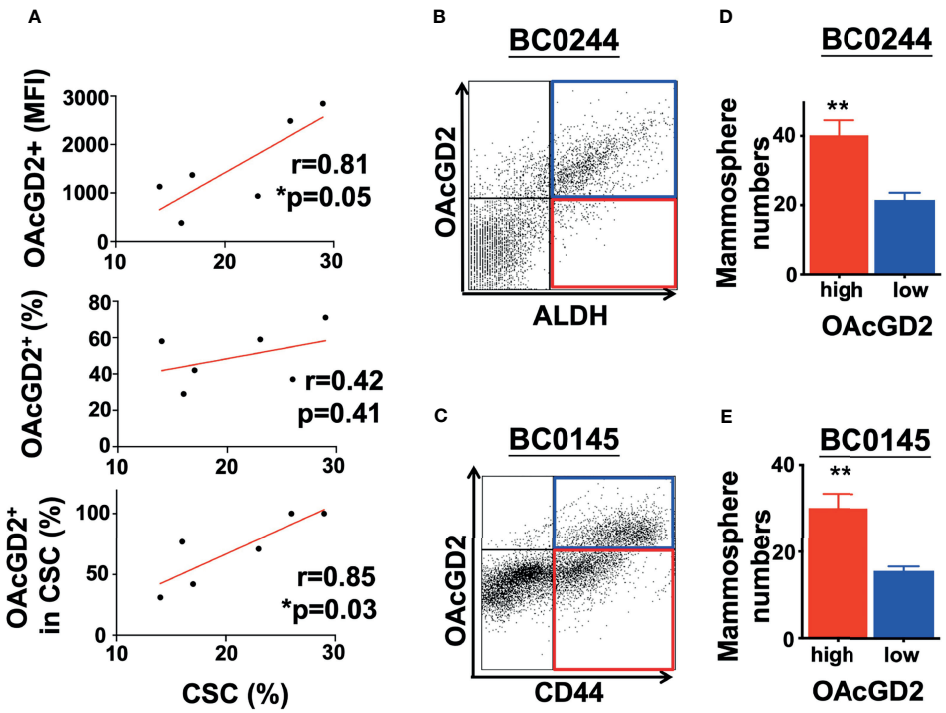


FIGURE 1 | OAcGD2-expressing breast cancer cells harvested from PDXs display enhanced mammosphere forming capacity. **(A)** Pearson correlation analysis between percent of CSCs population and OAcGD2 expression in PDXs as shown in . Pearson correlation coefficient (r) and *p*-value (p) were shown. **(B)** BC0244 cells were stained with anti-OAcGD2 antibody and *ALDEFLUOR*™ kit. The OAcGD2^{high}ALDH$^+$ and OAcGD2^{low}ALDH$^+$ BC0244 cells were sorted by FACSAria II and seeded at 1 × 10^3/well into a 96-well ultra-low attachment plate containing mammosphere growth medium to determine their mammosphere formation ability. **(C)** BC0145 cells were stained with anti-OAcGD2, anti-CD44-APC, and anti-CD24-FITC. OAcGD2^{high}CD44$^+$CD24$^-$ and OAcGD2^{low}CD44$^+$CD24$^-$ BC0145 cells were sorted to examine their mammosphere formation ability. **(D, E)** Seven days after culture, the number of mammosphere was counted under a bright-field microscope. The results were presented as mean ± SD of the mammosphere numbers. Data were analyzed by Pearson correlation analysis and Student's t-test. *$p < 0.05$, **$p < 0.01$.

TABLE 2 | Generation of tumors by OAcGD2^{low} and OAcGD2^{high} breast cancer cells *in vivo*.

	ALDH$^+$ BC0244		CD44$^+$CD24$^-$ BC0145	
	OAcGD2^{low}	OAcGD2^{high}	OAcGD2^{low}	OAcGD2^{high}
10^4	0/3	2/3	0/3	3/3
10^3	0/3	2/3	0/3	2/3
10^2	0/3	0/3	0/3	0/3
Frequency	N.D.	1:4,747	N.D.	1:1,072

OAcGD2$^-$ and OAcGD2$^+$ PDXs cells were sorted by FACSAria II, and 1,000, 100, or 10 cells of the sorted cells were injected into the mammary fat pad of NOD/SCID mice. Tumor formation was observed 8 weeks after transplantation. The frequencies were calculated using Extreme Limiting Dilution Analysis (http://bioinf.wehi.edu.au/software/elda/) based on tumor formation frequency data. N.D., not determined.

dose levels. In contrast, two out of three mice inoculated with 10^3 or 10^4 OAcGD2^{high} BC0244 BCSCs grew tumor. Similar results were found in mice injected with OAcGD2^{high} BC0145 BCSCs. These findings support the notion that OAcGD2 is a marker for BCSCs.

Treatment With mAb8B6 Suppresses Breast Cancer Growth and Reduces Cancer Stem Cells

Next, we evaluated the antitumor efficacy of mAb8B6, an anti-OAcGD2, on two PDXs of breast cancer, BC0244 and BC0145. Tumor cells were injected into the mammary fat pad of NOD/SCID mice. When the tumor volume reached 100 mm^3, mice were treated with mAb8B6 (5 mg/Kg), 2 mg/kg of doxorubicin (DOX-high), 0.5 mg/kg of doxorubicin (DOX-low), or PBS once a week for 4 weeks. All animals were sacrificed 3 days after the last administration. As shown in **Figure 2A**, the growth of BC0244 and BC0145 tumors of individual mice and the slope of tumor growth for each group were significantly suppressed by DOX and mAb8B6, with mAb8B6 compared to PBS, p<0.0001 in BC0244 and p=0.0012 in BC0145. Treatment with DOX showed dose-dependent inhibition-(DOX-high *vs.* DOX-low, p=0.0039 in BC0244, p=0.0236 in BC0145), and mAb8B6 was as effective as DOX-high in tumor growth inhibition (p=0.4195). At the time of sacrifice, the tumor volume of BC0244 in the DOX-low, DOX-high, and mAb8B6 groups was significantly reduced to 56.0 ± 8.6%, 34.1 ± 4.9%, and 38.7 ± 6.5%, respectively, of the control group treated with PBS (p<0.001), although the reduction in tumor volume of BC0145 was significant only in the mAb8B6 group (25.8 ± 7.4% of the PBS, p=0.0012), but not in the DOX-low (60.5 ± 12.5%), or DOX-high 43.3 ± 23.9% (**Figure 2B**). Thus, based on tumor growth rate or tumor volume harvested after treatment, anti-OAcGD2 treatment significantly suppressed the tumor growth of both PDXs. In addition, we examined the BCSCs (ALDH$^+$ for BC0244 and CD44$^+$ CD24$^-$ for BC0145) in the harvested tumors. BCSCs in both mAb8B6 treated BC0244 and BC0145 tumors decreased to 80.0 ± 17.9% (p=0.049) and 68.5 ± 15.6% (p=0.0017), respectively, of PBS control group (**Figure 2C**). Although DOX at 2 mg/kg reduced tumor volume, it had no effect on BCSCs when compared to the

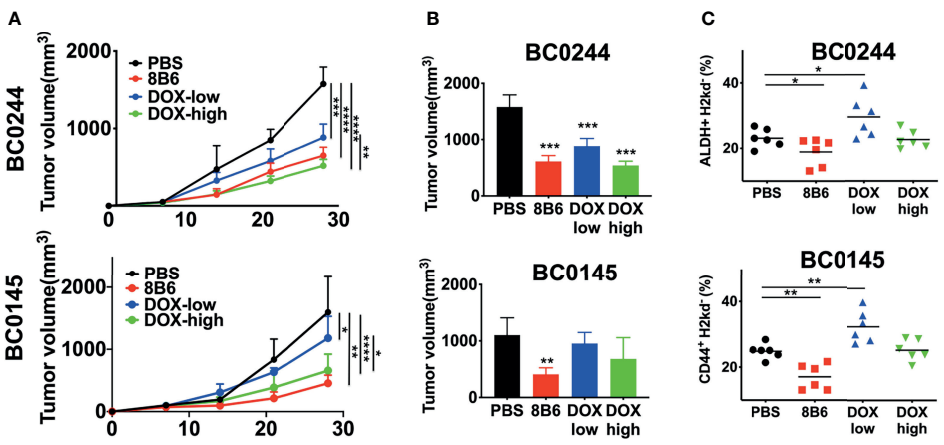


FIGURE 2 | Treatment with mAb8B6 suppresses breast cancer growth and reduces cancer stem cells. **(A)** Growth inhibition of BC0244 and BC0145 PDX tumors in NOD/SCID mice. Mice (n = 6/group) were inoculated with PDX cells (5 × 10^5 cells) at the mammary fat pad. When the tumor volume reached 100 mm^3, these mice were intravenously injected with either PBS, 2 mg/kg doxorubicin (DOX-high), 0.5 mg/kg doxorubicin (DOX-low), or mAb8B6 (5 mg/kg) weekly for four times. Tumor volumes were measured every 3 days, and tumor growth was depicted as mean tumor volume for each group. The slope of tumor growth was determined by linear regression. **(B)** Tumor volume was measured before sacrifice (3 days after the last treatment). **(C)** BCSCs frequency in BC0244- and BC0145-derived tumors was identified with *ALDEFLUOR*^{TM} kit and CD44-APC/CD24-FITC, respectively. Data representative of three experiments and values are expressed as mean ± SD and analyzed by one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

PBS control group. Interestingly, treatment with 0.5 mg/kg of DOX increased the BCSCs in both BC0145 and BC0244 tumors to 130.9 ± 19.7% (p=0.006) and 127.2 ± 26.7% (p=0.034) of control group, consistent with the reported relative resistance of BCSCs to chemotherapy.

To further confirm that anti-OAcGD2 treatment can target BCSCs, we tested the frequency of tumor-initiating cells by limiting dilution engraftment assay. BC0244 and BC0145 tumor cells isolated from the mAb8B6- and DOX-treated mice were inoculated into NOD.SCID mice at three different cell doses: 10^4, 10^5, and 10^6. Tumor formation was monitored for 2 months (**Table 3**). As expected, the frequency of tumor-initiating cells in BC0244 tumor treated with mAb8B6 (1:94,752) was significantly lower than those treated with PBS (1:14,241), DOX-low (1:9,100), and DOX-high (1:14,241). Similarly, in BC0145-bearing mice, the frequency of tumor-initiating cells from the mAb8B6 group (1:66,954) was much lower than those treated with PBS (1:14,241), DOX-low (1:5,581), and DOX-high (1:21,636) groups. In line with the increased percent of BCSCs as determined by surface markers, treatment of the BC0244 and BC0145 tumor-bearing mice with 0.5 mg/kg DOX increased the frequency of tumor-initiating cells.

Anti-OAcGD2 Treatment Inhibits Proliferation and Mammosphere Formation and Induces Apoptosis of BCSCs *In Vitro*

We further investigate the *in vitro* effect of the anti-OAcGD2 on the proliferation of OAcGD2^{high} and OAcGD2^{low} BC0145/BC0244, as determined by AlamarBlue assay. As shown in **Figure 3A**, there was no difference in the proliferation rate between OAcGD2^{high} and OAcGD2^{low} subpopulations of both PDXs, but treatment with mAb8B6 inhibited proliferation of OAcGD2^{high} BC0145/BC0244 cells only (left panel), not OAcGD2^{low} BC0145/BC0244 cells (right panel). We next examined the effects of mAb8B6 on the properties of BCSCs. Mammosphere-forming capacity of sorted ALDH$^+$OAcGD2^{low} and ALDH$^+$OAcGD2^{high} BC0244 cells was assessed in the absence/presence of mAb8B6 at 25 or 50μg/ml. The presence of mAb8B6 significantly decreased mammosphere formation of ALDH$^+$OAcGD2$^+$ in a dose-dependent manner, while only slightly attenuated the mammosphere formation of ALDH$^+$OAcGD2^{low} cells (**Figures 3B, C**).

It has been reported that mAb8B6 inhibited the growth of neuroblastoma, small cell lung cancer, and lymphoma cell lines, which was mediated by ADCC/CDC and induction of apoptosis (16, 27). To determine whether mAb8B6 exerts direct cytotoxicity on breast cancer, we examined apoptosis of BC0244 after incubation with mAb8b6 or isotype control antibody for 24 h by flow cytometry. As shown in **Figure 4A**, mAb8B6 induced greater early apoptosis (16.7 and 21.8% at 10 and 50 μg/ml, respectively) and late apoptosis (8.3 and 11.1% at 10 and 50 μg/ml, respectively) of OAcGD2^{high} BC0244 cells as compared to the isotype control antibody (early apoptosis: 4.9%; late apoptosis: 0.7% at 50 μg/ml). On the other hand, mAb8B6 did not induce obvious apoptosis of OAcGD2^{low} BC0244 cells. These results demonstrated the ability of mAb8B6 in inducing programed cell death in OAcGD2-expressing cells.

Anti-OAcGD2 Treatment *In Vivo* Abrogates Tumor Growth of Isolated BCSCs

To further ascertain whether mAb8B6 can inhibit tumor growth of OAcGD2-expressing cells *in vivo*, we inoculated 1x10^5 OAcGD2^{high} BC0244 cells into NOD/SCID mice. When the tumor volume reached 100 mm^3, mice were randomly divided into two groups for treatment with mAb8B6, or PBS control every week by i.v. injection. It is noteworthy that 50% of mAb8B6 (3 of 6) were completely tumor-free. At 4 weeks, tumors of the remaining three mice of the mAb8B6 group were reduced to 9.4 ± 2.5% (p<0.001) of PBS control (**Figure 4B**). Moreover, the remaining tumors from mice treated with mAb8B6 contained significantly less ALDH$^+$ BCSCs when compared to those treated with PBS (33.3 ± 15.7% of PBS control, p=0.009) (**Figure 4C**). To determine whether the anti-proliferative and apoptotic activities

TABLE 3 | Estimated frequencies of tumor-initiating cells in PDXs of breast cancer treated with Doxorubicin or mAb8B6.

BC0244: Mice with tumor/total mice				
	PBS	**8B6[a]**	**DOX-low[b]**	**DOX-high[b]**
10^6	6/6	6/6	6/6	6/6
10^5	6/6	3/6	6/6	6/6
10^4	3/6	2/6	4/6	3/6
Frequency	1:14,241	1:94,752	1:9,100	1:14,241
BC0145: Mice with tumor/total mice				
	PBS	8B6[a]	DOX-low[b]	DOX-high[b]
10^6	6/6	6/6	6/6	6/6
10^5	6/6	4/6	6/6	6/6
10^4	3/6	2/6	5/6	2/6
Frequency	1:14,241	1:66,954	1:5,581	1:21,636

The frequencies were calculated using Extreme Limiting Dilution Analysis.
(http://bioinf.wehi.edu.au/software/elda/) based on tumor formation frequency data.
[a]*5mg/kg 8B6.*
[b]*2 mg/kg doxorubicin (DOX-high), 0.5 mg/kg doxorubicin (DOX-low).*

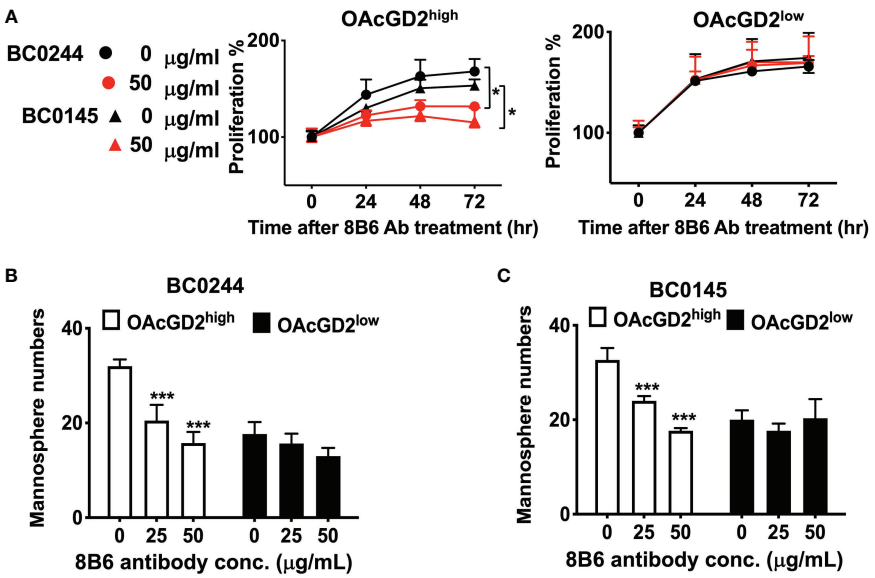


FIGURE 3 | Anti-OAcGD2 mAb8B6 inhibits mammosphere formation and proliferation of PDX cells. **(A)** OAcGD2^{high}ALDH^{+} BC0244, OAcGD2^{high}CD44^{+}CD24^{-} BC0145 cells, OAcGD2^{low}ALDH^{+} BC0244, and OAcGD2^{low}CD44^{+}CD24^{-} BC0145 cells were sorted and treated for 72 h with the indicated concentrations of mAb8B6. Cell proliferation was assessed by the AlamarBlue assay. Optical density was recorded at 570 nm and was expressed as proliferation % normalized to time 0 hr. **(B)** OAcGD2^{low}ALDH^{+} and OAcGD2^{low}ALDH^{+} BC0244 cells and **(C)** OAcGD2^{low}CD44^{+}CD24^{-} and OAcGD2^{low}CD44^{+}CD24^{-} BC0145 cells were sorted and plated at 1 × 10^{3}/well in 96-well ultra-low attachment plates containing mammosphere growth medium. Cells were incubated for 7 days with the indicated concentrations of mAb8B6, and the number of mammosphere was counted under a light microscope. The data are presented as the mean ± SD of three independent experiments, each in triplicate. *** $p < 0.001$ compared to cells without 8B6 treatment. *$p < 0.05$ by Student's t-test.

of mAb8B6 observed *in vitro* is mimicked *in vivo*, we examined the percentage of Ki67^{+} cells in harvested tumors (**Figure 4D**). The Ki67^{+} cell of tumors obtained from mice treated with mAb8B6 was 12.3± 4.0%, which was significantly lower than the PBS control group (49.8 ± 8.2%, p = 0.002). TUNEL-staining revealed extremely low levels of apoptosis in the tumors from PBS-treated mice (5.0 ± 2.0%) (**Figure 4E**). The percentage of apoptotic cells was significantly higher in the tumors from mice treated with mAb8B6 (66.3 ± 11.6%; p = 0.002). These findings indicate that mAb8B6 can target CSCs by inducing apoptosis and suppress tumor growth as illustrated in **Figure 5**.

DISCUSSION

In this study, we identified OAcGD2 as a marker for BCSCs. Specifically, OAcGD2 was found to be expressed predominantly on CSCs-enriched population (ALDH^{+} or CD44^{+}CD24^{-} cells) harvested from PDXs of different molecular subtypes of breast cancer. Functionally, OAcGD2^{+} CSC demonstrated greater tumor-initiating ability, suggesting their capability for proliferation instead of remaining in a quiescent state. Phenotypically, OAcGD2 expression levels correlated closely with the CSC population in PDX. Our findings provide the first evidence that OAcGD2 is a novel CSC marker for breast cancer. This is in line with the previous report of OAcGD2 as a CSC marker for glioblastoma. The antitumor activity of mAb8B6 against glioblastoma was shown to involve three different mechanisms: (1) induction of antibody-dependent cell cytotoxicity (16), (2) induction of complement cellular cytotoxicity (16), and (3) direct cytotoxicity by inducing pro-apoptosis signal (27). Our results demonstrated that mAb8B6 induced apoptosis of OAcGD2^{+} cells *in vitro*. Treatment of immune-compromised mice bearing PDXs with mAb8B6 *in vivo* resulted in a significant suppression of tumor growth, along with increased apoptotic cells and reduced number of BCSCs. Although NK cell deficit is apparent in NOD-SCID mice, the remnant NK activity may contribute to the observed anticancer effect of mAb8B6 *via* ADCC as reported. These results suggest that anti-OAcGD2 might be an ideal immunotherapeutic agent for BCSCs-targeted therapy of breast cancer.

GD2 has been reported as a specific cell surface marker of BCSCs in triple-negative breast cancer (TNBC) (12, 28–30). In these studies, an anti-GD2 antibody 14G2a was used to identify the GD2^{+} cells. In fact, mAb14G2a has been known to cross-react with OAcGD2 (15). Thus, OAcGD2^{+} cells exhibiting stem cell properties might be included in the cells reactive with mAb14G2a in these reports. On the other hand, mAb8B6 does not cross-react with GD2 (16). Therefore, our studies using mAb8B6 support the notion that OAcGD2 is a bona fide marker for BCSCs and is not limited to TNBC. The amount of OAcGD2 is much more pertinent to stemness than its percentage. The mechanism underlying the contribution of

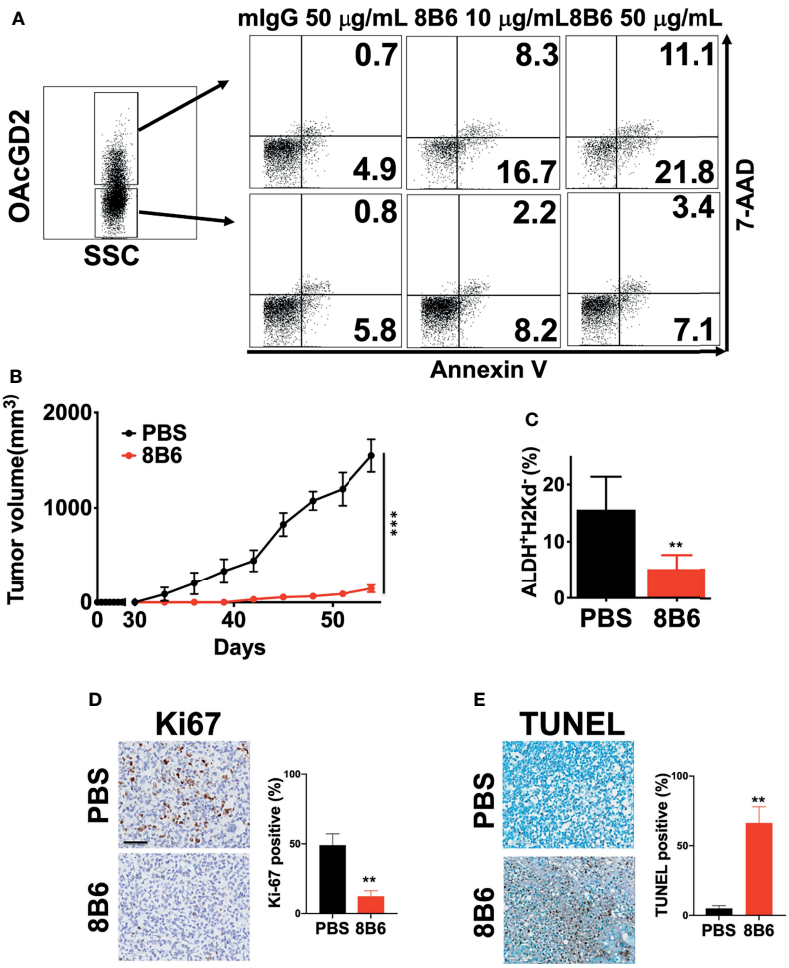


FIGURE 4 | MAb8B6 induces apoptosis in breast cancer cells harvested from PDXs *in vitro* and abrogates tumor growth of OAcGD2-expressing BCSCs in NOD/SCID mice. **(A)** Apoptosis of BC0244 cells after incubation with either 10 or 50 μg/ml of mAb8B6 or mIgG antibody for 24 h was determined by staining with 7-AAD and Annexin V-PE. **(B)** Inhibition of *in vivo* tumor growth of BCSCs sorted from BC0244 in NOD/SCID mice by mAb8B6. Mice (n = 6/group) were inoculated with sorted 1×10^5 $OAcGD2^+ALDH^+$ BC0244 cells at the mammary fat pad. Once the tumor volume reached 100 mm^3, mice were treated with PBS or mAb8B6 (5 mg/kg) weekly ×4. Tumor volumes were measured every 3 days, and average of the tumor volumes for each group was presented. Tumors were completely abrogated in 3/6 mice treated with mAb8D6. **(C)** Four weeks after tumor inoculation, the BCSCs in BC0244-derived tumors was determined by flow cytometry with *ALDEFLUOR*™ kit and mouse $H2K^d$. **(D)** Ki67 and **(E)** TUNEL staining of tumor sections after mAb8B6 treatment. Scale bars, 60 μm. Ki67- and TUNEL-staining-positive cells were counted, and the percentage of positive cells out of the total number of cancer cells was calculated. $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's t-test.

OAcGD2 to CSC properties has yet to be delineated. Currently, the synthetic OAcGD2 is not commercially available, which hampers the progress in this research field. Since the stemness property of GD2 involves HGF-MET (31) and EGFR signaling (28), it may be worthwhile to explore whether these pathways contribute to the stemness property of OAcGD2. In addition, it may be helpful to identify the OAcGD2-binding proteins by immunoprecipitation or OAcGD2-activating genes by RNA-seq. These endeavors may facilitate our understanding of the roles of OAcGD2/mAb8B6 in CSC.

CSCs show functional heterogeneity and hierarchical organization. It is known that CSCs contribute to chemotherapy resistance across a broad range of malignancies (4, 32). Most CSCs are in a quiescent state with a low proliferation rate and thus escape killing by cytotoxic agents that target proliferating cells (33). CSCs possess active drug-efflux machinery, such as ATP-binding cassette family transporters, to pump out chemotherapeutic agents. In addition, overexpression of DNA-repair mechanisms, including homologous recombination, non-homologous end-joining (34), and base-excision repair through increased poly (ADP-ribose) polymerase 1 activity, are very common in CSCs (35). Moreover, CSCs can escape from programmed cell death (36) and acquire an epithelial-to-mesenchymal transition phenotype (37), which facilitate cancer progression and metastasis, respectively. Thus, CSCs have become important targets for cancer treatment.

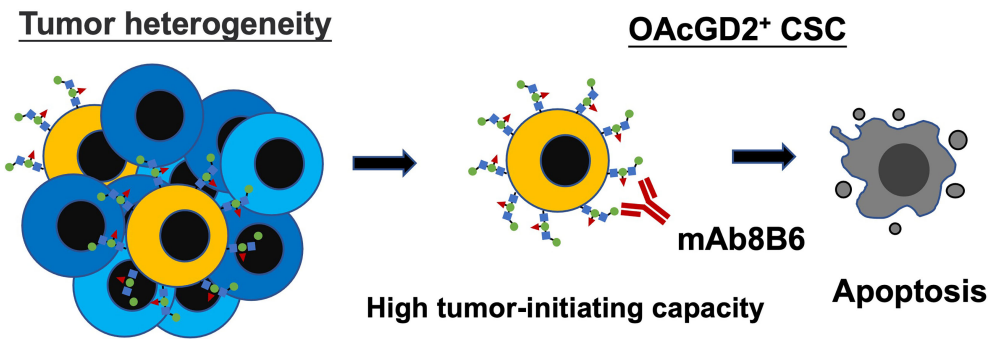


FIGURE 5 | Graphical abstract showing OAcGD2 as a novel marker for CSC which can be targeted with mAb8B6 to suppress tumor growth by inducing apoptosis.

Several therapeutic strategies to target CSCs have emerged, such as the development of a bispecific antibody that brings cytotoxic T cells to $CD133^+$ CSCs in pancreatic and hepatic cancers and blockade of CD47 to target CSCs in leukemia. CD47 is a ligand for signal-regulatory protein-α expressed on phagocytic cells and functions to inhibit phagocytosis. Thus, blockade of CD47 has been shown to be an effective strategy for targeting leukemia CSCs in PDX models (38). These CSC-targeting strategies are under clinical development.

Combination of anticancer antibody with chemotherapy is a well-known strategy to enhance the antitumor efficacy. A well-documented chemo-immunotherapy is the combination of CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) with Rituximab, which is an effective treatment for aggressive B-cell non-Hodgkin lymphoma (39). Recently, combination of Irinotecan and Temozolomide with an anti-GD2, Dinutuximab, has induced impressive clinical responses in patients with relapsed/refractory neuroblastoma (40). Along this line, preclinical study of the combination of temozolomide and mAb8B6 effectively suppressed the growth of glioma *in vivo* by reducing the temozolomide-resistant stem-like cell pool in glioma (41). This is consistent with our findings of the CSC-targeting capacity of mAb8B6 in breast cancer and suggests that future studies of anti-OAcGD2 in combination with chemotherapy should be explored in breast cancer.

Dinutuximab was approved for the treatment of high-risk neuroblastoma in the setting of minimal residual disease (42) and recently in neuroblastoma patients with refractory/resistant disease (43). However, dinutuximab is associated with dose-limiting neuropathic pain. The lack of allodynic properties of mAb8B6 and abundant expression of OAcGD2 in neuroblastoma (44) make mAb8B6 an attractive option for immunotherapy of OAcGD2-expressing tumors, including neuroblastoma. Future clinical development of mAb8B6 for the treatment of neuroblastoma is warranted. Recent reports have shown that combination of anti-GD2 with PD-1 blockade resulted in synergistic anticancer effects on GD2-expressing tumors in mice, which were attributable to upregulation of immune checkpoint molecules, PD-1/PD-L1, in neuroblastoma by anti-GD2 (45), and induction of immunogenic cell death (submitted manuscript). With the approval of immune checkpoint blockade for the treatment of breast cancer (46), it may be worthwhile to explore whether anti-OAcGD2 may also enhance the anticancer efficacy of anti-PD1/PD-L1 in breast cancer.

In summary, we have demonstrated that OAcGD2 is a marker for CSCs in breast cancer, which can be targeted by mAb8B6 *in vitro* and *in vivo*. Our findings provide strong rationales for the development of anti-OAcGD2 as a novel immunotherapeutic agent for CSC-targeted therapy of breast cancer.

MATERIALS AND METHODS

Cell Culture and Reagent

Human clinical breast cancer specimens were obtained from patients at the time of initial surgery and were fully encoded to protect patient confidentiality. Clinical specimens were utilized under a protocol approved by the Institutional Review Board of the Human Subjects Research Ethics Committee of Academia Sinica, Tri-Service General Hospital, and Veterans General Hospital (Taipei, Taiwan). Isolation of the primary tumor cells from clinical specimens was described previously (24). Five patient-derived xenografts (PDXs) were successfully established from patients BC0145, BC0244, BC0350, and BC0634. BCSC subpopulation was delineated as $CD24^-CD44^+$ cells in BC0145, and $ALDH^+$ cells in BC0244, BC350, and BC0634, according to their tumorigenicity (22, 25). All PDXs were maintained throughout xenograft passages. Monolayer cultures of $H\text{-}2Kd^-ALDH^+$ BC0244, sorted from xenograft tumors of human primary breast cancer, were designated as AS-B244 cells as described previously (22, 25). The anti-OAcGD2 mAb8B6 is kindly provided by OGD2 Pharma, France.

FACS Analysis and Sorting

Cell surface OAcGD2 expression on tumor cell lines was assessed by indirect immunofluorescence. Cells were incubated with either mAb8B6 or mouse IgG3 (isotype control antibody) at 10 μg/ml for 30 min at 4°C in 0.1% BSA-PBS. After the reaction,

these cells were incubated with the FITC-conjugated goat anti-mouse IgG as a second antibody (Biolegend) for 30 min at 4°C. BCSCs were defined as $CD44^+CD24^-$ or $ALDH^+$ cells. ALDH activity was determined by the *ALDEFLUOR*™ kit (Stem Cell Technologies) according to the manufacturer's instructions. All stained cells were then examined by EC800 flow cytometer (SONY). For sorting, the cells were collected using a BD FACSAria II flow cytometer (BD Biosciences).

Mammosphere Formation Assay

The sphere culture was performed as previously described (47) with some modifications. $OAcGD2^-$ or $OAcGD2^+$ CSCs from BC0244 or BC0145 cells were FACS sorted using antibodies against OAcGD2, ALDH, CD44, and CD24. The sorted cells ($1x10^3$) were incubated in a mammosphere growth medium in ultra-low-attachment 96-well plates (Corning). All cells grew at a density of 1×10^4 cells/ml in serum-free Dulbecco's Modified Eagle's Medium/F12 supplemented with 20 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 0.4% bovine serum albumin, 100 U/ml Pen/Strep, and 2% B27. Monoclonal antibodies were diluted and added to each well containing 1,000 tumor cells in 96-well plates to give the final concentrations of 0, 25, and 50 μg/ml. After 7 days, the resulting mammospheres were counted.

Cell Growth Inhibition

Cell viability was measured using the Alamarblue assay. Briefly, sorted cells were incubated with/without mAb8B6 (50 μg/ml) for 72 h at 37°C with 5% CO_2. Absorbance was measure at 570 nm on a SpectraMAX (M3). The proliferation rate was calculated by normalizing to 0 h.

Apoptosis

Cells (2×105 cells) were plated in six-well plates for 24 h at 37°C in a humidified atmosphere containing 5% CO_2, and then treated with 50 μg/ml of mAb8B6 and mIgG for 24 h. After incubation, cells were stained with FITC-conjugated goat anti-mouse IgG (Jackson) as described above. After washing twice with PBS, we resuspended these cells in 500 μl of a binding buffer with Annexin V in the dark for 20 min, according to the manufacturer's protocol (BD).

PDXs Tumor Model

NOD/SCID mice were purchased from Jackson Lab and maintained at the animal facility of the Chang Gung University (IACUC number: CGU106-055). Animal studies were conducted by the guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. BC0244 and BC0145 cells (5×10^5) mixed with 100 μl of 2 mg/ml Matrigel were injected at the base of the nipple of the fourth abdominal fat pad of female mice (4- to 6-week-old). Ear numbering system was used to create a unique identifier; the tumor-free mouse was exclusive. When tumor size reached 100 mm^3, mice (n = 6/group) were randomly assigned to each group and i.v. injected with 5 mg/kg of mAb8B6, 2 mg/kg of doxorubicin (DOX-high), 0.5 mg/kg of doxorubicin (DOX-low), and PBS once a week for 4 weeks. Sorted $OAcGD2^+ALDH^+$ BC0244 cells (1×10^5) mixed with 100 μl of 2 mg/ml Matrigel were injected at the base of the nipple of the fourth abdominal fat pad of female mice. When tumor size reached 100 mm^3, mice (n = 6/group) were randomly assigned to each group and treated with 5 mg/kg of mAb8B6 or PBS once a week for 4 weeks with or without PBMC intraperitoneally at 1×10^7/mouse as effector cell. Tumor volume was monitored using a vernier caliper twice a week for up to 8 weeks and calculated according to the equation: $V = 1/2*W^2*L$, where L is the length and W the width of a tumor. For ethical considerations, mice had to be euthanized once tumor volume had reached 2,000 mm^3, which was considered the endpoint for each mouse. In addition, $OAcGD2^{high}$ of $ALDH^+$ cells from BC0244 cells and $OAcGD2^{high}$ of $CD44^+CD24^-$ cells from BC0145 cells were sorted using FACSAria II cell sorter (BD) and then inoculated in NOD/SCID mice (n=5). These mice were treated with mAb8B6 or PBS as described above.

In Vivo Tumor Initiation Assay

To obtain single cells from the tumors, we sliced a tumor into square fragments of 1 mm^2 and then digested these fragments by incubation in a MEM medium containing collagenase (1,000 U/ml), hyaluronidase (300 U/ml), and DNase I (100 μg/ml) at 37°C for 1 h. Single cells (10^4, 10^5, or 10^6) isolated from tumor-bearing mice treated with mAb8B6, DOX-high, DOX-low, or PBS were injected at the mammary fat pad. Animals were examined for tumor formation after 1 week. The frequency of tumorigenic cells and the 95% confidence interval were calculated using Extreme Limiting Dilution Analysis (48).

Ki-67 Immunostaining

Tumor tissue sections were deparaffinized followed by antigen retrieval by autoclave for 121°C, 5 min in AR-10 solution (Biogenex). Endogenous peroxidase was quenched before saturating with H_2O_2 blocking solution (Dako). Sections were stained with mouse anti-human Ki67 mAb (Leica, Cat No. NCL-L-Ki67-MM1). Bound antibody was detected by polymer-HRP IHC detection system (Biogenex). Digital images were captured by Aperio ScanScope XT Slide Scanner (Aperio Technologies, Vista, CA, USA) under 20× magnification. Positive and negative stained cells were counted on five random fields for each tumor. Data were expressed as cells positive for Ki67 staining/total cells.

TUNEL Immunostaining

The extent of apoptosis in the tumors was measured by TUNEL using the TUNEL assay kit (Abcam, ab206386) following the manufacturer's protocol. Data were expressed as cells positive for TUNEL staining/total cells.

Statistical Analysis

Statistical analysis was performed using Prism (GraphPad Software). All values are presented as means ± SD. Three independent experiments were performed, and representative results were shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. P-value was calculated by using the Student t-test or one-way ANOVA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Human Subjects Research Ethics Committee of Academia Sinica. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Chang Gung University.

AUTHOR CONTRIBUTIONS

J-YC, J-TH, and AY conceived and designed the study. JL, J-RH, and S-PC conducted the experiment study. F-YL performed the outcome assessment. Y-HW and R-JL conducted the PDX establishment. J-CW conducted the mice management. J-TH, JY, and AY provided the funding. J-TH, J-YC, and AY analyzed the data and wrote the manuscript with contributions from all authors. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants CMRPG3K2391 from CGMH at Linkou of Taiwan to J-TH, and OMRPG3C0017 and OMRPG3C0018 from CGMH at Linkou of Taiwan to AY, and MOST106-3114-B-182A-001 and 107-2321-B-182A-005 to JY from the Ministry of Science and Technology of Taiwan.

ACKNOWLEDGMENTS

Anti-OAcGD2 antibody mAb8B6 and control isotype antibody were generously provided by OGD2 Pharma, France. We thank Li-Chun, Lai for her excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.791551/full#supplementary-material

REFERENCES

1. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, et al. Mesenchymal Stem Cells Within Tumour Stroma Promote Breast Cancer Metastasis. *Nature* (2007) 449:557–63. doi: 10.1038/nature06188
2. Greaves M, Maley CC. Clonal Evolution in Cancer. *Nature* (2012) 481:306–13. doi: 10.1038/nature10762
3. Korkaya H, Kim GI, Davis A, Malik F, Henry NL, Ithimakin S, et al. Activation of an IL6 Inflammatory Loop Mediates Trastuzumab Resistance in HER2+ Breast Cancer by Expanding the Cancer Stem Cell Population. *Mol Cell* (2012) 47:570–84. doi: 10.1016/j.molcel.2012.06.014
4. Oskarsson T, Batlle E, Massague J. Metastatic Stem Cells: Sources, Niches, and Vital Pathways. *Cell Stem Cell* (2014) 14:306–21. doi: 10.1016/j.stem.2014.02.002
5. Kreso A, Dick JE. Evolution of the Cancer Stem Cell Model. *Cell Stem Cell* (2014) 14:275–91. doi: 10.1016/j.stem.2014.02.006
6. Ge G, Zhou C, Ren Y, Tang X, Wang K, Zhang W, et al. Enhanced SLC34A2 in Breast Cancer Stem Cell-Like Cells Induces Chemotherapeutic Resistance to Doxorubicin *via* SLC34A2-Bmi1-ABCC5 Signaling. *Tumour Biol J Int Soc Oncodevelopmental Biol Med* (2016) 37:5049–62. doi: 10.1007/s13277-015-4226-0
7. Alakhova DY, Zhao Y, Li S, Kabanov AV. Effect of Doxorubicin/Pluronic SP1049C on Tumorigenicity, Aggressiveness, DNA Methylation and Stem Cell Markers in Murine Leukemia. *PloS One* (2013) 8:e72238. doi: 10.1371/journal.pone.0072238
8. Samanta D, Gilkes DM, Chaturvedi P, Xiang L, Semenza GL. Hypoxia-Inducible Factors Are Required for Chemotherapy Resistance of Breast Cancer Stem Cells. *Proc Natl Acad Sci USA* (2014) 111:E5429–38. doi: 10.1073/pnas.1421438111
9. Wang Y, Zong X, Mitra S, Mitra AK, Matei D, Nephew KP. IL-6 Mediates Platinum-Induced Enrichment of Ovarian Cancer Stem Cells. *JCI Insight* (2018) 3:e122360. doi: 10.1172/jci.insight.122360
10. Singh JK, Simoes BM, Howell SJ, Farnie G, Clarke RB. Recent Advances Reveal IL-8 Signaling as a Potential Key to Targeting Breast Cancer Stem Cells. *Breast Cancer Res* (2013) 15:210. doi: 10.1186/bcr3436
11. Levina V, Su Y, Nolen B, Liu X, Gordin Y, Lee M, et al. Chemotherapeutic Drugs and Human Tumor Cells Cytokine Network. *Int J Cancer* (2008) 123:2031–40. doi: 10.1002/ijc.23732
12. Battula VL, Shi Y, Evans KW, Wang RY, Spaeth EL, Jacamo RO, et al. Ganglioside GD2 Identifies Breast Cancer Stem Cells and Promotes Tumorigenesis. *J Clin Invest* (2012) 122:2066–78. doi: 10.1172/JCI59735
13. Liang YJ, Ding Y, Levery SB, Lobaton M, Handa K, Hakomori SI. Differential Expression Profiles of Glycosphingolipids in Human Breast Cancer Stem Cells vs. Cancer Non-Stem Cells. *Proc Natl Acad Sci USA* (2013) 110:4968–73. doi: 10.1073/pnas.1302825110
14. Li Y, Li A, Glas M, Lal B, Ying M, Sang Y, et al. C-Met Signaling Induces a Reprogramming Network and Supports the Glioblastoma Stem-Like Phenotype. *Proc Natl Acad Sci USA* (2011) 108:9951–6. doi: 10.1073/pnas.1016912108
15. Sjoberg ER, Manzi AE, Khoo KH, Dell A, Varki A. Structural and Immunological Characterization of O-Acetylated GD2. Evidence That GD2 Is an Acceptor for Ganglioside O-Acetyltransferase in Human Melanoma Cells. *J Biol Chem* (1992) 267:16200–11. doi: 10.1016/S0021-9258(18)41986-2
16. Alvarez-Rueda N, Desselle A, Cochonneau D, Chaumette T, Clemenceau B, Leprieur S, et al. A Monoclonal Antibody to O-Acetyl-GD2 Ganglioside and Not to GD2 Shows Potent Anti-Tumor Activity Without Peripheral Nervous System Cross-Reactivity. *PloS One* (2011) 6:e25220. doi: 10.1371/journal.pone.0025220
17. Malisan F, Franchi L, Tomassini B, Ventura N, Condo I, Rippo MR, et al. Acetylation Suppresses the Proapoptotic Activity of GD3 Ganglioside. *J Exp Med* (2002) 196:1535–41. doi: 10.1084/jem.20020960
18. Mukherjee K, Chava AK, Mandal C, Dey SN, Kniep B, Chandra S, et al. O-Acetylation of GD3 Prevents Its Apoptotic Effect and Promotes Survival of Lymphoblasts in Childhood Acute Lymphoblastic Leukaemia. *J Cell Biochem* (2008) 105:724–34. doi: 10.1002/jcb.21867
19. Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and Deacetylation of Non-Histone Proteins. *Gene* (2005) 363:15–23. doi: 10.1016/j.gene.2005.09.010
20. Cavdarli S, Dewald JH, Yamakawa N, Guerardel Y, Terme M, Le Doussal JM, et al. Identification of 9-O-Acetyl-N-Acetylneuraminic Acid (Neu5,9Ac2) as

Main O-Acetylated Sialic Acid Species of GD2 in Breast Cancer Cells. *Glycoconjugate J* (2019) 36:79–90. doi: 10.1007/s10719-018-09856-w
21. Williams SA, Anderson WC, Santaguida MT, Dylla SJ. Patient-Derived Xenografts, the Cancer Stem Cell Paradigm, and Cancer Pathobiology in the 21st Century. *Lab Invest J Tech Methods Pathol* (2013) 93:970–82. doi: 10.1038/labinvest.2013.92
22. Fu CH, Lin RJ, Yu J, Chang WW, Liao GS, Chang WY, et al. A Novel Oncogenic Role of Inositol Phosphatase SHIP2 in ER-Negative Breast Cancer Stem Cells: Involvement of JNK/vimentin Activation. *Stem Cells* (2014) 32:2048–60. doi: 10.1002/stem.1735
23. Chan YT, Lai AC, Lin RJ, Wang YH, Wang YT, Chang WW, et al. GPER-Induced Signaling Is Essential for the Survival of Breast Cancer Stem Cells. *Int J Cancer* (2020) 146:1674–85. doi: 10.1002/ijc.32588
24. Chang WW, Lee CH, Lee P, Lin J, Hsu CW, Hung JT, et al. Expression of Globo H and SSEA3 in Breast Cancer Stem Cells and the Involvement of Fucosyl Transferases 1 and 2 in Globo H Synthesis. *Proc Natl Acad Sci USA* (2008) 105:11667–72. doi: 10.1073/pnas.0804979105
25. Chang WW, Lin RJ, Yu J, Chang WY, Fu CH, Lai A, et al. The Expression and Significance of Insulin-Like Growth Factor-1 Receptor and Its Pathway on Breast Cancer Stem/Progenitors. *Breast Cancer Res* (2013) 15:R39. doi: 10.1186/bcr3423
26. Batlle E, Clevers H. Cancer Stem Cells Revisited. *Nat Med* (2017) 23:1124–34. doi: 10.1038/nm.4409
27. Cochonneau D, Terme M, Michaud A, Dorvillius M, Gautier N, Frikeche J, et al. Cell Cycle Arrest and Apoptosis Induced by O-Acetyl-GD2-Specific Monoclonal Antibody 8B6 Inhibits Tumor Growth *In Vitro* and *In Vivo*. *Cancer Lett* (2013) 333:194–204. doi: 10.1016/j.canlet.2013.01.032
28. Liang YJ, Wang CY, Wang IA, Chen YW, Li LT, Lin CY, et al. Interaction of Glycosphingolipids GD3 and GD2 With Growth Factor Receptors Maintains Breast Cancer Stem Cell Phenotype. *Oncotarget* (2017) 8:47454–73. doi: 10.18632/oncotarget.17665
29. Ho MY, Yu AL, Yu J. Glycosphingolipid Dynamics in Human Embryonic Stem Cell and Cancer: Their Characterization and Biomedical Implications. *Glycoconjugate J* (2017) 34:765–77. doi: 10.1007/s10719-016-9715-x
30. Ly S, Anand V, El-Dana F, Nguyen K, Cai Y, Cai S, et al. Anti-GD2 Antibody Dinutuximab Inhibits Triple-Negative Breast Tumor Growth by Targeting GD2(+) Breast Cancer Stem-Like Cells. *J Immunother Cancer* (2021) 9: e001197. doi: 10.1136/jitc-2020-001197
31. Cazet A, Bobowski M, Rombouts Y, Lefebvre J, Steenackers A, Popa I, et al. The Ganglioside G(D2) Induces the Constitutive Activation of C-Met in MDA-MB-231 Breast Cancer Cells Expressing the G(D3) Synthase. *Glycobiology* (2012) 22:806–16. doi: 10.1093/glycob/cws049
32. Plaks V, Kong N, Werb Z. The Cancer Stem Cell Niche: How Essential Is the Niche in Regulating Stemness of Tumor Cells? *Cell Stem Cell* (2015) 16:225–38. doi: 10.1016/j.stem.2015.02.015
33. Chen W, Dong J, Haiech J, Kilhoffer MC, Zeniou M. Cancer Stem Cell Quiescence and Plasticity as Major Challenges in Cancer Therapy. *Stem Cells Int* (2016) 2016:1740936. doi: 10.1155/2016/1740936
34. Srivastava M, Raghavan SC. DNA Double-Strand Break Repair Inhibitors as Cancer Therapeutics. *Chem Biol* (2015) 22:17–29. doi: 10.1016/j.chembiol.2014.11.013
35. Schiewer MJ, Knudsen KE. Transcriptional Roles of PARP1 in Cancer. *Mol Cancer Res* (2014) 12:1069–80. doi: 10.1158/1541-7786.MCR-13-0672
36. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma Stem Cells Promote Radioresistance by Preferential Activation of the DNA Damage Response. *Nature* (2006) 444:756–60. doi: 10.1038/nature05236
37. Singh A, Settleman J. EMT, Cancer Stem Cells and Drug Resistance: An Emerging Axis of Evil in the War on Cancer. *Oncogene* (2010) 29:4741–51. doi: 10.1038/onc.2010.215
38. Liu J, Wang L, Zhao F, Tseng S, Narayanan C, Shura L, et al. Pre-Clinical Development of a Humanized Anti-CD47 Antibody With Anti-Cancer Therapeutic Potential. *PloS One* (2015) 10:e0137345. doi: 10.1371/journal.pone.0137345
39. Czuczman MS. CHOP Plus Rituximab Chemoimmunotherapy of Indolent B-Cell Lymphoma. *Semin Oncol* (1999) 26:88–96.
40. Mody R, Yu AL, Naranjo A, Zhang FF, London WB, Shulkin BL, et al. Irinotecan, Temozolomide, and Dinutuximab With GM-CSF in Children With Refractory or Relapsed Neuroblastoma: A Report From the Children's Oncology Group. *J Clin Oncol Off J Am Soc Clin Oncol* (2020) 38:2160–9. doi: 10.1200/JCO.20.00203
41. Fleurence J, Bahri M, Fougeray S, Faraj S, Vermeulen S, Pinault E, et al. Impairing Temozolomide Resistance Driven by Glioma Stem-Like Cells With Adjuvant Immunotherapy Targeting O-Acetyl GD2 Ganglioside. *Int J Cancer* (2020) 146:424–38. doi: 10.1002/ijc.32533
42. Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, et al. Anti-GD2 Antibody With GM-CSF, Interleukin-2, and Isotretinoin for Neuroblastoma. *N Engl J Med* (2010) 363:1324–34. doi: 10.1056/NEJMoa0911123
43. Lode HN, Valteau-Couanet D, Gray J, Luksch R, Wieczorek A, Castel V, et al. Randomized Use of Anti-GD2 Antibody Dinutuximab Beta (DB) Long-Term Infusion With and Without Subcutaneous Interleukin-2 (scIL-2) in High-Risk Neuroblastoma Patients With Relapsed and Refractory Disease: Results From the SIOPEN LTI-Trial. *J Clin Oncol* (2019) 37:10014–4. doi: 10.1200/JCO.2019.37.15_suppl.10014
44. Terme M, Dorvillius M, Cochonneau D, Chaumette T, Xiao W, Diccianni MB, et al. Chimeric Antibody C.8B6 to O-Acetyl-GD2 Mediates the Same Efficient Anti-Neuroblastoma Effects as Therapeutic Ch14.18 Antibody to GD2 Without Antibody Induced Allodynia. *PloS One* (2014) 9:e87210. doi: 10.1371/journal.pone.0087210
45. Siebert N, Zumpe M, Juttner M, Troschke-Meurer S, Lode HN. PD-1 Blockade Augments Anti-Neuroblastoma Immune Response Induced by Anti-GD2 Antibody Ch14.18/CHO. *Oncoimmunology* (2017) 6:e1343775. doi: 10.1080/2162402X.2017.1343775
46. Thomas R, Al-Khadairi G, Decock J. Immune Checkpoint Inhibitors in Triple Negative Breast Cancer Treatment: Promising Future Prospects. *Front Oncol* (2020) 10:600573. doi: 10.3389/fonc.2020.600573
47. Fu C-H, Lin R-J, Yu J, Chang W-W, Liao S-S, Chang W-Y, et al. A Novel Oncogenic Role of Inositol Phosphatase SHIP2 in ER-Negative Breast Cancer Stem Cells: Involvement of JNK/Vimentin Activation. *Stem Cells* (2014) 32 (8):2048–60. doi: 10.1002/stem.1735
48. Hu Y, Smyth GK. ELDA: Extreme Limiting Dilution Analysis for Comparing Depleted and Enriched Populations in Stem Cell and Other Assays. *J Immunol Methods* (2009) 347:70–8. doi: 10.1016/j.jim.2009.06.008

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 14 March 2022
doi: 10.3389/fimmu.2022.837262

IL25 Enhanced Colitis-Associated Tumorigenesis in Mice by Upregulating Transcription Factor GLI1

Junxi Liu[1], Bingxiu Qian[2], Lin Zhou[1], Gang Shen[3], Yandan Tan[1], Siqi Liu[2], Zewei Zhao[1,2], Jianglin Shi[2], Weiwei Qi[1], Ti Zhou[1], Xia Yang[1], Guoquan Gao[1*] and Zhonghan Yang[1,2*]*

[1] Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, China, [2] Department of Biochemistry, Molecular Cancer Research Center, School of Medicine, Sun Yat-sen University, Shenzhen, China, [3] Department of Laboratory Medicine, Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

OPEN ACCESS

Edited by:
Cristina Maccalli, Sidra Medicine, Qatar

Reviewed by:
Kun Yang, East Tennessee State University, United States
Michal Kuczma, Georgia State University, United States

***Correspondence:**
Zhonghan Yang
yangzhh@mail.sysu.edu.cn
Guoquan Gao
gaogq@mail.sysu.edu.cn
Xia Yang
yangxia@mail.sysu.edu.cn

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *16 December 2021*
Accepted: *09 February 2022*
Published: *14 March 2022*

Citation:
Liu J, Qian B, Zhou L, Shen G, Tan Y, Liu S, Zhao Z, Shi J, Qi W, Zhou T, Yang X, Gao G and Yang Z (2022) IL25 Enhanced Colitis-Associated Tumorigenesis in Mice by Upregulating Transcription Factor GLI1. Front. Immunol. 13:837262. doi: 10.3389/fimmu.2022.837262

Interleukin-25 (IL17E/IL25) plays a critical role in colitis and intestinal homeostasis. However, the expression and biological role of IL25 in colorectal cancer is not properly understood. In this study, we show that IL25 is mainly expressed by cancer stem cells in the colorectal cancer microenvironment. Genetic deletion of IL25 inhibited tumor formation and growth and prolonged survival in AOM/DSS-treated mice. IL25 stimulated cancer organoid and cancer cells sphere formation and prevented the tumor from chemotherapy-induced apoptosis. Mechanistically, IL25 upregulated stem cell genes LGR5, CD133, and ABC transporters *via* activating the Hedgehog signaling pathway. IL25 inhibited phosphorylation of AMPK and promoted GLI1 accumulation to maintain cancer stem cells. Moreover, IL25 expression was associated with poor survival in patients with metastatic colorectal cancer. Taken together, our work reveals an immune-associated mechanism that intrinsically confers cancer cell stemness properties. Our results first demonstrated that IL25, as a new potent endogenous Hedgehog pathway agonist, could be an important prognostic factor and therapeutic target for CRC.

Keywords: IL25, colorectal cancer, AOM/DSS model, cancer stem cell, GLI1

INTRODUCTION

Colorectal cancer (CRC) is the third most occurring malignancy and the third most common cause of cancer death worldwide (1). As the third-generation platinum drug, oxaliplatin is the first-line treatment for patients with metastatic colorectal cancer (mCRC) (2). Unfortunately, the 5-year survival rates for patients with metastasis are approximately 14% (3, 4). One of the major reasons for treatment failure and poor prognosis is drug resistance (5). Therefore, it is essential for us to clarify the mechanism of chemotherapy resistance and to develop approaches to prevent or reverse drug resistance for patients with mCRC.

Cancer stem cells (CSCs) play a major role in tumor growth, progression and can resist chemotherapeutic agents by increasing drug efflux ABC transporters and activating DNA repair

machinery (6). It was noted that platinum drugs could be the substrates for selected ABC transporters ABCC2 and ABCC5 (7). Additionally, colorectal cancer cells could upregulate ABC transporters, such as ABCC2 to promote oxaliplatin resistance by activating the stem cell Hedgehog–GLI1 signal pathway (8). Furthermore, upregulation of CD44 and Lgr5 in CRC cells led to increased CSCs resistance to oxaliplatin and 5-FU (9). Besides, the decline of CSC markers, CD44, LGR5, and CD133 in the CRC organoids was more sensitive to oxaliplatin and 5-FU (10). Recently, it was suggested that differentiated cancer cells or progenitor cells can revert to CSCs through the cancer cell niche signals like WNT and EGF (11). However, it remains uncertain that the potent molecular lets the CRC cells differentiate into colorectal CSCs and gain the ability of chemotherapy resistance. Therefore, targeting some cancer niche secrete signals might be an effective strategy to eliminate colorectal CSCs and improve CRC patient prognosis.

Interleukin-25 (IL25), also known as IL-17E, is a member of IL-17 cytokine family, which includes IL-17A to IL-17F (12). It was found that IL25 was upregulated in DSS-induced colitis and played a significant role in intestinal parasitic infection and type 2 immunity (13–16). Furthermore, as the receptor of IL25, IL-17RB was found to be a marker of colorectal CSCs (17). In our previous study, we found that IL25 could promote liver cancer metastasis by inducing macrophages to secrete CXCL-10 (18). Besides, IL25 also promoted breast cancer liver metastasis by inducing macrophage M2 polarization (19). Conversely, Saori et al. reported that a high level of IL25 promoted IL-17RB$^+$ breast cancer apoptosis (20). These studies imply that IL25 is closely related to cancer development, but the exact role of IL25 in colorectal cancer is unclear and controversial. Last but not least, outside of colitis, much less is known about the roles of IL25 in CRC.

Given that there is a strong correlation between colitis and CRC, we hypothesized that IL25 was continuously upregulated in CRC and promoted cancer development. In this study, we aimed to identify the effects of IL25 on decreasing colorectal cancer sensitivity to oxaliplatin by maintaining colorectal cancer stemness and the underlying mechanism.

MATERIALS AND METHODS

Human Samples

A total of 49 cases of CRC tissue samples with survival information were collected from the Sun Yat-sen University Cancer Center. Informed consent of all patients has been obtained before surgery, and the use of medical records and histological sections has also been approved by the ethics committee in SYUCC. CRC tissue microarray (HColA150CS02, 74 cases) was purchased from the Shanghai Outdo Biotech (Shanghai, China). All procedures were performed under consensus agreements and following the Chinese Ethical Review Committee.

Animals and Models for AOM-DSS-Induced CRC

Wild-type C57BL/6J mice were acquired from the center of laboratory animal of Sun Yat-sen University. The IL25 gene knockout (IL25KO) mice with C57BL/6J genetic background were acquired from the model animal research center of Nanjing University. All mice were maintained under 12 h light-dark cycles with a designed environmental temperature (21°C ± 1°C). All animal studies were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University (approval number: SCXK2019-0209) meted with the China guideline of GB/T 35892-2018. This study was conducted following the ethical principles derived from the Declaration of Helsinki and the Belmont Report and was approved by the review board of Sun Yat-sen University (Guangzhou, China). Colorectal cancer (CRC) was induced by intraperitoneal injection of AOM (10 mg/kg; Sigma, A5486) combined with the Dextran sulfate sodium salt (DSS; MP, 160110) stimulus, resulting in tumor development restricted to the colon in mice as previously described (21). After injection of AOM on day 0, mice were given three rounds of a 2% DSS solution in their drinking water for 7 days starting on days 7, 28, and 49. Weight change during the experiment was calculated as the percent change in weight compared with the baseline measurement. The weight of the mice was monitored weekly. Mice were intraperitoneally injected with vehicle (5% glucose solution) or oxaliplatin (5 mg/kg once a week; Selleck, S1224) for two weeks.

Cell Culture

The human CRC cell lines (SW48, CaCO2, LoVo, SW620, HT-29) and normal colonic epithelial cell lines CCD 841 were obtained from the American Type Culture Collection. Cell lines were authenticated by Cellcook Biotech. All cells were cultured and grown in DMEM supplemented with 10% FBS. After starvation for 6 h, CRC cells were treated with recombinant IL25 (R&D Systems; 8134-IL-025) in a dose-dependent manner. The GLI1 inhibitor, GANT-58 (MCE; HY-13282), the SMO inhibitor, Vismodegib (MCE; HY-10440), and the AMPK activator, Metformin (Sigma Aldrich, 1115-70-4) were added in serum-free DMEM medium for 24 h. For RNAi experiments, CRC cells were transfected with HiPerFect reagent (QIAGEN, #301705) using siRNA molecules (Generay, Shanghai, China).

Cell Viability Assay

The viability of CRC cells was determined by Cell Counting Kit-8 (CCK-8) assay (Dojindo, CK04), following the instructions of the manufacturer. Briefly, 5,000–8,000 CRC cells per well were seeded in 96-well plates overnight. After starvation for 6 h, CRC cells were treated with or without IL25 for 36 h. Oxaliplatin (Selleck, S1224) was added in a dose-dependent manner for 48 h. Cell viability was measured by adding 10 μl of CCK-8 to each well. After 2 h of incubation at 37°C in a humidified incubator containing 5% CO_2, the OD value was determined by absorbance at 450 nm using the Sunrise microplate reader (TECAN, Mäannedorf, Switzerland).

Histology and Immunohistochemistry

Colorectal cancer tissue was fixed in 4% paraformaldehyde. Tissues were embedded in paraffin and sectioned by microtome. The slides were stained with hematoxylin and

eosin (H&E) and immunohistochemical staining was conducted following standard protocol. Briefly, the sections were deparaffinized, rehydrated in a gradient of ethanol, and pretreated with 0.01 M citrate buffer (pH 6.0) through the high-pressure method. Then the sections were immersed in 3% H_2O_2 for 30 min to quench endogenous peroxidase. For IL25 immunohistochemistry, slides of various tissues were blocked with goat serum for 1 h. Subsequently, the slides were incubated with the following primary antibodies: IL25 (1:200; Novus Biologicals, NB100-56541) and LGR5 (1:100; Abcam, ab75732) antibody overnight at 4°C following incubation with HRP-conjugated secondary antibody for 1 h at room temperature and then stained with the DAB Horseradish Peroxidase Color Development Kit. Hematoxylin was used as counterstain. Sections were photographed through a slide scanner (Axio Scan. Z1, ZEISS). The degree of IL25 immunostaining was determined by the staining index (SI) which was reported elsewhere (22). The SI was calculated as the product of the grade of tumor cell proportions and the staining intensity score.

Immunofluorescence

For immunofluorescence staining, slides were incubated with the following primary antibodies: GLI1 (1:100; Santa Cruz sc-515781), CD133(1:100; eBioscience 14-1331-82), LGR5 (1:100; Abcam, ab75732), DCAMKL1 (1:200; Abcam, ab31704), IL25 (1:200; Novus Biologicals, NB100-56541) overnight at 4°C, followed by staining with a mixture of secondary antibodies containing an Alex Flour 488-Donkey anti-rat IgG (H+L) (1:200; A21208) and an Alex Flour 594-Donkey anti-rabbit IgG (1:200; R37119) for 1 h at 37°C temperature. The cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. The slides were observed with a confocal laser scanning microscope.

Western Bolting

Tissues and cells were lysed in SDS buffer supplemented with 1 mM phenylmethanesulfonyl fluoride (Beyotime, ST506). The protein concentration was determined by the BCA protein assay kit (KeyGen, KGP902) and total cellular protein (30 ug) was subject to western blot analysis. The protein was transferred to 0.45 μm PVDF membrane (Millipore) and then the membranes were blocked with 7% of defatted milk in TBST (20 mM of Tris–HCl pH 7.4, 500 mM of NaCl, and 0.1% of Tween-20) for 1 h at room temperature. The membranes were incubated with the following primary antibodies: p-AMPKα (1:1,000; Thr172) (4188), AMPK (1:1,000; 2532), CD133 (1:1,000; 86781), BTRC (1:1,000; 4394) from Cell Signaling Technology; GLI1 (1:500; sc-515781), Smo (1:500; sc-166685), MRP2 (1:500; sc-59611), MRP5 (1:500; sc-376965), PTCH1 (1:500; sc-518102), IL-17E (1:500; sc-52933), SHH (1:500; sc-365112) CD133 (1:1,000; sc-365537) from SANTA CRUZ BIOTECHNOLOGY; LGR5 (1:1,000; ab75732), DCAMKL1 (1:1,000; ab31704) from Abcam; ALDH1A3 (1:1,000; Novus Biologicals, NBP2-15339), CD44 (1:1,000,15675-1-AP) and GAPDH (1:5,000,60004-1-Ig) from Proteintech Group. After incubation at 4°C overnight, membranes were probed with HRP-conjugated anti-rabbit IgG (Cell Signaling Tech, #7074) or anti-mouse IgG (Sigma-Aldrich, AP308P), then developed by ECL substrate (Merck Millipore) and visualized using the Bio-Rad ChemiDoc Touch Imaging System.

Real-Time PCR

Total RNA from tissue or cells was extracted with TRIZOL reagent (Invitrogen, #15596026). RNA concentration was measured by the spectrometer. Approximately 1,000 ng total RNA was reverse transcribed into cDNA by PrimeScript reverse transcription reagent (TaKaRa, RR036A) following the instructions of the manufacturer. Real-time PCR analysis using SYBR Green PCR Mix (TakaRa, RR420A) was performed on the CFX96 PCR system (BioRad). ACTB was used as an internal normalization control. The normalized fold change of gene mRNA levels was calculated using the $2^{-\Delta\Delta Ct}$. The PCR primer sequences are listed in **Table S2**.

Measurement of Free Fatty Acids and Cholesterol

Cholesterol (CHO) and free fatty acids (FFA) in plasma were measured by the TG assay kit (A111-1-1) and FFA assay kit (EFFA-100). All measurements were performed with standard manufacture protocol.

Sphere Formation Assay

CRC cells were plated in 96-well ultralow attachment plates (Corning) in DMEM/F12 serum-free medium supplemented with 2% B27 (Thermo Scientific, 12587010), 20 ng/ml epidermal growth factor (EGF, Beyotime, P5552), 20 ng/ml basic fibroblast growth factor (bFGF, Beyotime, P6443) at a density of 1,000 viable cells/well. CRC cells were treated with recombinant IL25 (R&D Systems; 8134-IL-025). The GLI1 inhibitor, GANT-58 (MCE; HY-13282), the SMO inhibitor, Vismodegib (MCE; HY-10440), and the AMPK activator, Metformin (Sigma Aldrich, 1115-70-4) were added in sphere culture for 1 week. Tumor spheres (tight, spherical, nonadherent masses >50 μm in diameter) were counted, and their images were captured under an inverted microscope (Leica DMI4000B).

Cancer Organoids Isolation

Intestinal fragments containing adenomas from WT or IL25KO AOM/DSS induced tumor or human colon tumor tissues were washed with PBS several times and incubated in Gentle cell dissociation reagent (STEMCELL, 07174) for 60 min on 37°C. intestinal adenomas were seeded in 24-well plates (500 crypts/fragments per 50 μl of Matrigel per well). The Matrigel was polymerized for 10 min at 37°C, and 500 μl/well basal culture medium (advanced Dulbecco's modified Eagle medium/F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, Glutamax, 1 × N2, 1 × B27 [all from Invitrogen], and 1 mmol/L N-acetylcysteine [Sigma]) (23).

Ubiquitin Conjugated Assay

Colorectal cancer cells were treated with 10 μM MG132 (Merck, Germany) for the indicated treatment and times. The cells were washed with cold PBS and lysed in the radioimmunoprecipitation assay (RIPA) buffer (Beyotime, P0013D) with 1 mM PMSF

(Beyotime, ST506), 1× phosphatase inhibitor (MCE, HY-K0021), and 1× cocktail (MCE, HY-K0010)) at 4°C overnight. After determining the total protein concentration, aliquots of equal amounts of protein were incubated with GLI1 antibody (1:100; sc-515781) overnight at 4°C. Next, Protein A/G PLUS-Agarose (SANTA CRUZ, sc-2003) were added and incubated for 4 h at 4°C. The beads were then centrifuged and washed with pre-cool basic RIPA buffer. After releasing with 2× SDS buffer, the precipitated proteins were subjected to western blot analysis with total cell lysates.

Protein Half-Life Determination

CRC cells were pretreated with or without 50 ng/ml IL25 overnight and then incubated with cycloheximide (50 μg/ml; CHX, Sigma) for the indicated time and were analyzed by western blot analysis. The intensity of the bands was quantified using Image J software.

Statistical Analysis

All data are presented as mean ± *SEMs*. The Student's t-test was used to compare between two groups. One-way analysis of variance (ANOVA) or Two-way ANOVA was applied to compare more than two different groups. The relationships between IL25 expression and clinicopathological characteristics were determined using the chi-square test. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test on GraphPad Prism 8.0 software. For each parameter of all data presented, NS (No Significance), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $p < 0.05$ is considered significant.

RESULTS

Elevated IL25 is Associated With Tumor Progression in CRC

To explore the critical role of IL25 in CRC prognosis, we analyzed the GEO dataset GSE17258. Although their difference was not statistically significant ($p = 0.1174$), it was shown that the OS of the IL25-high expression group was even 29.1 months shorter than that of the low expression group in Stage III & IV patients (**Figures S1A, B**). To further verify the crucial role of IL25 in the progression of CRC, we analyzed the protein level of IL25 by CRC Tissue Microarray. Impressively, compared with adjacent specimens (IHC-Score = 1.810), the expression of IL25 was remarkably elevated in CRC specimens (IHC-Score = 4.608; **Figure 1A**). Similar to the results in CRC tissues, IL25 expression was augmented in CRC cells compared with normal colon epithelial cell lines (**Figure 1B**). Meanwhile, we retrospectively studied the medical records of 123 CRC patients and identified that IL25 expression increased along with the progression of CRC clinical stages (**Figure 1C**). The 5-year OS rate of the IL25-high group (n = 15; median = 19.76) was significantly lower than that of the IL25-low group in Stage IV patients (n = 34; median = 38.65; $p = 0.0133$) (**Figure 1D**). Taken together, the upregulation of IL25 was closely relevant with progression and poor prognosis of CRC. To model colitis-associated colon cancer, we established wild-type female C57BL/6J (WT) AOM/DSS-induced mouse CRC models. Impressively, compared with control and adjacent colon specimens (IHC-Score = 1.33 and 1.50), the expression of IL25 had a rising trend in WT 10-week tumor (IHC-Score = 2.6) and was remarkably elevated in WT 16-week tumor (IHC-Score = 6.5) (**Figure 1E**).

IL25 Promoted the Progression of Colitis-Associated Cancer (CAC) *In Vivo*

To further verify the decisive role of IL25 in the progression of CRC, we also treated IL25KO mice with AOM/DSS to induce CRC. During the challenge, female IL25KO mice exhibited less weight loss and a higher survival rate than WT controls (**Figures 2A, B**). During AOM and DSS challenge, genetic deletion of IL25 had retarded the development of colitis-associated cancer (**Figures 2C–E**). While total tumor numbers were no obvious change, the tumor size of IL25KO mice was smaller than WT in 10-week (**Figure 2D**). We further examined the efficacy of oxaliplatin in WT and IL25KO AOM/DSS-induced CRC models (**Figure 2F**). Surprisingly, IL25 deletion significantly decreased tumor numbers and size in 16-week and the tumors of IL25KO mice were further attenuated by oxaliplatin, while there was only a decreased trend in tumors of WT mice whose diameters were smaller than 2 mm (**Figures 2F–H**). Furthermore, enhanced apoptosis showed by Tunel staining was observed in tumors of IL25KO mice (**Figure 2J**). Through GSEA gene enrichment analysis, we found that ABCC2 and ABCC5 were significantly increased in IL25-high CRC and had a positive correlation with IL25 (**Figures S1C, D**). By western blotting, we found that IL25 associated with ABCC2 and ABCC5 were increased after oxaliplatin treatment (**Figure S2C**). *In vitro*, HT-29 cells treated with IL25 were less sensitive to the oxaliplatin treatment (**Figure S2D**). To further validate whether IL25 regulates ABC transporters, real-time PCR was utilized to detect the change of ABC transporters which play essential roles in drug resistance. Impressively, we found that ABCC2 and ABCC5 mRNA and protein levels were significantly upregulated by IL25 (**Figures S2E, F**). Whereas, ABCC2 and ABCC5 were downregulated in IL25 silenced LoVo cells (**Figures S2G, H**). Moreover, IL25 silenced LoVo cells were more sensitive to the oxaliplatin treatment than the cells transfected with NC control siRNA (**Figure S2I**). Together, these data suggested that IL25 decreased the sensitivity of oxaliplatin in CRC by upregulating ABC transporters.

IL25 Maintained Colorectal Cancer Stemness

To further clarify the role of IL25 in CRC development, we performed Ki67 immunohistochemical staining in WT and IL25KO tumors slices. Since the proliferation, apoptosis, and colon length had no difference between WT and IL25KO (**Figures 2D, I, J** and **Figure S2A**), we found IL25 and DCLK1 were upregulated in oxaliplatin-treated tumors (**Figure S2C**). We also found $DCLK1^+$ or $CD133^+$ cells could secrete IL25 (**Figures S3E, F**). This finding indicated that IL25 may be involved in CSCs maintaining. Therefore, we analyzed the

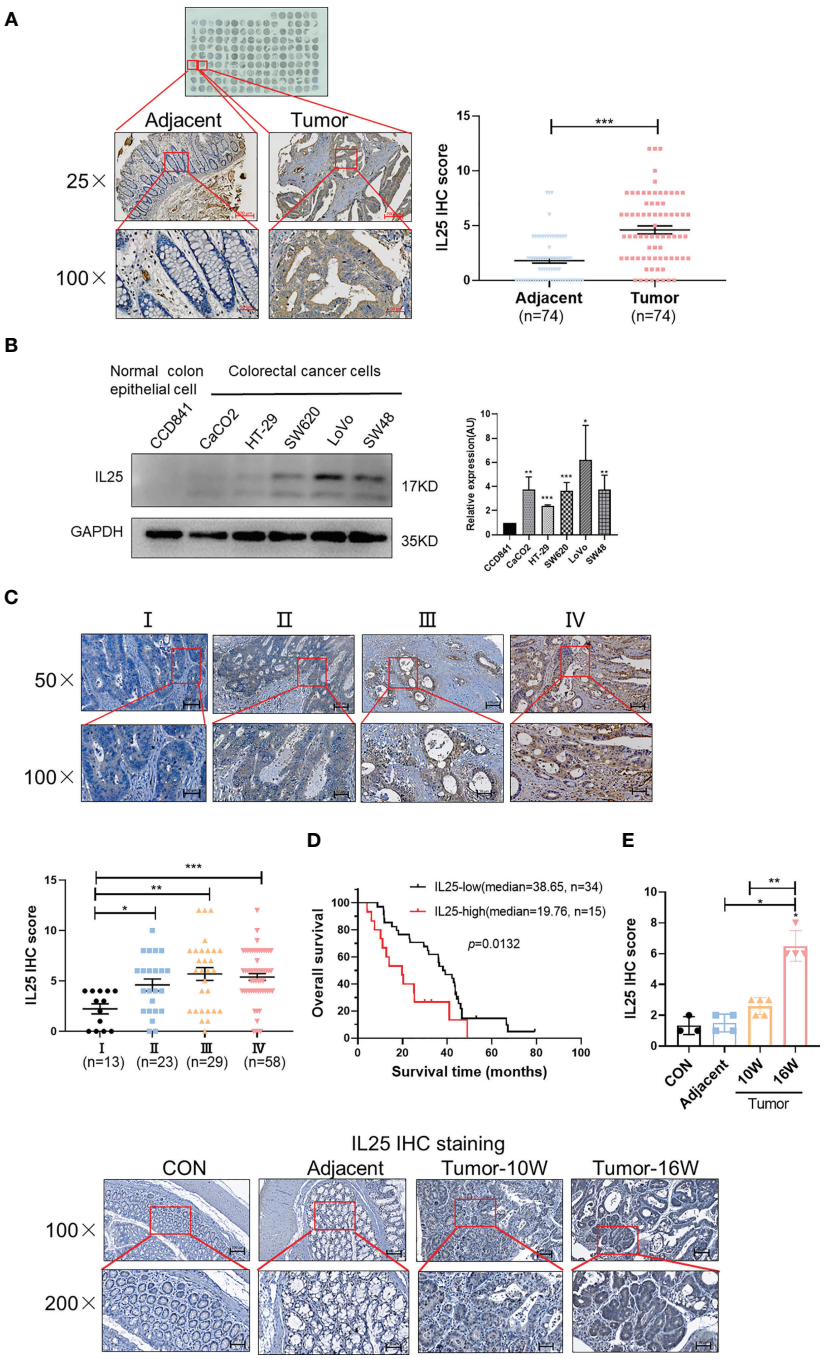


FIGURE 1 | Overexpression of IL25 was found in CRC patients and predicts a poor prognosis. **(A)** Immunohistochemistry (IHC) staining of IL25 was performed in a tissue microarray consisting of 74 CRC tumor tissues and adjacent colon tissues (left). Statistical analysis of IL25 staining in adjacent specimens and CRC specimens (right). **(B)** Protein levels of IL25 were detected by Western blotting in normal intestinal cells (CCD841) and CRC cell lines (left). The right panel showed the quantitative analysis of the gray scan. The ImageJ software was used for gray scanning. **(C)** Representative images of IL25 IHC staining at different clinical stages (up). Correlation between IL25 expression and various clinical stages (down). **(D)** Overall survival curves of 49 CRC patients in correlation with intra-tumor IL25 IHC-scores. High IL25 expression was considered IHC-Score >6. The patients with CRC were divided into 2 groups according to the intra-tumor IL25 IHC-score: low group (n = 34), high group (n = 15). **(E)** Representative images of IL25 IHC staining from WT colon and AOM/DSS induced tumors on weeks 10 and 16 (down). Statistical analysis of IL25 staining in con colon, adjacent tissues, and AOM/DSS-induced CRC tissues (up). Data present as mean ± *SEM*; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

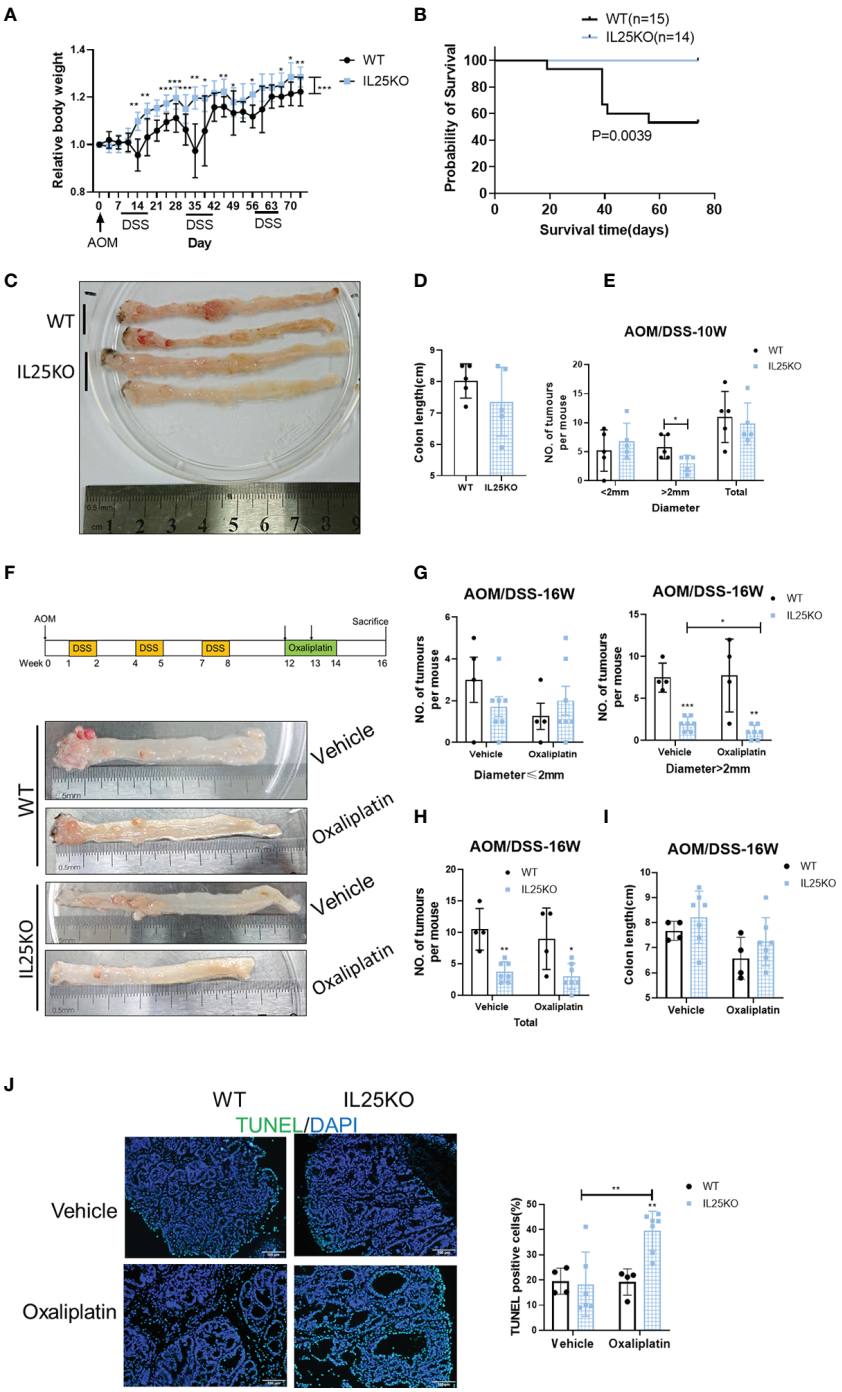


FIGURE 2 | Genetic deletion of IL25 inhibited the progression of the Colitis-Associated Cancer (CAC) Model. IL25KO or WT control mice were given an intraperitoneal injection of AOM on day 1, 2.5% DSS in drinking water for 7 days starting on days 7, 28, and 42, and euthanized on days 70 and 112. **(A)** Bodyweight change during colitis-associated colorectal cancer with AOM/DSS as a percentage of initial weight. **(B)** Overall survival curves of WT and IL25KO mice. **(C)** Representative images of colonic tumors from WT and IL25KO mice in 10 weeks. **(D)** Total number and size of tumors along the colon in WT (n = 5) and IL25KO (n = 5). **(E)** Colon length in mice treated with the indicated treatment in 10 weeks. **(F)** Effect of oxaliplatin on WT and IL25KO AOM-DSS-induced CRC mouse models. The colon was removed, cut lengthwise, washed with PBS, and digitally photographed. **(G)** Size of individual tumors along the colon in WT treated with vehicle (n = 4) or oxaliplatin (n = 4) and IL25KO treated with vehicle (n = 7) or oxaliplatin (n = 7). **(H)** Total number and size of tumors. **(I)** Colon length in mice treated with the indicated treatment on 16 weeks. **(J)** Representative immunofluorescent stains for TUNEL in colonic sections from WT and IL25KO treated with vehicle or oxaliplatin (left). Statistical analysis of Tunel staining in WT and IL25KO tumors (right). Data present as mean ± *SEM*; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

RNA sequence in colorectal cancer in the GEO dataset GSE17538. The gene signatures of cancer stem cells were positively correlated with IL25 expression (**Figure S1D**). In addition, LoVo spheres sorted by sphere formation also had higher IL25 expression than control LoVo cells (**Figure S3D**). Then we analyzed the mRNA levels of stemness-related markers in IL25 treated HT-29 and SW620 cells, which showed that expression of stemness-related markers, especially, CD133, LGR5, and OCT-4 were elevated in IL25 treated CRC cells (**Figure 3A** and **Figure S3G**). Meanwhile, western blotting results revealed that CD133 and LGR5 were upregulated in IL25 treated CRC cells in a dose and time-dependent manner (**Figures 3B, C**). To determine whether IL25 affects the frequency of colorectal CSCs, a cancer organoid model was established from freshly isolated primary tumors from colon cancer patients, which showed that IL25 increased the frequency of cancer organoid formation (**Figure 3D**). Then, the sphere formation assays were carried out to inspect the influence of IL25 on the self-renewal capability of CRC cells. After a 7-day culture, the numbers and sizes of spheres in the IL25 treated CRC cells were more remarkable than that of the control group (**Figure 3E**).

On the contrary, LGR5 and CD133 positive cells were decreased in IL25KO tumors (**Figures 4A, B**) and the expression of stemness-related markers, especially, LGR5, Myc, and Sox2 were downregulated in IL25KO tumors (**Figure 4C**). Meanwhile, we observed the stemness-related markers, CD44, ALDH1, DCLK1 were downregulated in IL25KO AOM/DSS induced tumors (**Figure 4D** and **Figure S3H**). *In vitro*, silencing IL25 reduced the stemness-related markers, CD133, LGR5, CD44, ALDH1, and DCLK1 (**Figure 4E** and **Figure S3I**). Moreover, deletion of IL25 decreased the frequency of cancer organoid formation, which was reversed by IL25 (**Figure 4F**). Notably, knockdown of IL25 in LoVo spheres substantially reduced the numbers and sizes of the formed spheres (**Figure 4G**). Collectively, these results indicated that IL25 maintained the stemness of CRC cells.

IL25 Mediates Stemness Through the Activation of the Hedgehog Signaling

To identify pathways that may regulate CRC stemness, gene set enrichment analysis (GSEA) was carried out, comparing the IL25-high group to IL25-low group from GEO dataset GSE17538 and GSE41258. Our analysis demonstrated the transcriptome of the IL25-high group to be enriched in gene sets associated with the Hedgehog signaling pathway (**Figure 5A** and **Figures S1E, F**). Meanwhile, mRNA transcription of the downstream targets of Hedgehog signaling, namely, GLI1, SMO, HHIP, WNT8A, were upregulated in IL25 treated CRC cells (**Figure 5B**). Meanwhile, GLI1 and WNT1 were downregulated in IL25KO tumors (**Figure 5C**). Next, IL25 dramatically increased the GLI1 nuclear signals showed by immunofluorescence assays whereas IL25 deletion reduced GLI1 nuclear translocation (**Figures 5D, E**). By western blotting, it was shown that GLI1, PTCH1, and SMO declined in IL25 knockdown LoVo cells (**Figure 5F** and **Figure S4A**). To further delineate the role of GLI1-dependent Hedgehog signaling in IL25 mediated stemness, CRC cells were treated with small molecule inhibitors of Hedgehog (the SMO inhibitor vismodegib and GLI1 inhibitor GANT-58). Inhibition of SMO and GLI1 blocked the increase of CD133 and LGR5 and spheres formation mediated by IL25 (**Figures 5G, H** and **Figure S4B**). These results indicated that IL25 enhanced the Hedgehog signaling by regulating GLI1.

IL25 Upregulates GLI1 Through Inhibiting p-AMPK

To investigate whether IL25 affects the protein stability of GLI1, we measured the half-life of GLI1 by using cycloheximide (CHX), which blocks protein synthesis *in vitro*. The GLI1 degradation was decelerated in the presence of IL25 with CHX, while GLI1 had a half-life of approximately 23 min (**Figure 6A**). To clarify whether IL25 increases GLI1 by inhibiting p-AMPK, we detected p-AMPK by Western blotting. IL25KO tumors showed higher p-AMPK levels than wild-type mice (**Figure 6B**). Moreover, it was shown that IL25 inhibited p-AMPK before GLI1 accumulation (**Figure 6C** and **Figure S4C**), which was reversed by adding AMPK activator A769662 and metformin (**Figures 6D, E** and **Figures S4D**, **S5E**). Besides, GLI1 nuclear signals increased by IL25 were also blocked by metformin (**Figure 6F**). Furthermore, MG132 treatment with IL25 significantly decreased GLI1 polyubiquitination levels which were reversed by metformin (**Figure 6G**). Inhibition of p-AMPK blocked increased spheres and organoid formation mediated by IL25 (**Figure 6H** and **Figure S4E**).

Additionally, we also found that IL25 increased N-SHH in a time-dependent manner (**Figure S5A**). Furthermore, IL25 upregulated HMGCR expression and cholesterol (**Figures S5B–D**). Similarly, IL25 promoted SHH-N through p-AMPK inhibition, which was reversed by activating p-AMPK (**Figure 6E**). IL25 increased SMO, GLI1, CD133, and SHH-N expression, which was reversed by silencing SHH receptor PTCH1 (**Figures S5E, F**). These results demonstrated that IL25 could activate the Hedgehog signaling pathway by inhibiting p-AMPK.

DISCUSSION

In this study, we identified that IL25 was strikingly elevated in the tissue of CRC patients and AOM/DSS-induced tumors, and high IL25 expression in CRC tissue was negatively correlated with survival rate. IL25 treated CRC cells substantially enhanced the expression levels of CD133 and LGR5, the formation of tumor organoid and sphere, thus decreasing the sensitivity to oxaliplatin of CRC cells. Consistently, silencing or deletion of IL25 *in vitro* and *in vivo*, decreased the formation of tumor organoid and sphere formation, thus enhancing the sensitivity to oxaliplatin of tumor. We first demonstrated that IL25 maintained CRC stemness through inhibiting p-AMPK and increased GLI1. This study provides evidence of a novel treatment strategy for CRC stemness by inhibition of IL25 centered pathway in CRC patients (**Figure 7**).

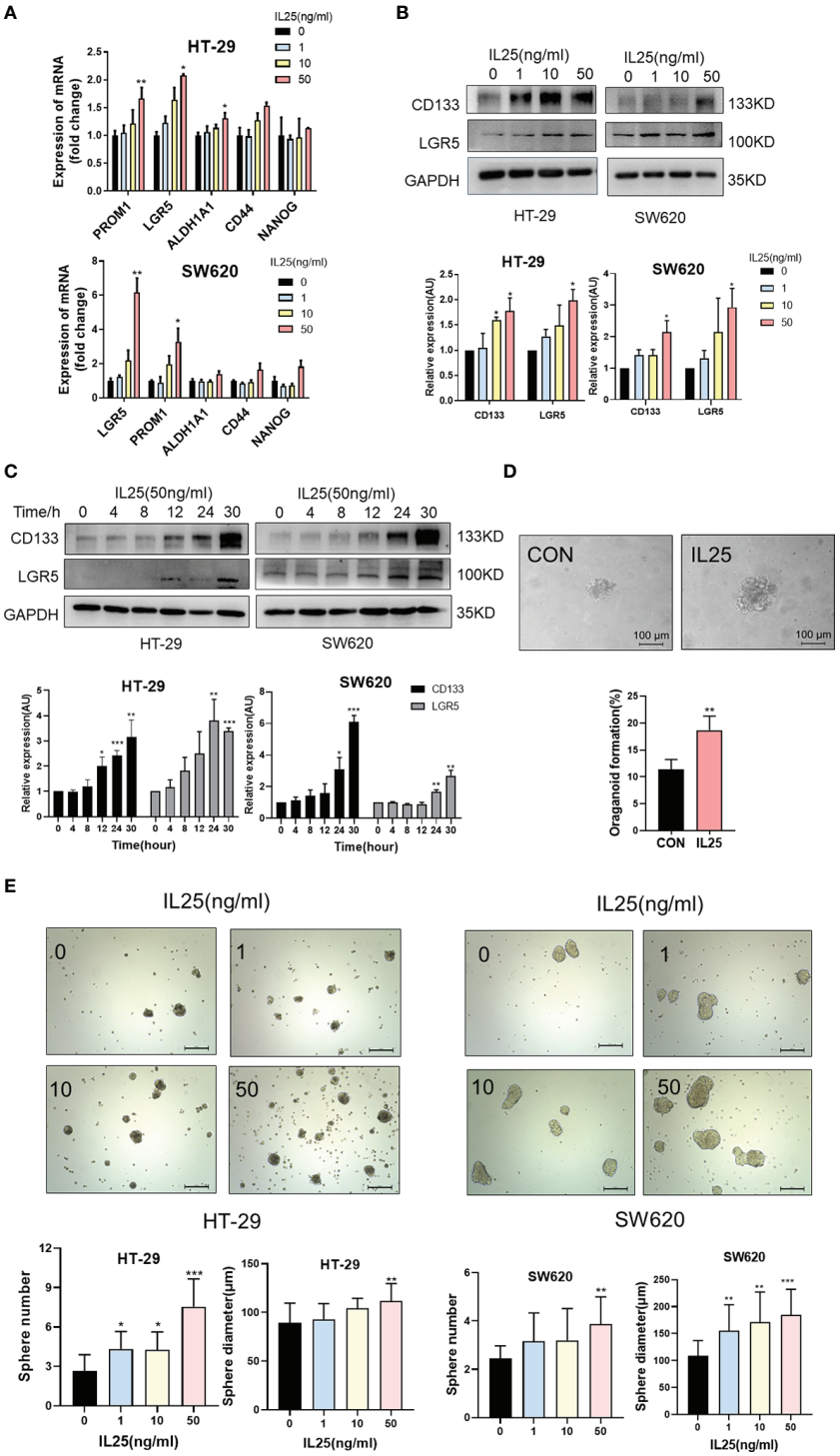


FIGURE 3 | IL25 promoted the stemness of CRC cells. **(A)** The expression levels of CSC markers, namely, CD133 and LGR5, were examined in HT-29 and SW620 cells treated with recombinant IL25 in a concentration-dependent manner by RT-PCR. **(B, C)** The expression levels of CD133 and LGR5 were examined in HT-29 and SW620 cells treated with recombinant IL25 in a concentration and time-dependent manner by Western blotting. **(D)** Frequency (down) and representative day-7 images (up) of human colon adenomatous organoids treated with/without IL25 (50 ng/ml). **(E)** Sphere formation analysis of HT-29 and SW620 cells treated with recombinant IL25 in a concentration-dependent manner. Representative images (up) and the mean numbers and sphere size (down) of spheres are shown. Scale bar, 200 μm. Data present as mean ± *SEM*; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

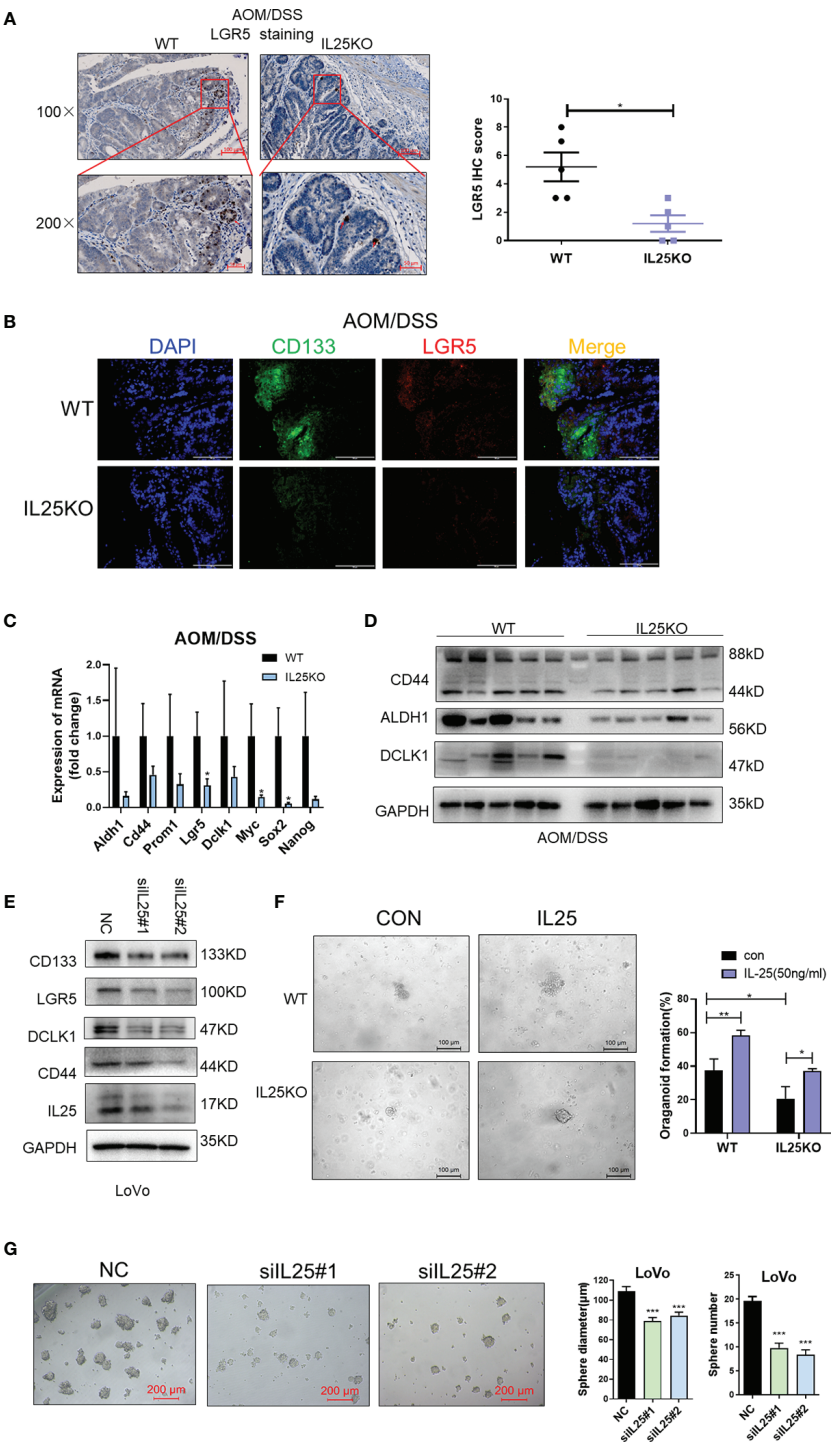


FIGURE 4 | IL25 Deficiency Induced loss of CRC stemness. **(A)** Representative images of LGR5 IHC staining (left). Statistical analysis of LGR5 staining in WT and IL25KO tumors (right). **(B)** Stem cell markers CD133 and LGR5 expression were detected by multiplexed fluorescence staining in the WT and IL25KO AOM-DSS-induced CRC mouse models. The representative images show the expression of LGR5 (red), DAPI (blue), CD133 (green). **(C)** The expression levels of stem cells markers were examined in WT and IL25KO tumors by RT-PCR. **(D)** The expression levels of CSC markers, namely, DCLK1, ALDH1, and CD44, were examined in tumor tissues by Western blotting. **(E)** Western blotting of Stem cell markers levels in LoVo cells following IL25 silencing. **(F)** Frequency (right) and representative day-7 images (left) of adenomatous organoids from WT and IL25KO mice treated with/without IL25 (50 ng/ml). **(G)** Sphere formation analysis of LoVo cells following IL25 silencing. Representative images (left) and the mean numbers and sphere size (right) of spheres are shown. Data present as mean ± *SEM*; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

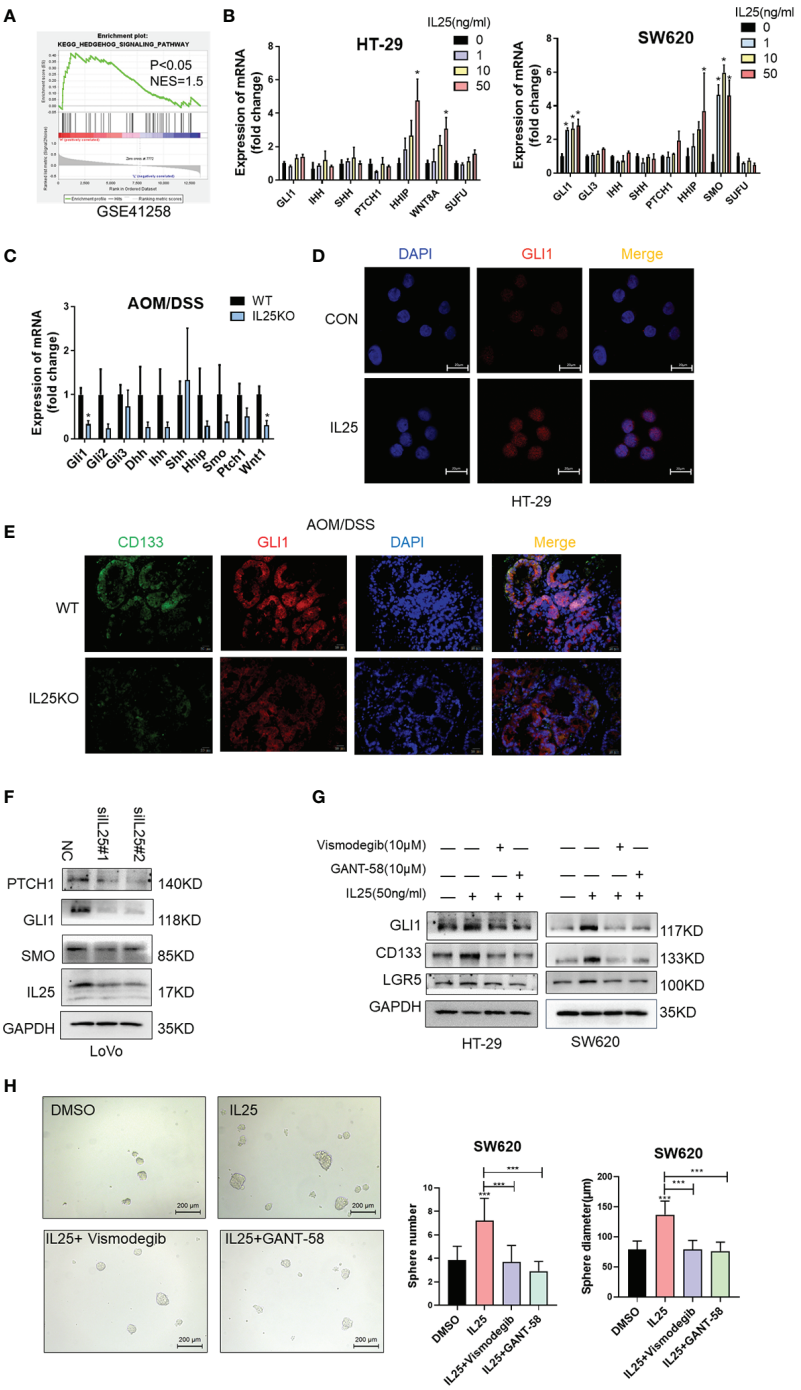


FIGURE 5 | IL25 promoted stemness *via* the Hedgehog signaling pathway in CRC cancer. **(A)** GSEA for Hedgehog signaling pathway (nominal $p < 0.05$) in IL25 high expression colorectal cancer compared with IL25 low expression colorectal cancer from GSE41258. **(B)** The expression levels of Hedgehog signaling genes were examined in HT-29 and SW620 cells treated with recombinant IL25 in a concentration-dependent manner by RT-PCR. **(C)** The expression levels of Hedgehog signaling genes were examined in WT and IL25KO AOM/DSS-induced CRC cancer tissues by RT-PCR. **(D)** GLI1 expression was detected by immunofluorescence staining in HT-29 cells treated with or without 50 ng/ml IL25. **(E)** GLI1 and CD133 expression were detected by multiplexed fluorescence staining in the WT and IL25KO AOM/DSS-induced tumor tissues. Representative images show the expression of GLI1 (red), DAPI (blue), and CD133 (green). **(F)** Western blotting of Hedgehog signaling genes in LoVo cells following IL25 silencing. **(G)** Western blotting of GLI1 and CD133 in HT-29 and SW620 cells treated with SMO inhibitor Vismodegib and GLI1 inhibitor GANT-58 following IL25 treatment. **(H)** Sphere formation analysis of SW620 cells treated with SMO inhibitor Vismodegib and GLI1 inhibitor GANT-58 following IL25 treatment. Representative images (left) and the mean numbers and sphere size (right) of spheres are shown. Data present as mean ± *SEM*; $^{*}p < 0.05$, $^{***}p < 0.001$.

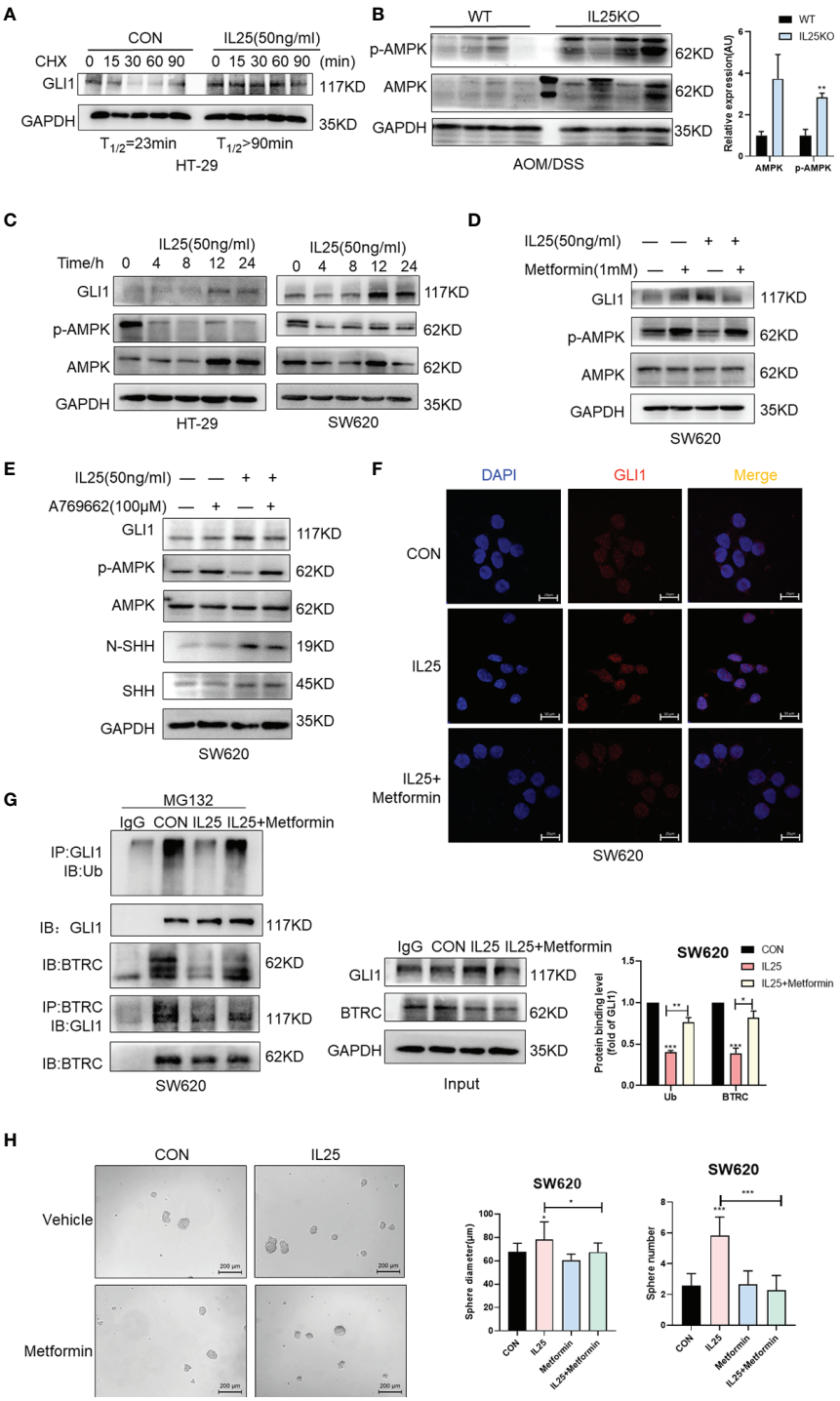


FIGURE 6 | IL25 upregulated GLI1 by inhibiting p-AMPK. **(A)** HT-29 cells were treated with cycloheximide (CHX, 50 μg/ml) for the indicated time, and cell lysates were analyzed by Western blotting with the indicated antibodies. **(B)** Western blotting of p-AMPK and AMPK in the WT and IL25KO AOM/DSS-induced tumor tissue. **(C)** The expression levels of GLI1, p-AMPK, and AMPK were examined in HT-29 and SW620 cells treated with recombinant IL25 in a time-dependent manner by Western blotting. **(D, E)** Western blotting of GLI1, p-AMPK, and AMPK in SW620 cells treated with AMPK activator A769662 and Metformin following IL25 treatment. **(F)** GLI1 expression was detected by immunofluorescence staining in SW620 cells treated with AMPK activator Metformin following IL25 treatment. **(G)** SW620 cells were treated with 10 μM MG132 and then incubated with or without 50 ng/ml recombinant IL25 and 1 mM Metformin, then immunoprecipitated with GLI1 antibody. GLI1 ubiquitination was determined using an anti-ubiquitin antibody. IP, immunoprecipitation. **(H)** Sphere formation analysis of SW620 cells treated with AMPK activator Metformin following IL25 treatment. Representative images (left) and the mean numbers and sphere size (right) of spheres are shown. Data present as mean ± *SEM;* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

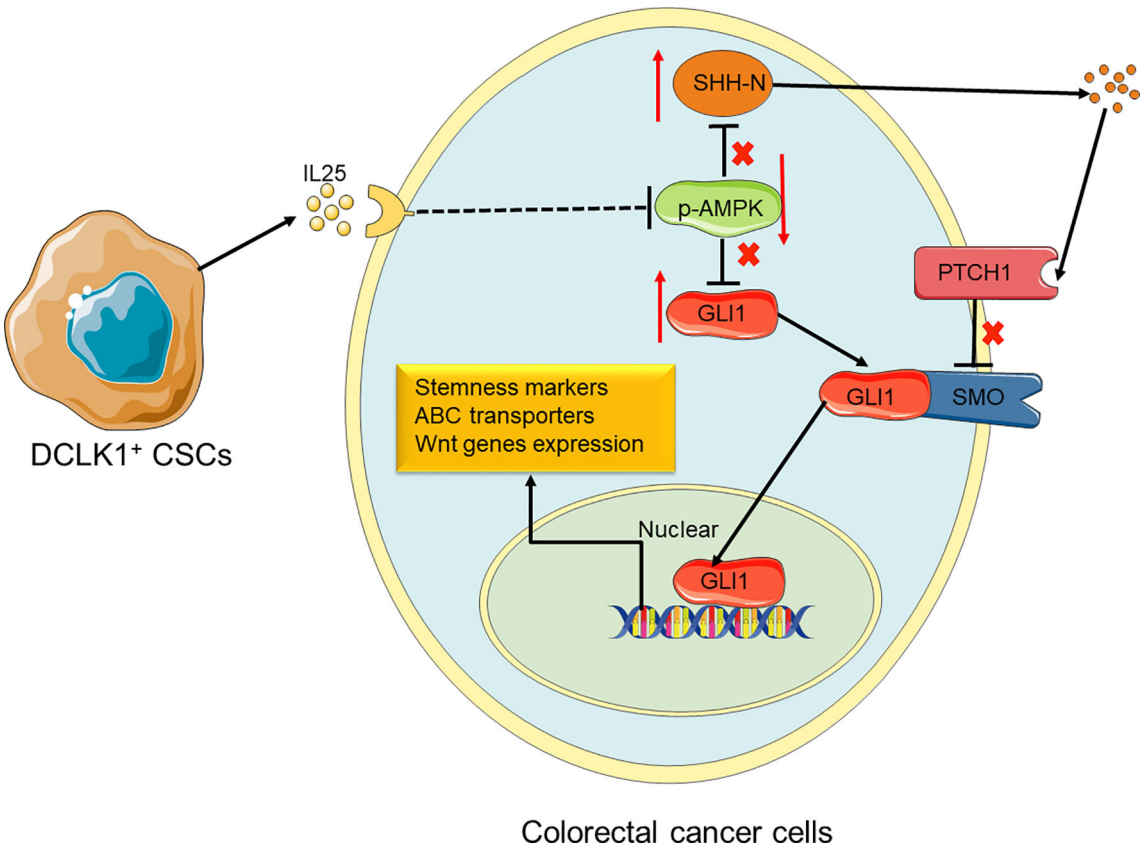


FIGURE 7 | Proposed model for the roles and functions of IL25 in promoting CRC stemness. IL25 can inhibit p-AMPK and lead to GLI1 accumulation. It also stimulates SHH secretion by the means that minus times minus equals plus, thereby GLI1 binding with SMO. In nuclear, GLI1 can promote stem cell markers and ABC transporters expression. Therefore, CRC cells gain the ability of stemness and oxaliplatin resistance.

In the past, IL25 was considered to induce strong type 2 immunity in the gastroenterological tract characterized by increased expression of IL-5, IL-13, etc. (24). However, our previous research showed that deletion of IL25 in C57BL/6J protected from DSS-induced colitis. Endogenous IL25 acts as a pro-inflammatory factor in DSS-induced colitis by upregulated IL33 but not IL13 (25). AOM/DSS-induced CRC models resemble many aspects of the pathogenic process of human ulcerative colitis and CAC (26–28). A previous study showed that genetic deletion of IL25 did not affect tumor burden caused by AOM and 2 cycles DSS treatment in BALB/c mice (29). However, in our study, with the progression of CRC by AOM and 3 cycles DSS treatment, the numbers and volumes of tumors were significantly decreased in 16 weeks IL25KO C57BL/6J mice. It is known that mice in C57BL/6J background are prone to Th1 immunity, whereas mice in BALB/c background are biased toward type 2 immunity. Genetically deficient of IL13 in C57BL/6J mice are more susceptible to acute DSS-induced colitis. On the contrary, IL13KO in a BALB/c background showed reduced severity of DSS-induced colitis (25). It is likely that IL25 promotes colitis in an IL33 dependent manner on C57BL/6J, but promotes IL13 in BALB/c, which leads to the differences between the disparate strains. Our previous study showed that IL25 was not directly affecting the growth, apoptosis, or migration in HCC, but promoted macrophages secret CXCL10 and led to cancer metastasis (18). Similarly, we also discovered that the proliferation and apoptosis were not different between WT and IL25KO tumors. Cancer stem cells play an important role in tumor initiation, propagation, and therapy resistance, which are thought to be quiescent and more resistant to chemotherapy (6). In chronic myeloid leukemia (CML), CSCs were quiescent and more resistant to chemotherapy (30). Moreover, the undifferentiated tumor with stem cell signaling overexpression is associated with lower immune infiltration and downregulated programmed cell death 1 ligand 1 (PD- L1) signaling predicted a poor response to immunotherapy (31–33). Continuing studies elucidate that CSCs recruit immune cells to modulate a favorable microenvironment through secreting chemokines, cytokines, and inflammatory factors. At the same time, the topology and dynamic behavior of CSCs are sculpted by chemokines, cytokines, and inflammatory factors such as IL-6, IL-8, IL-22, and IL-33 (11, 34, 35). In addition, a previous study found that IL25 expression in breast cancer was a positive correlation with infiltrating $CD4^+$T cells and macrophages, whereas IL25

blockade decreased type 2 T cells and macrophages in the primary tumor microenvironments and inhibited lung metastasis (19). Moreover, IL25 could promote proliferation and sustain self-renewal of NANOG positive hepatocellular carcinoma by activating NF-κB and JAK/Stat3 pathways (36). These data suggested that IL25 may be a key linkage between CSCs and the tumor microenvironment thus promoting CRC development.

$DCLK1^+$ tuft cells which are the main producers of IL25 in the intestine had stem cell properties and played an important role in colitis and CRC initiation (37). In the intestine, tuft cells are the main producers of IL25 in the steady state. Besides, in mice DSS-induced APC lacking colonic adenocarcinoma, long-lived intestinal tuft cells serve as colon cancer-initiating cells (37). Moreover, tuft cells marker DCLK1 was especially expressed in intestinal tumor stem cells, whereas, it was hardly expressed in the colon steady stage (38, 39). However, how tuft cells drive tumor initiation and development remains unknown. Our data showed that IL25 was secreted by CD133 or DCLK1 positive cells (**Figures S3E, F**). At the same time, we found DCLK1 and IL25 were upregulated in tumors after oxaliplatin injection (**Figure S2C**). A previous study showed that high-fat diet increased intestinal stem cells and progenitor cells (40). To our surprise, free fatty acid and cholesterol were increased in AOM/DSS-induced mice serum which was injected with oxaliplatin (**Figures S3A, B**). Moreover, oleic acid could upregulate DCLK1 and IL25 expression *in vitro* (**Figure S3C**). These data suggested that oxaliplatin increased $DCLK1^+$ CSCs through lipid. Read in conjunction, IL25 may play a curial role in chemotherapy resistance of CRC derived by tuft or cancer stem cells. On the other hand, CSCs markers are commonly shared by normal stem cells. Thus, therapies that target these markers may cause severe injury to normal colonic tissues. Our data demonstrated that IL25 was especially expressed in colorectal CSCs and is essential for maintaining cancer stemness. Targeting the IL25 signaling pathway may offer the potential to upgrade chemotherapy efficiency for colorectal cancer without damaging normal stem cells.

Our study comprehensively explored the mechanism involved in the process of IL25 enhanced CRC stemness. As GSEA analysis, we found IL25 could activate the Hedgehog signaling pathway. The Hedgehog signaling pathway plays a critical role in tissue-patterning during embryonic development and the repair of normal tissues, and cancer development (41). GLI1, GLI2, and GLI3 are the key transcription factors of the Hedgehog signaling pathway (42). However, we found GLI2 and GLI3 were barely detectable in colorectal cancer cells. A previous study showed that GLI1 was mainly phosphorylated by PKA, GSK3β, CK and degraded mediated by Ub (43). In the presence of SHH ligand, SMO was associated with β-arrestin and the microtubule motor KIF3A and prevented GLI1 from degradation (44). However, a recent study showed that p-AMPK could phosphorylate GLI1, thereby promoting GLI1 degradation, even in the presence of SHH ligand (45). Meanwhile, GLI1 could promote chemoresistance by upregulating ABC transporters in CRC cells (8). However, the relationship between IL25 and GLI1 had never been reported. It was also found that inhibition of p-AMPK could upregulate the key enzyme of cholesterol synthesis HMGCR and promote SHH CHLation, then increased N-SHH activated GLI1 binding target genes (46). Our research first identified that IL25 could activate the Hedgehog signaling pathway by inhibiting p-AMPK, and the induction of CRC stemness mainly depends on increasing SHH and nuclear GLI1. The most clinical inhibitor targeting the Hedgehog pathway is vismodegib, which was approved by the US FDA in 2012 and the European Medicines Agency (EMA) in 2013 for the treatment of metastatic basal cell carcinoma (BCC) or locally advanced BCC in patients who are not candidates for surgery or radiotherapy (47). However, the addition of vismodegib to combination treatment with FOLFOX (5-fluorouracil [5-FU], folinic acid and oxaliplatin) or FOLFIRI (5-FU, folinic acid, and irinotecan) chemotherapy plus bevacizumab did not increase PFS or the overall response rate (ORR) in metastatic colorectal cancer (48). Our research provides a novel strategy for the treatment of augmented levels of IL25 metastatic CRC by targeting the Hedgehog pathway by vismodegib.

Upregulation of IL25 may lead to inflammatory disorders such as atopic dermatitis, psoriasis, or asthma (49). Encouragingly, IL25 monoclonal antibody XKH001 was the first IL25 inhibitor which was approved by the FDA in 2021 for the clinical trial of inflammatory associated diseases, and IL25 blockade inhibited lung metastasis in breast cancer (19). Based on our findings, injection of IL25 neutralizing antibody would reduce the tumor in AOM/DSS treated mice. In the future, IL25 inhibition will be a new clinical strategy for colitis-associated cancer. In conclusion, our research first demonstrated that IL25 was elevated in CRC and inhibited p-AMPK, and upregulated GLI1, thereby maintaining stemness. This study indicated that IL25 would be a significant prognostic factor. Targeting IL25 by neutralizing antibodies and Hedgehog signaling pathway may improve chemotherapy efficacy and serve as a potential treatment for CRC patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Sun Yat-sen University Cancer Center. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University (approval number: SCXK2019-0209).

AUTHOR CONTRIBUTIONS

JL, BQ performed the experiments and wrote the first draft of the manuscript. LZ and GS revised the manuscript and performed the animal breeding and identification experiments. YT, SL, ZZ, JS: worked together on the collection of associated data and their interpretation and collaborated with all other authors. WQ and TZ discussed the data. ZY, XY and GG: conceptualization, supervision. All authors read and approved the final manuscript.

FUNDING

This research was funded by the National Nature Science Foundation of China (Grant numbers: 81570764, 81872165, and 82070888); the National Key R&D Program of China (Grant number: 2018YFA0800403); the Guangdong Provincial Key R&D Program (Grant number: 2018B030337001); the Key Project of Nature Science Foundation of Guangdong Province, China (Grant number: 2019B1515120077); the Guangzhou Science and Technology Project (Grant number No: 201807010069), the Shenzhen Science and Technology Project (Grant number: JCYJ20190807154205627), the Guangdong Natural Science Fund (Grant numbers: 2020A1515010365, 2021A1515010434), the Guangdong Provincial Key Laboratory of Precision Medicine and Clinical Translation Research of Hakka Population (Grant number No: 2018B030322003KF01), the Key Sci-Tech of Hakka Population (Grant number No: 2018B030322003KF01), Key Sci-Tech. The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.837262/full#supplementary-material

Supplementary Figure 1 | GEO dataset analysis. **(A, B)** Overall survival analysis of CRC patients from GEO dataset GSE17538 in correlation with tumor IL25 expression. **(C)** Heat map showing core enrichment hallmark ABC transporters signaling genes in IL25 high expression colorectal cancer compared with IL25 low expression colorectal cancer from GSE17538. **(D)** Correlation analysis between IL25 and ABC transporters or stem cell markers from GSE17538. **(E)** Heat map showing core enrichment hallmark Hedgehog signaling genes in IL25 high expression colorectal cancer compared with IL25 low expression colorectal cancer from GSE41258. **(F)** GSEA for Hedgehog signaling pathway in IL25 high expression colorectal cancer compared with IL25 low expression colorectal cancer from GSE17538.

Supplementary Figure 2 | IL25 expression was positively associated with chemotherapy drug transporters expression. **(A)** Representative images of Ki67 IHC staining. **(B)** Representative images of hematoxylin and eosin (H&E) from tumors in WT and IL25KO mice. **(C)** The expression levels of ABCC2, ABCC5, and IL25 were examined in tumor tissues by Western blotting. **(D)** HT-29 cells cultured with or without IL25 (50 ng/mL) for 24 hours and were subsequently exposed to oxaliplatin for 48 hours in a concentration-dependent. The cell viability was determined by the CCK-8 assay. **(E, F)** The expression levels of ABC transporters in HT-29 and SW620 cells treated with recombinant IL25 in a concentration-dependent manner by RT-PCR and Western blotting. **(G)** Silencing effect of various siRNA of IL25 in LoVo cells. **(H)** Western blotting of ABCC2 and ABCC5 in LoVo cells following IL25 silencing. **(I)** LoVo cells treated with oxaliplatin for 48 hours in a concentration-dependent following IL25 silencing. Data present as mean ± *SEM*; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure 3 | IL25 was positively associated with stem cell genes expression. **(A, B)** Serum cholesterol and free fatty acid content in mice treated with the indicated treatment. **(C)** CRC cells were treated with 100 and 200μM oleic acid for 24h and cell lysates were analyzed by Western blotting with the indicated antibodies. **(D)** LoVo spheres were sorted from sphere formation assays by passaging spheres three times. LoVo cells and LoVo spheres lysates were analyzed by Western blotting. **(E)** IL25 and DCLK1 expression were detected by multiplexed fluorescence staining in the CRC patients and WT AOM/DSS-induced tumor tissues. The representative images show the expression of DCLK1 (red), DAPI (blue), IL25 (green). **(F)** IL25 and CD133 expression was detected by multiplexed fluorescence staining in the WT colon and WT AOM/DSS-induced tumor tissues. The representative images show the expression of IL25 (red), DAPI (blue), CD133 (green). **(G)** The expression levels of pluripotent stem cells markers, including NANOG, SOX2, MYC, POU5F1 were examined in HT-29 cells treated with recombinant IL25 in a concentration-dependent manner by RT-PCR. **(H)** Quantitative analysis of the western blotting of stem cells markers was examined in WT and IL25KO tumors. **(I)** Quantitative analysis of the western blotting of stem cells markers was examined in LoVo cells following IL25 silencing. Data present as mean ± *SEM*; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure 4 | IL25 promoted stemness *via* GLI1 accumulation in CRC cancer. **(A)** Quantitative analysis of the western blotting of Hedgehog signaling genes in LoVo cells following IL25 silencing. **(B)** Quantitative analysis of western blotting in HT-29 and SW620 cells treated with SMO inhibitor Vismodegib and GLI1 inhibitor GANT-58 following IL25 treatment. **(C)** Quantitative analysis of the expression levels of GLI1, p-AMPK, and AMPK were examined in HT-29 and SW620 cells. **(D)** Quantitative analysis of western blotting of GLI1, p-AMPK, and AMPK in SW620 cells treated with AMPK activator Metformin following IL25 treatment. **(E)** Frequency (right) and representative day-7 images (left) of adenomatous organoids from mice treated with SMO inhibitor Vismodegib, GLI1 inhibitor GANT-58, and AMPK activator Metformin following IL25 treatment. Data present as mean ± *SEM*; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure 5 | IL25 activated Hedgehog signaling pathway *via* SHH-N upregulation in CRC cancer. **(A)** The expression levels of SHH and PTCH1 in HT-29 and SW620 cells treated with recombinant IL25 in a time-dependent manner by Western blotting. **(B, C)** Cholesterol synthesis-related genes expression in HT-29 and SW620 cells treated with recombinant IL25 by RT-PCR. **(D)** Total cholesterol content in SW620 cells treated with IL25. **(E)** Quantitative analysis of western blotting of GLI1, p-AMPK, and AMPK in SW620 cells treated with AMPK activator A769662 following IL25 treatment. **(F)** Silencing effect of various siRNA of PTCH1 in SW620 cells. **(G)** Hedgehog signaling pathway genes expression was detected by Western blotting in SW620 following PTCH1 silencing. Data present as mean ± *SEM*; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2021. *CA: A Cancer J Clin* (2021) 71:7–33. doi: 10.3322/caac.21654
2. Cremolini C, Loupakis F, Antoniotti C, Lupi C, Sensi E, Lonardi S, et al. FOLFOXIRI Plus Bevacizumab Versus FOLFIRI Plus Bevacizumab as First-Line Treatment of Patients With Metastatic Colorectal Cancer: Updated Overall Survival and Molecular Subgroup Analyses of the Open-Label, Phase 3 TRIBE Study. *Lancet Oncol* (2015) 16:1306–15. doi: 10.1016/S1470-2045(15)00122-9
3. Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, et al. Colorectal Cancer. *Nat Rev Dis Primers* (2015) 1:15065. doi: 10.1038/nrdp.2015.65
4. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, et al. Colorectal Cancer Statistics, 2020. *CA: A Cancer J Clin* (2020) 70:145–64. doi: 10.3322/caac.21601

5. Vasan N, Baselga J, Hyman DM. A View on Drug Resistance in Cancer. *Nature* (2019) 575:299–309. doi: 10.1038/s41586-019-1730-1
6. Lytle NK, Barber AG, Reya T. Stem Cell Fate in Cancer Growth, Progression and Therapy Resistance. *Nat Rev Cancer* (2018) 18:669–80. doi: 10.1038/s41568-018-0056-x
7. Fletcher JI, Haber M, Henderson MJ, Norris MD. ABC Transporters in Cancer: More Than Just Drug Efflux Pumps. *Nat Rev Cancer* (2010) 10:147–56. doi: 10.1038/nrc2789
8. Po A, Citarella A, Catanzaro G, Besharat ZM, Trocchianesi S, Gianno F, et al. Hedgehog-GLI Signalling Promotes Chemoresistance Through the Regulation of ABC Transporters in Colorectal Cancer Cells. *Sci REP-UK* (2020) 10(1):13988. doi: 10.1038/s41598-020-70871-9
9. Pothuraju R, Rachagani S, Krishn SR, Chaudhary S, Nimmakayala RK, Siddiqui JA, et al. Molecular Implications of MUC5AC-CD44 Axis in Colorectal Cancer Progression and Chemoresistance. *Mol Cancer* (2020) 19 (1):37. doi: 10.1186/s12943-020-01156-y
10. Elbadawy M, Hayashi K, Ayame H, Ishihara Y, Abugomaa A, Shibutani M, et al. Anti-Cancer Activity of Amorphous Curcumin Preparation in Patient-Derived Colorectal Cancer Organoids. *BioMed Pharmacother* (2021) 142:112043. doi: 10.1016/j.biopha.2021.112043
11. Batlle E, Clevers H. Cancer Stem Cells Revisited. *Nat Med* (2017) 23:1124–34. doi: 10.1038/nm.4409
12. Song X, Qian Y. IL-17 Family Cytokines Mediated Signaling in the Pathogenesis of Inflammatory Diseases. *Cell Signal* (2013) 25:2335–47. doi: 10.1016/j.cellsig.2013.07.021
13. Reynolds JM, Lee Y, Shi Y, Wang X, Angkasekwinai P, Nallaparaju KC, et al. Interleukin-17b Antagonizes Interleukin-25-Mediated Mucosal Inflammation. *Immunity* (2015) 42:692–703. doi: 10.1016/j.immuni.2015.03.008
14. Gerbe F, Sidot E, Smyth DJ, Ohmoto M, Matsumoto I, Dardalhon V, et al. Intestinal Epithelial Tuft Cells Initiate Type 2 Mucosal Immunity to Helminth Parasites. *Nature* (2016) 529:226–30. doi: 10.1038/nature16527
15. Howitt MR, Lavoie S, Michaud M, Blum AM, Tran SV, Weinstock JV, et al. Tuft Cells, Taste-Chemosensory Cells, Orchestrate Parasite Type 2 Immunity in the Gut. *Sci (American Assoc Advancement Science)* (2016) 351:1329–33. doi: 10.1126/science.aaf1648
16. von Moltke J, Ji M, Liang H, Locksley RM. Tuft-Cell-Derived IL-25 Regulates an Intestinal ILC2–epithelial Response Circuit. *Nature* (2016) 529:221–5. doi: 10.1038/nature16161
17. Goto N, Fukuda A, Yamaga Y, Yoshikawa T, Maruno T, Maekawa H, et al. Lineage Tracing and Targeting of IL17RB + Tuft Cell-Like Human Colorectal Cancer Stem Cells. *Proc Natl Acad Sci - PNAS* (2019) 116:12996–3005. doi: 10.1073/pnas.1900251116
18. Li Q, Ma L, Shen S, Guo Y, Cao Q, Cai X, et al. Intestinal Dysbacteriosis-Induced IL-25 Promotes Development of HCC *via* Alternative Activation of Macrophages in Tumor Microenvironment. *J Exp Clin Canc Res* (2019) 38 (1):303. doi: 10.1186/s13046-019-1271-3
19. Jiang Z, Chen J, Du X, Cheng H, Wang X, Dong C. IL-25 Blockade Inhibits Metastasis in Breast Cancer. *Protein Cell* (2017) 8:191–201. doi: 10.1007/s13238-016-0345-7
20. Furuta S, Jeng YM, Zhou L, Huang L, Kuhn I, Bissell MJ, et al. IL-25 Causes Apoptosis of IL-25r-Expressing Breast Cancer Cells Without Toxicity to Nonmalignant Cells. *Sci Transl Med* (2011) 3:31r–78r. doi: 10.1126/scitranslmed.3001374
21. Thaker AI, Shaker A, Rao MS, Ciorba MA. Modeling Colitis-Associated Cancer With Azoxymethane (AOM) and Dextran Sulfate Sodium (DSS). *J Visualized Experiments* (2012) 67:4100. doi: 10.3791/4100
22. Yin H, Gao T, Xie J, Huang Z, Zhang X, Yang F, et al. FUBP1 Promotes Colorectal Cancer Stemness and Metastasis *via* DVL1-Mediated Activation of Wnt/β-Catenin Signaling. *Mol Oncol* (2021) 12:3490–512. doi: 10.21203/rs.3.rs-152458/v1
23. Sato T, Stange DE, Ferrante M, Vries RGJ, van Es JH, van den Brink S, et al. Long-Term Expansion of Epithelial Organoids From Human Colon, Adenoma, Adenocarcinoma, and Barrett's Epithelium. *Gastroenterology* (2011) 141:1762–72. doi: 10.1053/j.gastro.2011.07.050
24. Zhao A, Urban JJ, Sun R, Stiltz J, Morimoto M, Notari L, et al. Critical Role of IL-25 in Nematode Infection-Induced Alterations in Intestinal Function. *J Immunol* (2010) 185:6921–9. doi: 10.4049/jimmunol.1000450
25. Wang A, Smith A, Li Y, Urban JJF, Ramalingam TR, Wynn TA, et al. Genetic Deletion of IL-25 (IL-17E) Confers Resistance to Dextran Sulfate Sodium-Induced Colitis in Mice. *Cell Bioscience* (2014) 4:72. doi: 10.1186/2045-3701-4-72
26. Wei T, Lin Y, Chen W, Luo P, Lin Y, Shun C, et al. Dual Targeting of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase and Histone Deacetylase as a Therapy for Colorectal Cancer. *Ebiomedicine* (2016) 10:124–36. doi: 10.1016/j.ebiom.2016.07.019
27. Li Z, Sun B, Gong T, Guo S, Zhang J, Wang J, et al. GNAI1 and GNAI3 Reduce Colitis-Associated Tumorigenesis in Mice by Blocking IL6 Signaling and Down-Regulating Expression of GNAI2. *Gastroenterology* (2019) 156:2297–312. doi: 10.1053/j.gastro.2019.02.040
28. Aguilar E, Esteves P, Sancerni T, Lenoir V, Aparicio T, Bouillaud F, et al. UCP2 Deficiency Increases Colon Tumorigenesis by Promoting Lipid Synthesis and Depleting NADPH for Antioxidant Defenses. *Cell Rep* (2019) 28:2306–16. doi: 10.1016/j.celrep.2019.07.097
29. Thelen TD, Green RM, Ziegler SF. Acute Blockade of IL-25 in a Colitis Associated Colon Cancer Model Leads to Increased Tumor Burden. *Sci Rep-UK* (2016) 6:25643. doi: 10.1038/srep25643
30. Ye H, Adane B, Khan N, Sullivan T, Minhajuddin M, Gasparetto M, et al. Leukemic Stem Cells Evade Chemotherapy by Metabolic Adaptation to an Adipose Tissue Niche. *Cell Stem Cell* (2016) 19:23–37. doi: 10.1016/j.stem.2016.06.001
31. Ji R, Chasalow SD, Wang L, Hamid O, Schmidt H, Cogswell J, et al. An Immune-Active Tumor Microenvironment Favors Clinical Response to Ipilimumab. *Cancer Immunol Immunother* (2012) 61:1019–31. doi: 10.1007/s00262-011-1172-6
32. Spranger S, Bao R, Gajewski TF. Melanoma-Intrinsic β-Catenin Signalling Prevents Anti-Tumour Immunity. *Nature* (2015) 523:231–5. doi: 10.1038/nature14404
33. Zaretsky JM, Garcia-Diaz A, Shin DS, Escuin-Ordinas H, Hugo W, Hu-Lieskovan S, et al. Mutations Associated With Acquired Resistance to PD-1 Blockade in Melanoma. *N Engl J Med* (2016) 375:819–29. doi: 10.1056/NEJMoa1604958
34. Fang M, Li Y, Huang K, Qi S, Zhang J, Zgodzinski W, et al. IL33 Promotes Colon Cancer Cell Stemness *via* JNK Activation and Macrophage Recruitment. *Cancer Res* (2017) 77:2735–45. doi: 10.1158/0008-5472.CAN-16-1602
35. Gronke K, Hernández PP, Zimmermann J, Klose CSN, Kofoed-Branzk M, Guendel F, et al. Interleukin-22 Protects Intestinal Stem Cells Against Genotoxic Stress. *Nature* (2019) 566:249–53. doi: 10.1038/s41586-019-0899-7
36. Luo Y, Yang Z, Su L, Shan J, Xu H, Xu Y, et al. Non-CSCs Nourish CSCs Through Interleukin-17E-Mediated Activation of NF-κb and JAK/STAT3 Signaling in Human Hepatocellular Carcinoma. *Cancer Lett* (2016) 375:390–9. doi: 10.1016/j.canlet.2016.03.012
37. Westphalen CB, Asfaha S, Hayakawa Y, Takemoto Y, Lukin DJ, Nuber AH, et al. Long-Lived Intestinal Tuft Cells Serve as Colon Cancer–Initiating Cells. *J Clin Invest* (2014) 124:1283–95. doi: 10.1172/JCI73434
38. Nakanishi Y, Seno H, Fukuoka A, Ueo T, Yamaga Y, Maruno T, et al. Dclk1 Distinguishes Between Tumor and Normal Stem Cells in the Intestine. *Nat Genet* (2013) 45:98–103. doi: 10.1038/ng.2481
39. Chandrakesan P, Yao J, Qu D, May R, Weygant N, Ge Y, et al. Dclk1, a Tumor Stem Cell Marker, Regulates Pro-Survival Signaling and Self-Renewal of Intestinal Tumor Cells. *Mol Cancer* (2017) 16(1):30. doi: 10.1186/s12943-017-0594-y
40. Beyaz S, Mana MD, Roper J, Kedrin D, Saadatpour A, Hong S, et al. High-Fat Diet Enhances Stemness and Tumorigenicity of Intestinal Progenitors. *Nature* (2016) 531:53–8. doi: 10.1038/nature17173
41. Rubin LL, de Sauvage FJ. Targeting the Hedgehog Pathway in Cancer. *Nat Rev Drug Discov* (2006) 5:1026–33. doi: 10.1038/nrd2086
42. Yang L, Shi P, Zhao G, Xu J, Peng W, Zhang J, et al. Targeting Cancer Stem Cell Pathways for Cancer Therapy. *Signal Transduct Target Ther* (2020) 5:8. doi: 10.1038/s41392-020-0110-5
43. Briscoe J, Thérond PP. The Mechanisms of Hedgehog Signalling and Its Roles in Development and Disease. *Nat Rev Mol Cell Bio* (2013) 14:416–29. doi: 10.1038/nrm3598
44. Clara JA, Monge C, Yang Y, Takebe N. Targeting Signalling Pathways and the Immune Microenvironment of Cancer Stem Cells — A Clinical Update. *Nat Rev Clin Oncol* (2020) 17:204–32. doi: 10.1038/s41571-019-0293-2

45. Li Y, Luo J, Mosley YC, Hedrick VE, Paul LN, Chang J, et al. AMP-Activated Protein Kinase Directly Phosphorylates and Destabilizes Hedgehog Pathway Transcription Factor GLI1 in Medulloblastoma. *Cell Rep* (2015) 12:599–609. doi: 10.1016/j.celrep.2015.06.054
46. Su K, Dai S, Tang Z, Xu M, Dai C. Heat Shock Factor 1 Is a Direct Antagonist of AMP-Activated Protein Kinase. *Mol Cell* (2019) 76:546–61. doi: 10.1016/j.molcel.2019.08.021
47. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, et al. Targeting Notch, Hedgehog, and Wnt Pathways in Cancer Stem Cells: Clinical Update. *Nat Rev Clin Oncol* (2015) 12:445–64. doi: 10.1038/nrclinonc.2015.61
48. Berlin J, Bendell JC, Hart LL, Firdaus I, Gore I, Hermann RC, et al. A Randomized Phase II Trial of Vismodegib Versus Placebo With FOLFOX or FOLFIRI and Bevacizumab in Patients With Previously Untreated Metastatic Colorectal Cancer. *Clin Cancer Res* (2013) 19:258–67. doi: 10.1158/1078-0432.CCR-12-1800
49. Borowczyk J, Shutova M, Brembilla NC, Boehncke W. IL-25 (IL-17E) in Epithelial Immunology and Pathophysiology. *J Allergy Clin Immun* (2021) 148:40–52. doi: 10.1016/j.jaci.2020.12.628

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 27 June 2022
doi: 10.3389/fimmu.2022.829057

Immune- and Stemness-Related Genes Revealed by Comprehensive Analysis and Validation for Cancer Immunity and Prognosis and Its Nomogram in Lung Adenocarcinoma

Mengqing Chen[1†], Xue Wang[1†], Wenjun Wang[1], Xuemei Gui[1] and Zhan Li[2,3*]*

[1] Department of Respiratory and Critical Care Medicine, the Affiliated Hospital of Southwest Medical University, Luzhou, China, [2] Department of Stem Cell and Regenerative Medicine, State Key Laboratory of Trauma, Burn and Combined Injury, Daping Hospital, Army Medical University, Chongqing, China, [3] Central Laboratory, State Key Laboratory of Trauma, Burn and Combined Injury, Daping Hospital, Army Medical University, Chongqing, China

OPEN ACCESS

Edited by:
Cristina Maccalli, Sidra Medicine, Qatar

Reviewed by:
Xinwei Han, Zhengzhou University, China
Ming Yi, Zhejiang University, China
Xishan Wang, Chinese Academy of Medical Sciences and Peking Union Medical College, China

***Correspondence:**
Zhan Li
lizhan6243@foxmail.com
Mengqing Chen
chenmq@swmu.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *04 December 2021*
Accepted: *20 May 2022*
Published: *27 June 2022*

Citation:
Chen M, Wang X, Wang W, Gui X and Li Z (2022) Immune- and Stemness-Related Genes Revealed by Comprehensive Analysis and Validation for Cancer Immunity and Prognosis and Its Nomogram in Lung Adenocarcinoma. Front. Immunol. 13:829057. doi: 10.3389/fimmu.2022.829057

Objective: Lung adenocarcinoma (LUAD) is a familiar lung cancer with a very poor prognosis. This study investigated the immune- and stemness-related genes to develop model related with cancer immunity and prognosis in LUAD.

Method: The Cancer Genome Atlas (TCGA) was utilized for obtaining original transcriptome data and clinical information. Differential expression, prognostic value, and correlation with clinic parameter of mRNA stemness index (mRNAsi) were conducted in LUAD. Significant mRNAsi-related module and hub genes were screened using weighted gene coexpression network analysis (WGCNA). Meanwhile, immune-related differential genes (IRGs) were screened in LUAD. Stem cell index and immune-related differential genes (SC-IRGs) were screened and further developed to construct prognosis-related model and nomogram. Comprehensive analysis of hub genes and subgroups, involving enrichment in the subgroup [gene set enrichment analysis (GSEA)], gene mutation, genetic correlation, gene expression, immune, tumor mutation burden (TMB), and drug sensitivity, used bioinformatics and reverse transcription polymerase chain reaction (RT-PCR) for verification.

Results: Through difference analysis, mRNAsi of LUAD group was markedly higher than that of normal group. Clinical parameters (age, gender, and T staging) were ascertained to be highly relevant to mRNAsi. MEturquoise and MEblue were found to be the most significant modules (including positive and negative correlations) related to mRNAsi *via* WGCNA. The functions and pathways of the two mRNAsi-related modules were mainly enriched in tumorigenesis, development, and metastasis. Combining stem cell index–related differential genes and immune-related differential genes, 30 prognosis-related SC-IRGs were screened *via* Cox regression analysis. Then, 16 prognosis-related SC-IRGs were screened to construct a LASSO regression model at last. In addition, the model was successfully validated by using TCGA-LUAD and GSE68465, whereas c-index and the

calibration curves were utilized to demonstrate the clinical value of our nomogram. Following the validation of the model, GSEA, immune cell correlation, TMB, clinical relevance, etc., have found significant difference in high- and low-risk groups, and 16-gene expression of the SC-IRG model also was tested by RT-PCR. *ADRB2*, *ANGPTL4*, *BDNF*, *CBLC*, *CX3CR1*, and *IL3RA* were found markedly different expression between the tumor and normal group.

Conclusion: The SC-IRG model and the prognostic nomogram could accurately predict LUAD survival. Our study used mRNAsi combined with immunity that may lay a foundation for the future research studies in LUAD.

Keywords: lung adenocarcinoma, cancer stem cell, stem cell index, immune, nomogram, muti-omics analysis, RT-PCR

INTRODUCTION

Until now, as an important branch of malignant tumors, lung cancer is still a conventional causation of tumor death (1), and about 83% of lung cancers are non–small cell lung cancer (NSCLC) (2). Lung adenocarcinoma (LUAD) is a major subtype of NSCLC, and its incidence has always been high (3).

Since targeted therapy and immunotherapy have made considerable progresses in recent years, patients with LUAD now have more chance to choose a better treatment. However, on account of lack of targeted gene mutations, low PD-L1 (CD274) expression rate, and resistance after targeted therapy, there are still a significant proportion of patients making a tumor progression and die (4). Among them, the important cause of death involves tumor growth and metastasis, and cancer stem cells (CSCs) are regarded as the key driver: CSC biology is still in its infancy, but a large amount of data shows that there was a strong correlation between the expression of stem cell–like cells and the drug resistance of lung cancer (5). This phenomenon does not only occur in patients undergoing chemotherapy, but resistance to targeted therapy may also be related to it (6–8). In addition to this, tumor cells with PD-L1 expression may occur immune escape (9). CSC can evade immune surveillance due to their immunomodulatory effects (10). CSCs also can affect the immune system, such as the immune microenvironment of tumor lymph nodes (11). However, anti-cancer therapies currently not only fail to eradicate CSC clones but also assist in the screening of resistant CSC clones from the CSC pool, leading to treatment resistance and relapse (5, 12). Moreover, with the rise of immunotherapy, opening a new era of tumor therapy may require better exploration of the interaction between the CSC and the tumor immune microenvironment (TIME) (13).

The stem cell index, also known as the stemness index, is proposed by researchers from the University of Sao Paulo to assess the degree of dedifferentiation of cancer tissues. The researchers have found that cancer stemness index have unexpected correlations with immune checkpoint expression and infiltration of immune system cells (14), and these indicators may help us identify new biomarkers. At present, many studies have used CSC index to mine new biomarkers in LUAD (15–18), but there were few research works studying the relationship between stem cell index and tumor immune infiltration and the combination of them in LUAD. Therefore, in our study, according to the definition of stem cell index, combined with the immune-related gene, using bioinformatics analysis, we screened the genes related to stem cell index and immunity, constructed the model, and verified subgroups through multi-omics aspects of bioinformatics analysis and reverse transcription polymerase chain reaction (RT-PCR), providing a new perspective for cancer immunity and prognosis of LUAD.

Abbreviations: NSCLC, non–small cell lung cancer; LUAD, lung adenocarcinoma; CSC, cancer stem cells; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; mRNAsi, mRNA stemness index; WGCNA, Weighted gene coexpression network analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; TOM, topological overlap matrix; RT-PCR, reverse transcription polymerase chain reaction; DMEM, Dulbecco's modified Eagle medium; CC, cellular component (CC); MF, molecular function; BP, biological process; DEGs, differential expression genes; TMB, tumor mutation burden; TIME, tumor immune microenvironment; SCIRGs, stem cell and immune-related differential genes; C-index, concordance index.

METHOD

Acquisition and Processing of Data

Getting Datasets; Survival Analysis and Clinical Correlation Analysis of Stem Cell Index

We downloaded the data from The Cancer Genome Atlas database (TCGA, https://portal.gdc.cancer.gov/), which contained transcript data of 535 tumor tissues with LUAD and 59 normal tissues (TCGA-LUAD) and clinical data of 522 patients with LUAD. We also download GSE68465 from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). Transcriptome profiling data of 443 tumor tissues with LUAD and 19 normal tissues in the GSE68465 dataset were used for further analysis.

Using mRNA stemness index (mRNAsi) as a variable, the R packages "survminer" was applied to analyze the correlation of mRNAsi with clinical parameters. Then, according to the median

of mRNAsi, the tumor components were separate into two groups (high–mRNAsi level and low–mRNAsi level group) for survival analysis. The mRNAsi index of LUAD was acquired from the supplemental information in the study of Malta et al. (14).

Screening for Differential Genes and WGCNA Module Function and Pathway Enrichment Analysis

After the analysis above, we first assessed the difference between tumor and normal group according to mRNAsi, and then we used the R package "limma" (the Wilcoxon test) to screen the differential expression genes (DEGs) related to LUAD. The DEGs were next used to construct a coexpression module using a weighted gene coexpression network analysis (WGCNA). The construction process includes the following main steps: (1) give a definition for similarity matrix; (2) use the function pickSoftThreshold to select the soft threshold powerβ; (3) convert the adjacency matrix into a topological overlap matrix (TOM); (4) execute hierarchical aggregation of dissTOM derived from TOM; (5) from the hierarchical clustering tree, use the dynamic tree cutting method to distinguish modules with identical expression profiles; (6) quantify the coexpression similarity of the entire modules and compute their characteristic genes, etc. (19). At last, we selected two modules with the highest absolute value associated with mRNAsi (including positive and negative correlations) for the following analysis.

For better understanding the functions and pathways of the two mRNAsi-related modules above in LUAD, each of them was analyzed for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEG)G enrichment, respectively. R package "colorspace", "stringi", and "ggplot2" were used. The GO enrichment analysis included three components: molecular function (MF), cellular component (CC), and biological process (BP). Choose the threshold as p-value <0.05 and q-value <0.05.

Intersection of Stem Cell Index–Related Differential Genes and Immune-Related Differential Genes and Univariate Cox Regression Analysis and Construct LASSO Regression Model

To discover the immune-related genes (IRGs) in LUAD, we first downloaded the IRG data from the immunology database and analysis website (ImmPort, https://www.immport.org/). By taking the intersection with the DEGs that we screened before, we extracted the LUAD immune-related DEGs for the next step. We further analyzed the intersection of mRNAsi-related DEGs and immune-related DEGs *via* Venn diagram.

Using the R package "survival", we further screened for prognosis-related hub genes by univariate Cox regression analysis. We selected the genes with $P < 0.05$ and $HR \neq 1$ from the univariate Cox analysis. The Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis is a popular algorithm, which was extensively utilized in medical studies (20, 21). Using the R package "glmnet" and "survival", the optimal model based on prognosis-related stem cell index–related differential genes and immune-related differential genes (SCIRGs) was subsequently identified utilizing LASSO regression analysis (22). The model formula is

$$Riskscore = \sum_{i=1}^{n}(\text{Coefi} \times \text{Ni})$$

where Coef refers to the regression coefficient of SCIRGs in LASSO Cox regression analysis, "Ni" is the expression value of the gene, and "n" is the number of SCIRGs.

Verify the Risk Score Model Based on SCIRGs and Construct a Prognostic Nomogram

To verify the predictive ability of the model, we assessed the model through the training set (TCGA-LUAD) and the validation set (GSE68465), respectively. Using R package "survival" and "survminer" for survival analysis, we drew a Kaplan–Meier curve in TCGA and GEO datasets, separately. To explore high- and low-risk hub genes in the model and the risk score distribution in LUAD, we used the "pheatmap" package to depict risk curves, survival status maps, and risk heat maps. In addition, using R package "survival" for an independent prognostic analysis of the training and validation set, these helped us to understand whether the risk score can be used as a prognostic factor independent of clinical parameters. We used R package "survivalROC" to draw a multi-index ROC curve to assess prediction accuracy of the model. Last, we further take risk score with clinical parameters to draw a nomogram. The clinical parameters included age, gender, TNM (TNM Classification of Malignant Tumors, UICC 8th edition), and stage. The nomogram was used to evaluate the 1-, 2-, and 3-year survival rates of patients. The predictive capability of the model was assessed by calculating the C-index and plotting the calibration curves.

Comprehensive Analysis of Molecular and Subgroups Characteristics in the Model

Gene set enrichment analysis (GSEA) and the frequency of gene mutations were analyzed in high- and low-risk groups by utilizing the Maftools package of R. Furthermore, the association of high- and low-risk groups with TIME was also validated. CIBERSORT (https://cibersort.stanford.edu/) was used to input the data and perform 1,000 iterations to explore the 22 immune cells' proportions. In addition to this, we compared the difference of 22 immune cells' related function between the two subgroups. The correlation between risk score and common oncogene (*EGFR*, *ALK*, *ROS1*, *KRAS*, and *TP53*), *CD274*, and stem cell index (DNAss and RNAss) were also explored. Then, correlation analysis was performed between tumor mutation burden (TMB) and risk score, and the difference analysis between TMB and the subgroups was also explored. We also explored the distribution of every samples classified by clinical parameters between the subgroups, and the difference of stage and immunophenotyping was further demonstrated. Finally, the drug sensitivity analysis of every hub gene was demonstrated using CellMiner.

To gain a deep understanding of the key genes in the model, we use Oncomine (https://www.oncomine.org), UALCAN

(http://ualcan.path.uab.edu), Kaplan–Meier (http://kmplot.com), TIMER (https://cistrome.shinyapps.io/timer/), GEPIA (https://gepia.cancer-pku.cn), and other web-based bioinformation tools to perform differential analysis, survival analysis, immune infiltration analysis, and correlation analysis in LUAD. In the correlation analysis, so as to represent the strength of the interrelationship between gene expression and tumor immune infiltration in TIMER, we categorized it as follows: 0.00–0.19, "very weak"; 0.20–0.39, "weak"; 0.40–0.59, "moderate"; 0.60–0.79, "strong"; and 0.80–1.0, "very strong".

Cell and Stem Cell Culture

We purchased human bronchial epithelial cells (Beas-2B) and human LUAD cell lines (A549 and HCC827) from American Type Culture Collection (USA). Beas-2B was cultured with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, USA). We utilized RPMI-1640 medium (Biological Industries, Israel) with 10% fetal bovine serum to sustain A549 and HCC827 cell lines. Further, we cultured cells at 37°C with an atmosphere of 5% CO2. Then, the pretreated cells (A549 and HCC827) were suspended in DMEM/F12 medium and added with 20 ng/ml EGF (Sigma), 20 ng/ml bFGF (BD Biosciences), and 2% B27 (Gibco; Thermo Fisher Scientific, USA) to further study stem cells. The mRNA expression levels of SCIRGs in the model were detected by RT-PCR.

Quantitative Real-Time Polymerase Chain Reaction

We take the cell line with a good growth status, using TRIzol reagent for total RNA extraction, and further transcribed into cDNA by reverse transcription. RT-PCR was performed using the SYBR qPCR mix (Takara Bio Inc) in the 7500 real-time PCR system (Thermo Fisher Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was selected as the standardized endogenous reference. See **Supplementary Table 1** for the primer sequences of GAPDH and SCIRGs in the model.

Statistical Analysis

All analyses were performed using R version 4.1.1. The purpose of every statistical analysis was described in the specific section in Method. Experiments in this study were performed in triplicate with the statistical results presented as means ± standard deviation (SD) using GraphPad Prism Software (version 9.3, CA, USA). Student t-test was applied to compare the differences between the two groups. Differences were considered statistically significant if the p-value was < 0.05.

RESULTS

Routinely Analyze the Characteristics of mRNAsi in LUAD

Figure 1 provides a flow blueprint of the analysis process. The overall process is mainly divided into method development, SCIRGs screening, model validation, and key gene identification.

After dividing tumor group into two subgroups according to the median (high–mRNAsi level and low–mRNAsi level groups), survival analysis did not show any considerable difference between them ($p > 0.05$) (**Figure 2A**). This suggested that we needed to explore the significance of mRNAsi in LUAD from other perspectives. We then mined the correlation of mRNAsi with clinical parameters (age, sex, and TNM). The results exhibited that the mRNAsi level of the group that was younger than 55 years old was higher than that of the group which was greater than 55 years old ($p < 0.05$) (**Figure 2B**); the mRNAsi level of the male was higher than that of the female ($p < 0.05$) (**Figure 2C**). Moreover, in terms of tumor stages, the mRNAsi level was markedly different in T stages ($p < 0.05$), and it showed a gradually increasing trend (**Figure 2D**). The relationship between mRNAsi and clinical factors laid the foundation for us to further screen for genes related with mRNAsi.

Most Significant Modules of mRNAsi *via* WGCNA and Module Function and Pathway Enrichment Analysis

Through difference analysis, we found that the mRNAsi level in the tumor group was markedly higher than in normal group ($p < 0.001$), and then the DEGs were screened out for the following analysis (**Figures 2E, F**). WGCNA was further executed on DEG to sort out gene coexpression modules. The power $\beta = 3$ was used to determine a scalefree topology index (R^2) of 0.97, and dynamic hierarchical tree cutting algorithm was adopted to detect coexpression module (**Supplementary Figures 1A–C**). Ten modules were obtained in mRNAsi (**Figure 3A**). MEturquoise ($R = 0.78$, $p < 0.001$) and MEblue($R = -0.6$, $p < 0.001$) had the most significant correlations with mRNAsi, and we finally selected genes whose module membership was greater than 0.8 and gene significance for mRNAsi was greater than 0.5 in the two modules for further analysis (**Figures 3B, C**).

Then, we analyzed the function and pathway enrichment of two modules, respectively. In GO enrichment analysis, it was found that the MEturquoise module more participated in tumor growth and reproduction than MEblue module. For example, in BP, the MEturquoise module was enriched in chromosome segregation, nuclear division, nuclear chromosome segregation, DNA replication, etc. In CC, MEturquoise module was enriched in chromosome region, spindle, condensed chromosome, etc. In MF, MEturquoise module was enriched in ATPase activity, tubulin binding, microtubule binding, DNA replication origin binding, etc. Whereas MEblue module was mainly enriched in tumor microenvironment such as vasculogenesis and may have some relationship in tumor metastasis (**Figures 3D, E**). Similarly, KEGG pathway enrichment analysis exhibited that in the MEturquoise module, genes were related to cell cycle, DNA Replication, p53 signaling pathway, cell senescence, mismatch repair, and base excision repair; whereas in the MEblue module, genes were enriched in cell adhesion molecules and vascular smooth muscle contraction, which may play an important role in tumor metastasis (**Figures 3F, G**).

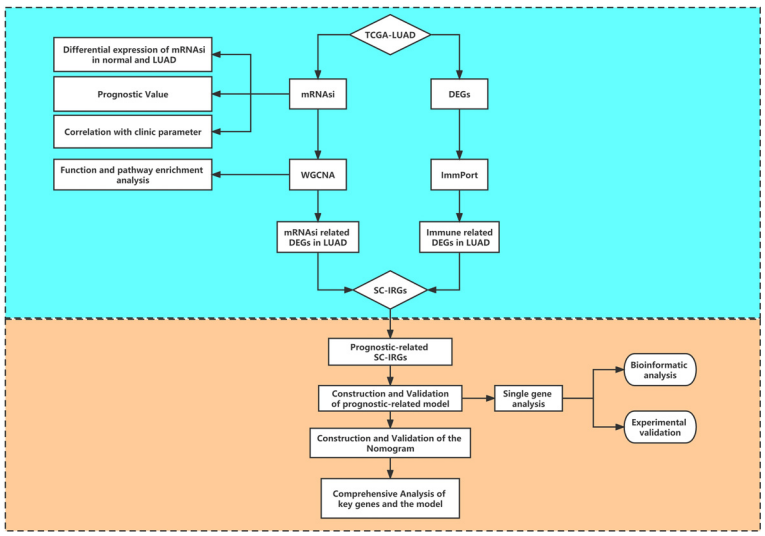


FIGURE 1 | Flow diagram of the study.

Screening for Prognosis-Related SCIRGs and Univariate COX Regression Analysis and Construct Model

Through the heat map and volcano map (**Figures 4A, B**), we found that in LUAD, there were 359 immune-related DEGs, including 168 downregulated genes and 191 upregulated genes. Then, we intersected these upregulated and downregulated immune-related DEGs with MEturquoise and MEblue modules, respectively. The intersection genes related to both mRNAsi and immunity were obtained (**Figure 4C**). Among them, the intersection of MEturquoise module and immune downregulated genes (IRDEG_down) contained 26 genes, whereas the intersection of MEturquoise module and immune upregulated genes (IRDEG_up) got 48 genes. The intersection of MEblue module and immune downregulated genes (IRDEG_down) contained 69 genes, whereas the intersection of MEblue module and immune upregulated genes (IRDEG_up) got 11 genes. The 154 genes were used for sorting out prognosis-related SCIRGs further.

Through univariate COX regression analysis, we sorted out the prognosis-related SCIRGs among the intersection genes. The

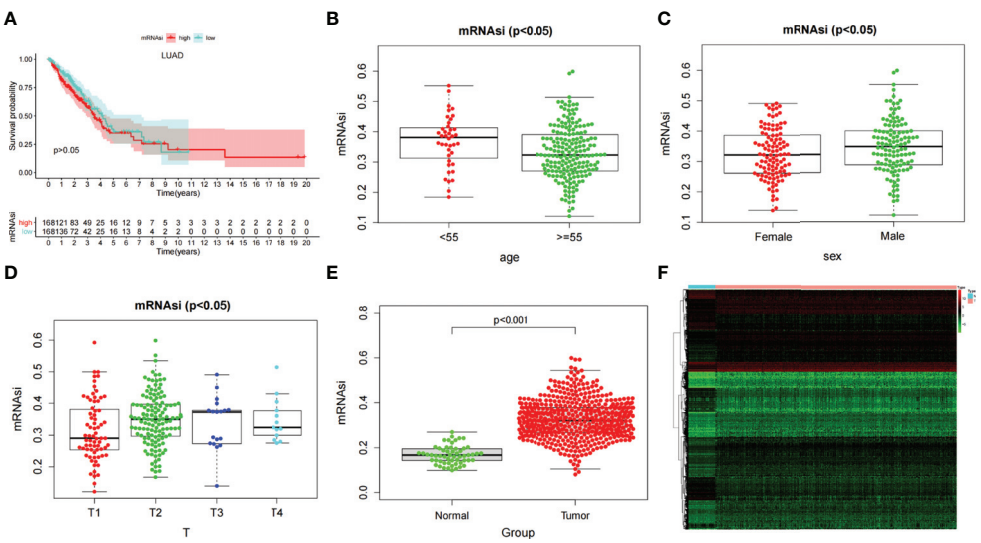


FIGURE 2 | **(A)** Kaplan–Meier displays no significant difference between the high- and low-mRNAsi groups. **(B–D)** The correlation of global mRNAsi profiles with LUAD clinical subtypes: **(B)** age, **(C)** sex, and **(D)** T staging. **(E)** Different analysis of the mRNAsi level between normal and LUAD tissues. **(F)** The heat map of DEGs in LUAD.

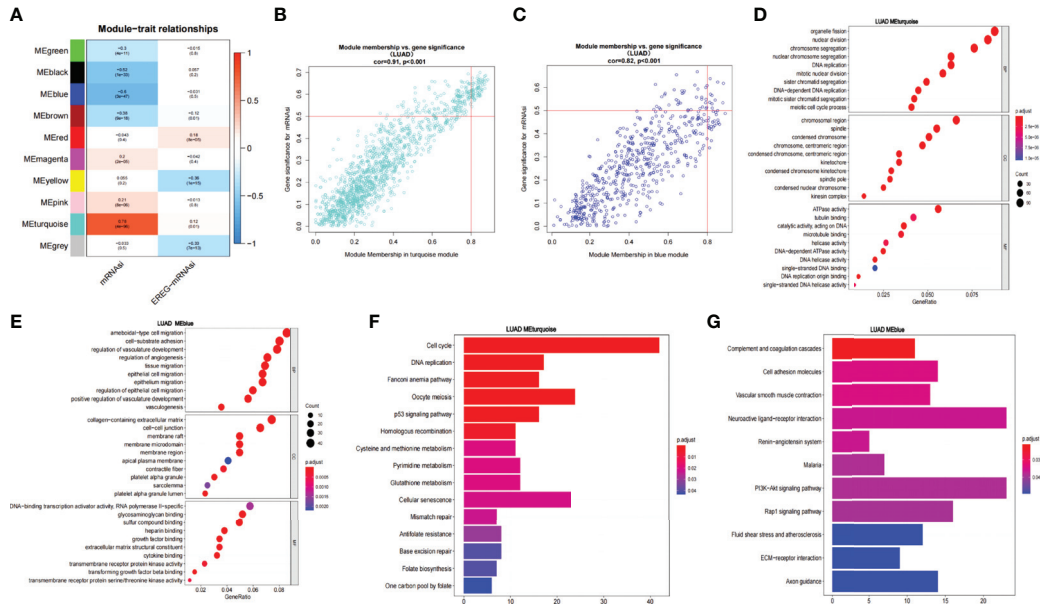


FIGURE 3 | WGCNA of LUAD and enrichment analysis of the significant modules. **(A)** Correlation of the gene module with mRNAsi and EREG-mRNAsi. **(B, C)** Scatter graph of the blue module (module membership vs. gene significance). Scatter graph of the turquoise module (module membership vs. gene significance). **(D, E)**. GO enrichment analysis of the blue and turquoise modules. **(F, G)** KEGG pathway enrichment analysis of the blue and turquoise modules.

HR of *ANGPTL7*, *ADRB2*, *SHC3*, *CX3CR1*, *VIPR1*, *CTSG*, *GDF10*, *ANGPT1*, *TEK*, *LIFR*, *IL3RA*, *TNFSF13*, *ARRB1*, *S1PR1*, *CAT*, *AGER*, *A2M*, and *SFTPD* were <1, which indicated that those genes were low-risk genes; whereas for the HR of *MET*, *HDGF*, *CRABP1*, *MIF*, *ANGPTL4*, *GPI*, *CBLC*, *BIRC5*, *PAK1*, *SEMA3A*, *GPER1*, and *BDNF*>1, it indicated that those genes were high-risk genes (**Figure 4D**). Furthermore, LASSO regression was executed to select the optimal predictive factors (genes), preventing overfitting, and then to build a LASSO Cox regression model.

We finally got 16 genes to construct LASSO Cox regression model (Supplementary **Figures 1D, E**). The formula for the model is as follows: risk score = 0.02733 * *BDNF* + 0.004734 * *GPI* + (−0.05939) * *CX3CR1* + 0.00120 * *MET* + 0.00960 * *SEMA3A* + 0.00503 * *GPER1* + (−0.00995) * *ARRB1* + (−0.02840) * *LIFR* + 0.00206 * *CRABP1* + 0.00804 * *PAK1* + (−0.02285) * *IL3RA* + (−0.05521) * *SHC3* + (−0.00924) * *VIPR1* + 0.00051 * *CBLC* + (−0.02154) * *ADRB2* + 0.00719 * *ANGPTL4* (23).

Validation of the Model and Construction and Validation of the Nomogram

To demonstrate whether the final model was robust in different populations, we singled out a cutoff value in the internal training set (TCGA-LUAD) and performed an identical formula in external validation set (GSE68465). According to the median risk value in the TCGA dataset, patients were separated into high-risk groups and low-risk groups. Comparing with the low-risk group, the high-risk group showed a better prognosis both in the TCGA and GEO datasets (**Figures 4E, F**).

Heat map shows that the expressions of *ANGPTL4*, *GPI*, *CBLC*, and *PAK1* are higher in the high-risk group than in the low-risk group, regardless of the training set or the validation set, which pointed out that they may be carcinogenesis. On the contrary, in the low-risk group, the expressions of *IL3RA*, *CX3CR1*, *ARRB1*, *LIFR*, and *VIPR1* were higher than those in the high-risk group, which signified that they have a tumor suppressor effect. Risk curves and survival status maps showed same trends in TCGA and GEO, patients with higher scores were more likely to have a poorer prognosis (**Figures 4G-I**; **Supplementary Figure 2**).

Univariate- and multivariate-independent prognostic analyses were carried out to explore the correlation between prognosis and clinical parameters and risk score and verified in the TCGA and GEO dataset, respectively. Through univariate-independent prognostic analysis, in the TCGA dataset, the clinical parameters T, N, and M staging, stage, and risk score were associated with prognosis; whereas in GEO dataset, gender, age, T and N staging, and risk score were related to prognosis (**Figures 5A, B**). Through multivariate-independent prognostic analysis, it revealed that in the TCGA dataset, risk score was related to prognosis; whereas in the GEO validation set, T and N staging and risk score were associated with prognosis (**Figures 5C, D**). These indicated that risk score was independent of clinical parameters to be a prognostic parameter. To go step further, we assessed the prediction accuracy of the model through ROC curve. The areas under curves (AUCs) of the risk score were 0.712 in TCGA and 0.661 in GEO dataset, respectively. Comparing with other clinical parameters, the model had the largest value of AUC in TCGA

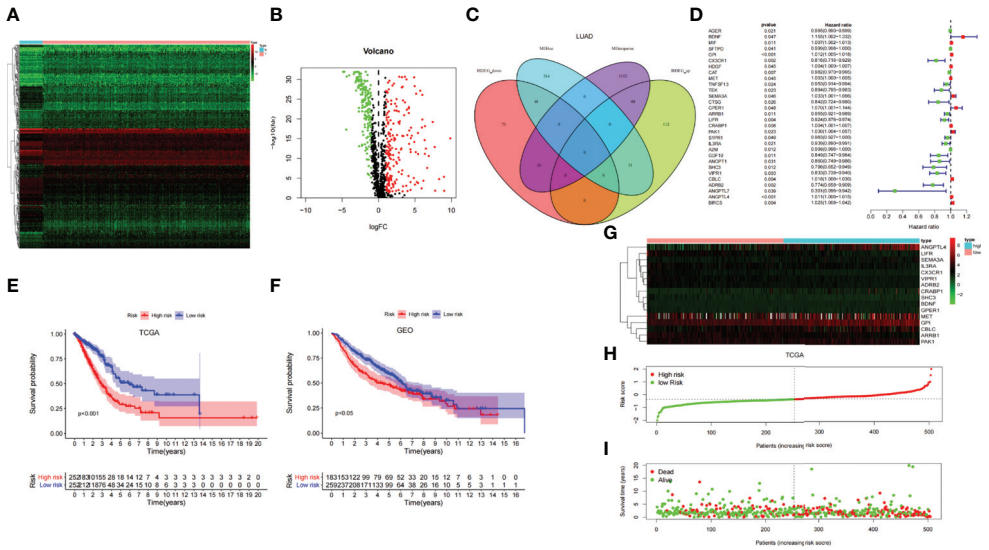


FIGURE 4 | **(A)** The heat map of immune-related DEGs in LUAD. **(B)** Volcano map of immune-related DEGs in LUAD. Green, downregulated genes; red, upregulated genes. **(C)** Venn diagram of the intersection genes related to both mRNAsi and immunity. **(D)** Univariate COX regression analysis of prognosis-related stem cell and immune-related differential genes (SCIRGs) in LUAD. **(E, F)** Kaplan–Meier curves show a considerable difference between the high- and the low-risk groups. **(G)** Heat maps of the hub genes' expression pattern, where the red to green means changes from high to low expression in TCGA. **(H)** Distribution of multi-genes signature risk score in TCGA datasets. **(I)** The survival status and interval of TCGA-LUAD patients.

dataset; whereas in GEO dataset, it also had the second largest value of AUC except for the N Staging (**Figures 5E, F**). This indicated that the risk score may be a better parameter with better sensitivity and specificity for predicting prognosis.

For the convenience of application, we have constructed a nomogram. Age, gender, TNM staging, stage, and risk score were utilized as predictive parameters to construct the nomogram (24), and we calculated the total points to obtain the 1-, 2-, and 3-year overall survival in LUAD (**Figure 6A**). Furthermore, for the accuracy of the model, we used the consistency index (C-index) and calibration curve to estimate. The C-index was 0.699 (0.649–0.749). The horizontal and

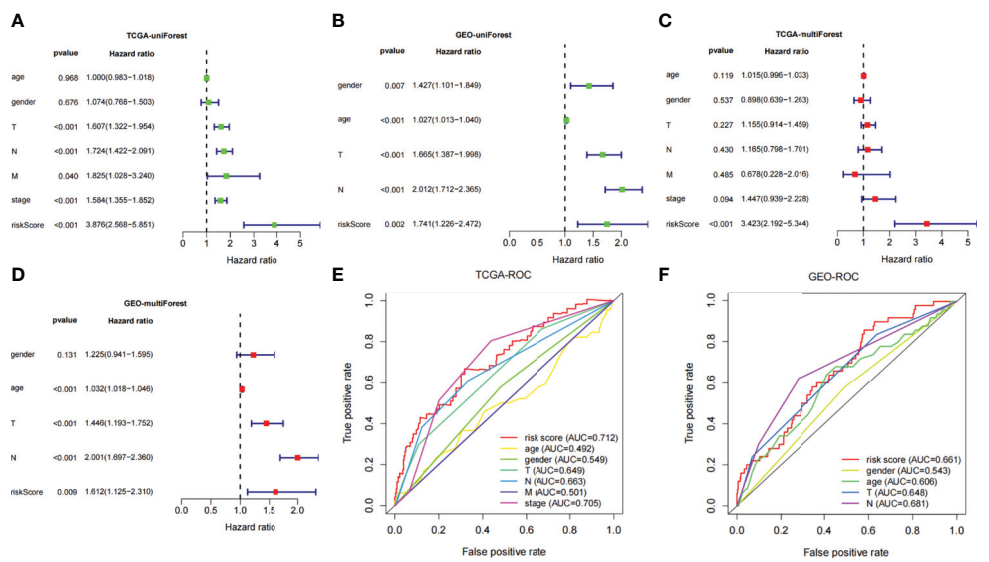


FIGURE 5 | **(A, B)** Univariate Cox regression analyses of overall survival in TCGA and GEO dataset. **(C, D)** Multivariate Cox regression analyses of overall survival in TCGA and GEO dataset. **(E, F)** Comparing the AUCs of the risk scores with other clinical parameters in TCGA and GEO dataset.

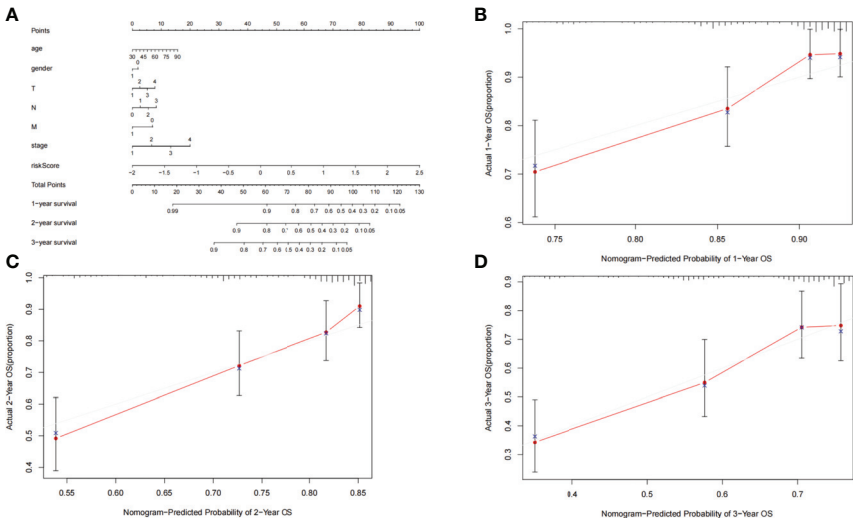


FIGURE 6 | **(A)** Nomogram was assembled by clinical parameters and risk signature for predicting survival of patients with LUAD. **(B)** One-year nomogram calibration curves. **(C)** Two-year nomogram calibration curves. **(D)** Three-year nomogram calibration curves.

vertical coordinates of every calibration curves represented the predicted probability and actual probability of every year overall survival (**Figures 6B–D**). The results of the calibration graph exhibited that the nomogram has a good capability to foresee the overall survival rate of patients with LUAD.

Comprehensive Analysis of Gene and Immune Characteristics in the Model

Immune Characteristics of the Key Genes and the Model

The infiltration proportion of every immune cell in the two risk groups is shown, respectively (**Figure 7A**). Plasma cells, CD8 T cells, activated memory CD4 T cells, M0 and M1 macrophages, and activated mast cells were more abundant in the high-risk subgroup; correspondingly, immune-related function like inflammation-promoting, MHC class I, NK cells, and Tfh were more frequency in the high-risk subgroup. Whereas memory B cells, memory resting CD4 T cells, monocytes, M2 macrophages cell, resting dendritic cells, resting mast cells, etc., were more abundant in the low-risk subgroup; correspondingly aDCs, B cells, DCs, HLA, mast cells, etc., were more common in the low-risk group (**Figures 7B, C**). The association of risk score with TIME shows that both immune score and stromal score were negatively relevant to risk score (**Figures 7D, E**).

Furthermore, to prove the relevance of these genes to immunity, we compared the correlation between hub genes and immune cells through TIMER. *ADRB2* has moderate correlation with dendritic cell; *CX3CR1* has moderate correlation with macrophage and neutrophil; IL3RA also has moderate correlation with neutrophil and dendritic cell. Apart from these genes, other genes also have weak correlation with immune cell (**Figures 7F–I**; **Supplementary Figure 3**).

Clinical Characteristics of the Key Genes and the Model

The relationship between risk score and TMB was further probed. The results exhibited that TMB was markedly higher in the high-risk group than in the low-risk group, and the higher the risk score, the larger the TMB (R = 0.31, p = 4e−12; **Figures 8A, B**). To explore the difference of every samples classified by clinical parameters between those two groups, clinical relevance heat map was used. In **Figure 8C**, age and T staging have markedly difference between the two groups. We also found that the proportion of stage IV samples has almost equal distributions between the two groups, and there were more samples in the high-risk subgroup and fewer samples in the low-risk subgroup in stages II–III, but there was an opposite result in stage I (p = 0.003, chi-square test) (**Figure 8D**). Then, 446 TCGA samples were further classified according to immune subtype. As shown in **Figure 8E**, there were more C3 subtypes in the SCIRG-low subgroup, whereas more C1 and C2 subtypes in SCIRG-high subgroup (p = 0.001, chi-square test).

Finally, the SCIRG gene was analyzed in combination with drug sensitivity, and the first 16 drugs with statistically significant differences were selected. The results uncovered that the expression level of *CX3CR1* was positively relevant to the sensitivity of Alectinib, LDK-378, Denileukin Diftitox Ontak, Estramustine, Nelfinavir, PF-06463922, and Carmustine. This indicated that the higher the expression of *CX3CR1*, the stronger the sensitivity to the abovementioned drugs. We also ascertained that the *CX3CR1* expression level was negatively relevant to the sensitivity of Irofulven. In addition, *CRABP1* expression level was positively relevant to the sensitivity of Bendamustine and Dexrazoxane. The expression of *MET* was negatively relevant to the sensitivity of Bendamustine and Dexrazoxane. The expression of *LIFR* and *BDNF* was negatively relevant to the

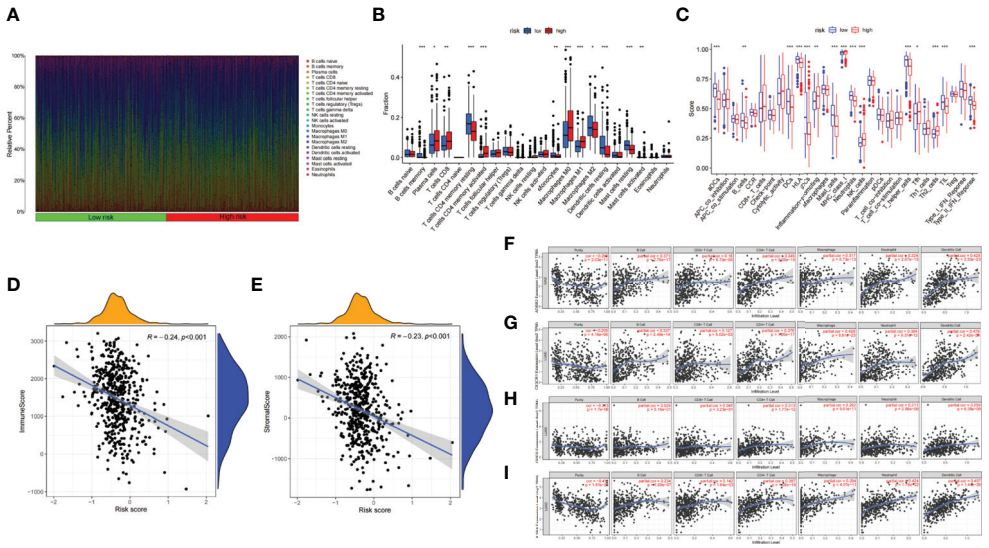


FIGURE 7 | **(A)** Bar plot presents the distribution of 22 kinds of TICs in LUAD tumor samples. Column names represent sample ID. **(B)** Bar plot presents the difference of TICs between the high- and low-risk groups. **(C)** Bar plot presents the difference of immune-related function between the high- and low-risk groups. **(D, E)** Association between tumor immune microenvironment (TIME) and risk score. **(D)** ImmuneScore; **(E)** StromalScore. **(F, I)** TIMER: Immune correlation analysis of SCIRGs in the model based on immune infiltration, **(F)** *ADRB2*, **(G)** *CX3CR1*, **(H)** *GPER* (*GPER1*), and **(I)** *IL3RA*. *p < 0.05, **p < 0.01, ***p < 0.001.

sensitivity of Tamoxifen, whereas the expression of *GPER1* was positively relevant to Procarbazine (**Supplementary Figure 4**).

General Characteristics of the Key Genes and the Model

We then implement GSEA analysis to find out in which function the two subgroups of genes were up- or downregulated. For example, the genes of the high-risk group were upregulated in chromosome segregation, cornification, DNA dependent, DNA replication, and epidermal cell differentiation, whereas they also upregulated in cell cycle, DNA replication, proteasome, pyrimidine metabolism, and spliceosome in KEGG. This tells us that the high-risk group was mainly correlated with proliferation in LUAD. On the other hand, the genes of the

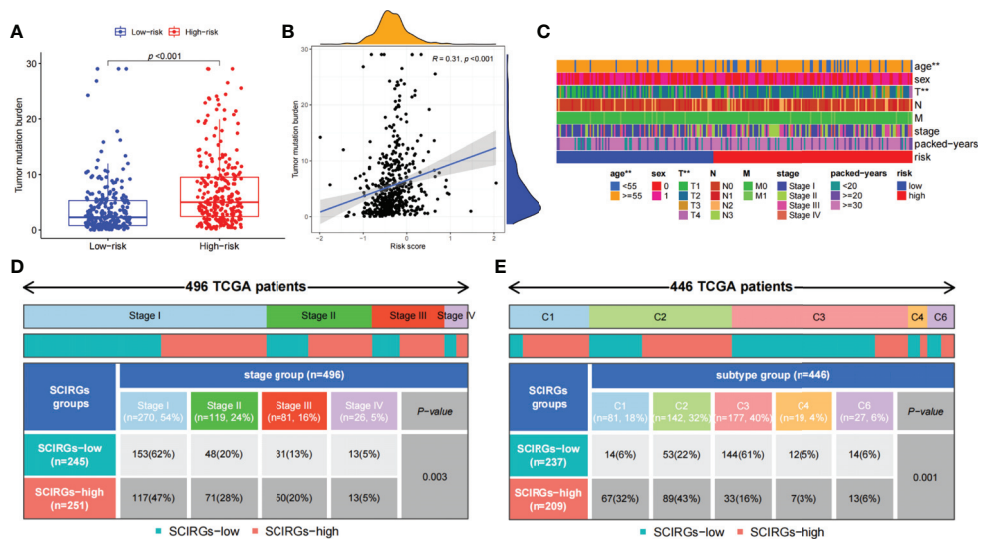


FIGURE 8 | **(A)** The difference of TMB between the high- and low-risk groups. **(B)** Association between TMB and risk score. **(C)** The proportion of clinical characteristics of every sample in relative risk group was presented in heat map. **(D)** Proportion of patients in different stages of high- and low-risk groups. **(E)** Proportion of patients in different immune sub-typing of high- and low-risk groups. *p < 0.05, **p < 0.01, ***p < 0.001.

low-risk group were downregulated in cilium movement, rDNA heterochromatin assembly, ciliary plasma, cilium, and DNA packaging complex in GO, whereas they also have the same performance in asthma, Fc epsilon RI signaling pathway, long-term depression, systemic lupus erythematosus, and vascular smooth muscle contraction in KEGG (**Supplementary Figures 5A–D**, $p < 0.05$). Next, we analyzed gene mutations to gain further insight in the charicteristics of the subgroups. We found 96.03% samples were altered in high-risk groups, whereas 80.58% samples were altered in low-risk groups. Missense variations were the most common mutation type (**Supplementary Figures 5E, F**). In addition to this, the risk score was also markedly relevant to common oncogenes expression, such as *ALK*, *ROS1*, *KRAS*, and *TP53*, but no markedly relevant to CD274 (**Supplementary Figure 6**).

Here, to find out potential biomarkers in LUAD, we explored the 16 genes in the model through bioinformatics. First, Oncomine was used to explore the overall difference of the above genes in lung cancer, and UALCAN was utilized to seek every gene differential expression between LUAD and normal. As shown in **Supplementary Figures 7**, **8**, whether in Oncomine or in UALCAN, *ADRB2*, *ARRB1*, *BDNF*, etc., had a lower expression in tumor group than normal tissues, whereas *CBLC*, *GPI*, and *PAK1* had a higher expression in tumor group than normal tissues. There were no studies of *ANGPTL4*, *CRABP1*, *MET*, and *SEMA3A* in Oncomine, but in UALCAN, they were frequently expressed in tumor group than normal tissues. Second, the survival analysis of key genes in LUAD was analyzed by the Kaplan–Meier method. The results exhibited that the high expression groups of *ANGPTL4*, *CBLC*, *CRABP1*, etc., have a poorer prognosis in LUAD than the low expression group, whereas the survival analysis of *ADRB2*, *CX3CR1*, *GPI*, etc., in LUAD exhibited that the prognosis was better in the high expression group (**Supplementary Figure 9**). To further understand the correlation between these key genes and common oncogenes such as *TP53*, *EGFR*, and *CD274*, we explored the correlation through GEPIA (spearman, P value < 0.05 and $R > \pm 0.1$). Genes related to TP53 include *ANGPTL4*, *ARRB1*, *CBLC*, etc.; genes related to *EGFR* include *ADRB2*, *ANGPTL4*, *ARRB1*, etc; genes related to *CD274* include *ADRB2*, *BDNF*, *CBLC*, etc. (**Supplementary Figures 10–12**).

Experimental Verification of the Key Genes

We respectively compared the expression of the SCIRGs in normal lung epithelial cells, lung cancer cells, and lung CSCs and repeatedly compared them in different cell lines (A549 and HCC827) (**Figure 9**; **Supplementary Figure 13**). The results showed that in the A549 cell line, the expression results of nine genes in the 16 genes were consistent with those in UALCAN (**Figure 9**; **Supplementary Figures 8**, **13**): *ADRB2*, *ANGPTL4*, *BDNF*, *CBLC*, *CRABP1*, *CX3CR1*, *GPI*, *IL3RA*, and *SCH3*; in the HCC827 cell line, the expression results of nine genes among the 16 genes were consistent with those in UALCAN: *ADRB2*, *ANGPTL4*, *BDNF*, *CBLC*, *CX3CR1*, *IL3RA*, *LIFR*, and *MET*. Therefore, half or more of the genes in our model were consistent with the gene expression results of external data.

DISCUSSION

Since De Maria et al. have found that the *CD133* undifferentiated cells in LUAD can produce tumor xenografts that have the same phenotype with the primary LUAD in mmunodeficient mice, more and more studies began to identify lung CSC-related biomarkers and explore the characteristics of stem cells in growth, reproduction, metastasis, drug resistance of lung caner, etc. (5, 25). In addition to the discovery that *CD133* and *ALDH1* can be as biomarkers of lung CSCs, there were many explorations on the self-renewal, metabolism, drug resistance of LUAD stem cells, and even gene expression profile analysis (26–29). The ability to produce differentiated cells and to self-renew was the characteristic of stem cells, and stemness was defined as the potential for self-renew and differentiation from the cell of origin (30). To define signatures to quantify stemness and to estimate the degree of carcinogenic dedifferentiation, previous studies utilized a set of logistic regression machine learning algorithms (OCLR) to generate a stemness index (14). In recent years, its significance had been confirmed by the bioinformatics analysis in various tumors (31, 32), which also included the stemness indices of LUAD (15–18). However, few studies have combined stemness indices and immunity to construct models and explore stem cell index and immune-related differential genes in LUAD. In recent years, tumor microenvironment infiltration and tumor immunotherapy have played an important role in LUAD (33, 34). Therefore, we combined stemness indices and immune-related differential genes to construct model and explore the significance of these genes in LUAD.

Throughout the current research on mRNAsi, many studies have found the difference of mRNAsi between tumor and normal group in NSCLC. The difference analysis, survival analysis, and clinical correlation analysis of mRNAsi also have certified that mRNAsi was indeed markedly higher in tumor group than in the normal group, and it has a certain correlation with various clinical parameters in LUAD. The module that contained the highest correlation with mRNAsi was found through WGCNA and finally found and verified hub genes of LUAD. Some studies further combined the key genes with clinical parameters to construct models to help predict prognosis (15–18). Previous studies have found that the stemness was a crucial part in anti-cancer immunity (35), but the abovementioned studies did not combined mRNAsi with immunity nor did it explore the high- and low-risk groups in the model. In addition, although some of the studies selected clinical samples for verified the model, they neither explain the subgroups characteristic in the model nor construct a nomogram. As the correlation between tumor prognosis, treatment and immunity have been demonstrated by current tumor immunotherapy, there was still a need for research to explore the relationship and mechanism between immunity and tumors. Therefore, in view of previous studies of mRNAsi in LUAD, our study combined mRNAsi with IRGs from the current immune database ImmPort, intersected the mRNAsi-related modules obtained by WGCNA with immune-related differential genes, finally obtained the SCIRGs. In the subsequent construction and verification of model, the previous studies did not carry out internal and external verification of the

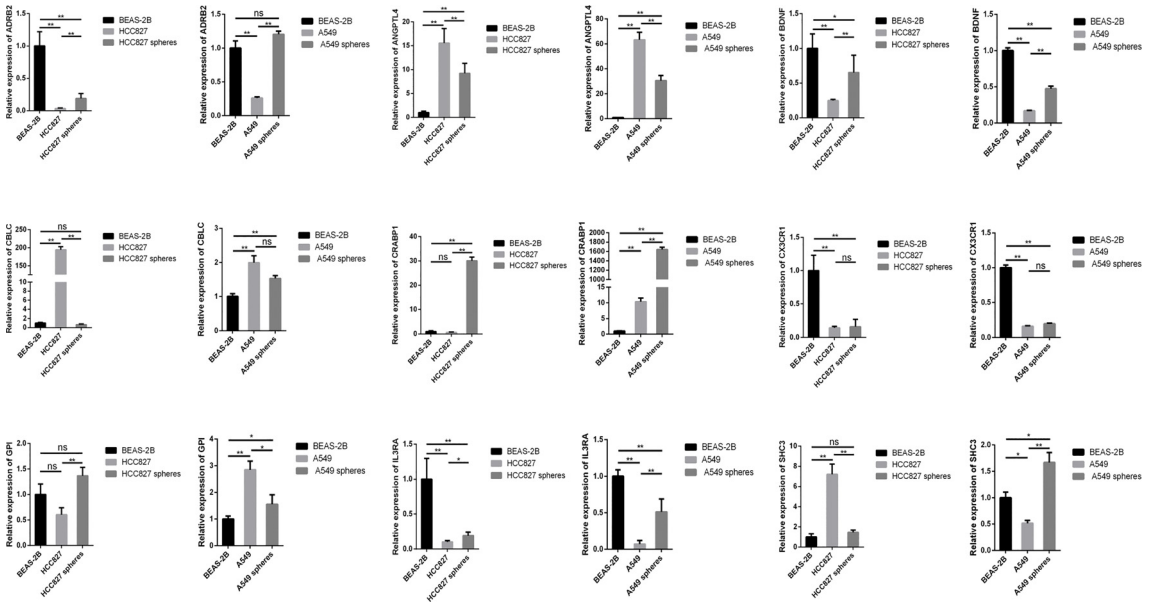


FIGURE 9 | The expression levels of SCIRGs in the model between Beas-2B, HCC827 cell lines, HCC827 cancer stem cell, and results of the RT-PCR to determine gene expression. *p < 0.05, **p < 0.01, ***p < 0.001; ns, no significance.

model. In contrast, our study used the TCGA and GEO datasets for internal and external verification, and the consistency of internal verification and external verification provided a reliable basis for the application of our model. We found risk score was regarded as a risk factor both in the risk curve of **Figure 6** and independent prognostic analysis of **Figure 7**, but when using Kaplan–Meier to verify the survival analysis of the model, we found that the prognosis of the high-risk group was better than the low-risk group no matter in the internal or external datasets. This suggested that it may have other factors that affect the prognosis of patients with risk score. The potential mechanism is worthwhile for further discussion in the future. In addition, we further explored the differences in function and enrichment, gene mutation frequency, immune cell type, immune-related function, and TMB and explored the differences of clinical features in the high- and low-risk groups. As a result, more immune-related functions were higher in the low-risk group than in the high-risk group, the risk score was negatively relevant to tumor immunity and positively relevant to tumor mutation burden. The relevant mechanisms of this phenomenon can be further explored in the future. As there were few similar studies at present, our study was enriched for the research on stem cell index combined with immunity in LUAD and confirmed the conclusions of previous studies. Incidentally, mining the correlation between classic oncogenes and immune genes and risk scores exploring the situation of each key gene in the model were also the difference between our study and current study.

For the key genes in the model, *c-Met* that is a part of *RTKs* family is a known CSC marker in previous study (36). *Met* and its ligand, *HGF*, were core roles in signaling pathways of the oncogenic process, which was included the regulation of angiogenesis, cell proliferation, invasion, and CSC regulation (37). In addition, in the previous study of NSCLC, *MET* amplification was particularly related to the inflammatory microenvironment, indicating that MET-amplified tumor might respond to ICIs (38). Over the years, previous studies have found and well replicated the roles of neurotrophins in tumor development. In particular, it was reported that nerve growth factor (*NGF*) and brain-derived neurotrophic factor (*BDNF*) could stimulate tumor cell proliferation, survival, migration, and/or invasion and was beneficial to tumor angiogenesis (39). Adrenergic receptors (*ARs*), especially *β-ARs*, are expressed in most mammalian cells and relevant to kinds of malignancies including lung cancer (40). *ADRB2* encodes β-2-adrenergic receptor. Previous study has found that Beta2-AR was highly expressed in both LUAD and LUSC but clearly highly expressed in LUAD when compared with LUSC and with their matched surrounding non-tumor tissue (41). In addition, the cross-talk between macrophages and cancer cells through *CX3CR1* and *CCR2* is the basic mechanism resulting to lung cancer (42). The knockdown of *PAK1* hinders the proliferation and invasion of NSCLC (43). *ANGPTL4* was relevant to NSCLC progression and regulated epithelial-mesenchymal transition *via* ERK pathway, indicating that *ANGPTL4* is vital for the proliferation and metastasis of lung cancer, and may regard as a brand-new target for the treatment of lung cancer (44). There are many studies showing the significance of key genes in our model in LUAD or CSC. Our RT-qPCR results found that even for the key genes in the model, there were significant differences of many gene expressions between CSCs and cancer cells. We speculate that the difference was related to the underlying mechanisms of CSCs.

Moreover, we ascertained that the different expression of *ADRB2*, *ANGPTL4*, *BDNF*, *CBLC*, *CX3CR1*, and *IL3RA* in tumor and normal group was consistent both in PCR and UALCAN. Combined with the previous analysis, it is indispensable to further analyze the underlying system of CSCs and the above genes in lung cancer in future research.

CONCLUSIONS

Our research explored genes to construct the current model from the perspective of combining stem cell index and immunity and analyzed and verified the model *via* multi-omics analysis. At the same time, it verified the characteristics of genes in the model through bioinformatics analysis and experiments. However, our study neither analyzes the mechanism of CSC through laboratory methods nor explores the mechanism of genes in the model in lung cancer through experimental methods. In addition, the robust of the prognostic model required more clinical samples and experiments for demonstration. In the future, more research studies are needed to explore from the above directions.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available in the TCGA, GEO under the accession number GSE68465, Oncomine, TIMER, UALCAN, Kaplan–Meier Plotter, and GEPIA.

AUTHOR CONTRIBUTIONS

Conception and design of the work: MC and ZL. Acquisition, analysis, and interpretation of data: XW, WW, and XG. Drafting and revising of the article: MC and ZL. Final approval of the manuscript and agreement to be accountable for all aspects of the work: all authors. All authors contributed to the article and approved the submitted version.

FUNDING

This work is supported by Grants from Natural Science Foundation of Chongqing (cstc2020jcyj-msxmX0227).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.829057/full#supplementary-material

Supplementary Figure 1 | (A, B).Network topology analysis for soft-thresholding powers. **(A)** the scale-free fit index, signed R^2(Y) and the soft threshold power(X). **(B)** the mean connectivity(Y) and the soft threshold power (X). Choose β=3 for the subsequent analysis. **(C)**. The cluster dendrogram. In the figure, each limb represents one gene, and every color below represents one coexpression module. **(C, D)**.LASSO coefficient profiles of 30 prognostic genes for LUAD.

Supplementary Figure 2 | (A). Heat maps of the hub genes' expression pattern, where the red to green means changes from high to low expression in TCGA and GEO. **(B)**. Distribution of multi-genes signature risk score in TCGA and GEO datasets. **(C)**. The survival status and interval of LUAD patients.

Supplementary Figure 3 | Immune correlation analysis of SCIRGs in the model based on immune infiltration.

Supplementary Figure 4 | Association between drug sensitivity and SCIRGs in the model.

Supplementary Figure 5 | (A, B).GSEA of the high and the low-risk group(GO). **(C, D)**. GSEA of the high- and low-risk groups(KEGG). **(E, F)**. The oncoPrint of high- and low-risk groups, the top 20 mutated genes and their mutational types and percentages are visualized in detail.

Supplementary Figure 6 | Association of risk score with classical gene expression and stem cell index. **(A)** *EGFR*, **(B)** *ALK*, **(C)** *ROS1*, **(D)** *KRAS*, **(E)** *TP53*, **(F)** *CD274*, **(G)** DNAss, **(H)** RNAss.

Supplementary Figure 7 | The expression level of SCIRGs in the model in different types of tumor and normal tissues *via* Oncomine.

Supplementary Figure 8 | The expression level of SCIRGs in the model from UALCAN.

Supplementary Figure 9 | Kaplan–Meier curves compare the OS time of the SCIRGs subgroups in LUAD.

Supplementary Figure 10 | The correlation between these key genes and *EGFR*.

Supplementary Figure 11 | The correlation between these key genes and *TP53*.

Supplementary Figure 12 | The correlation between these key genes and CD274.

Supplementary Figure 13 | the expression levels of SCIRGs in the model between Beas-2B, A549 cell lines, A549 cancer stem cell, results of the RT-PCR to determine gene expression.

Supplementary Table 1 | The RT-PCR primers sequences of SCIRGs in the model.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2020. *CA: Cancer J Clin* (2020) 70(1):7–30. doi: 10.3322/caac.21590
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: Cancer J Clin* (2018) 68 (6):394–424. doi: 10.3322/caac.21492
3. Kadara H, Kabbout M, Wistuba II. Pulmonary Adenocarcinoma: A Renewed Entity in 2011. *Respirology* (2012) 17(1):50–65. doi: 10.1111/j.1440-1843.2011.02095.x
4. NCCN.org. National Comprehensive Cancer Network(NCCN) Clinical Practice Guidelines in Oncology. In: *Non-Small Cell Lung Cancer Version 1.2022* (2021).
5. MacDonagh L, Gray SG, Breen E, Cuffe S, Finn SP, O'Byrne KJ, et al. Lung Cancer Stem Cells: The Root of Resistance. *Cancer Lett* (2016) 372(2):147–56. doi: 10.1016/j.canlet.2016.01.012
6. Wu Z, Liu Z, Jiang X, Mi Z, Meng M, Wang H, et al. Depleting PTOV1 Sensitizes non-Small Cell Lung Cancer Cells to Chemotherapy Through Attenuating Cancer Stem Cell Traits. *J Exp Clin Cancer Res CR* (2019) 38 (1):341. doi: 10.1186/s13046-019-1349-y

7. Si J, Ma Y, Bi JW, Xiong Y, Lv C, Li S, et al. Shisa3 Brakes Resistance to EGFR-TKIs in Lung Adenocarcinoma by Suppressing Cancer Stem Cell Properties. *J Exp Clin Cancer Res CR* (2019) 38(1):481. doi: 10.1186/s13046-019-1486-3
8. Shien K, Toyooka S, Yamamoto H, Soh J, Jida M, Thu KL, et al. Acquired Resistance to EGFR Inhibitors is Associated With a Manifestation of Stem Cell-Like Properties in Cancer Cells. *Cancer Res* (2013) 73(10):3051–61. doi: 10.1158/0008-5472.CAN-12-4136
9. Noguchi T, Ward JP, Gubin MM, Arthur CD, Lee SH, Hundal J, et al. Temporally Distinct PD-L1 Expression by Tumor and Host Cells Contributes to Immune Escape. *Cancer Immunol Res* (2017) 5(2):106–17. doi: 10.1158/2326-6066.CIR-16-0391
10. Chang WH, Lai AG. Aberrations in Notch-Hedgehog Signalling Reveal Cancer Stem Cells Harbouring Conserved Oncogenic Properties Associated With Hypoxia and Immunoevasion. *Br J Cancer* (2019) 121(8):666–78. doi: 10.1038/s41416-019-0572-9
11. Raniszewska A, Vroman H, Dumoulin D, Cornelissen R, Aerts J, Domagała-Kulawik J. PD-L1(+) Lung Cancer Stem Cells Modify the Metastatic Lymph-Node Immunomicroenvironment in Nsclc Patients. *Cancer immunology immunotherapy CII* (2021) 70(2):453–61. doi: 10.1007/s00262-020-02648-y
12. Leon G, MacDonagh L, Finn SP, Cuffe S, Barr MP. Cancer Stem Cells in Drug Resistant Lung Cancer: Targeting Cell Surface Markers and Signaling Pathways. *Pharmacol Ther* (2016) 158:71–90. doi: 10.1016/j.pharmthera.2015.12.001
13. Clara JA, Monge C, Yang Y, Takebe N. Targeting Signalling Pathways and the Immune Microenvironment of Cancer Stem Cells - a Clinical Update. *Nat Rev Clin Oncol* (2020) 17(4):204–32. doi: 10.1038/s41571-019-0293-2
14. Malta TM, Sokolov A, Gentles AJ, Burzykowski T, Poisson L, Weinstein JN, et al. Machine Learning Identifies Stemness Features Associated With Oncogenic Dedifferentiation. *Cell* (2018) 173(2):338–54.e315. doi: 10.1016/j.cell.2018.03.034
15. Zhao M, Chen Z, Zheng Y, Liang J, Hu Z, Bian Y, et al. Identification of Cancer Stem Cell-Related Biomarkers in Lung Adenocarcinoma by Stemness Index and Weighted Correlation Network Analysis. *J Cancer Res Clin Oncol* (2020) 146(6):1463–72. doi: 10.1007/s00432-020-03194-x
16. Zhang Y, Tseng JT, Lien IC, Li F, Wu W, Li H. Mrnasi Index: Machine Learning in Mining Lung Adenocarcinoma Stem Cell Biomarkers. *Genes* (2020) 11(3). doi: 10.3390/genes11030257
17. Liao Y, Wang Y, Cheng M, Huang C, Fan X. Weighted Gene Coexpression Network Analysis of Features That Control Cancer Stem Cells Reveals Prognostic Biomarkers in Lung Adenocarcinoma. *Front Genet* (2020) 11:311. doi: 10.3389/fgene.2020.00311
18. Zeng H, Ji J, Song X, Huang Y, Li H, Huang J, et al. Stemness Related Genes Revealed by Network Analysis Associated With Tumor Immune Microenvironment and the Clinical Outcome in Lung Adenocarcinoma. *Front Genet* (2020) 11:549213. doi: 10.3389/fgene.2020.549213
19. Langfelder P, Horvath S. WGCNA: An R Package for Weighted Correlation Network Analysis. *BMC Bioinf* (2008) 9:559. doi: 10.1186/1471-2105-9-559
20. Liu Z, Liu L, Weng S, Guo C, Dang Q, Xu H, et al. Machine Learning-Based Integration Develops an Immune-Derived lncRNA Signature for Improving Outcomes in Colorectal Cancer. *Nat Commun* (2022) 13(1):816. doi: 10.1038/s41467-022-28421-6
21. Liu Z, Xu H, Weng S, Ren Y, Han X. Stemness Refines the Classification of Colorectal Cancer With Stratified Prognosis, Multi-Omics Landscape, Potential Mechanisms, and Treatment Options. *Front Immunol* (2022) 13:828330. doi: 10.3389/fimmu.2022.828330
22. Wang Z, Zhu L, Li L, Stebbing J, Wang Z, Peng L. Identification of an Immune Gene-Associated Prognostic Signature in Patients With Bladder Cancer. *Cancer Gene Ther* (2022). doi: 10.1038/s41417-022-00438-5
23. Yan C, Liu Q, Jia R. Construction and Validation of a Prognostic Risk Model for Triple-Negative Breast Cancer Based on Autophagy-Related Genes. *Front Oncol* (2022) 12:829045. doi: 10.3389/fonc.2022.829045
24. He J, Chen Z, Xue Q, Sun P, Wang Y, Zhu C, et al. Identification of Molecular Subtypes and a Novel Prognostic Model of Diffuse Large B-Cell Lymphoma Based on a Metabolism-Associated Gene Signature. *J Trans Med* (2022) 20 (1):186. doi: 10.1186/s12967-022-03393-9
25. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, et al. Identification and Expansion of the Tumorigenic Lung Cancer Stem Cell Population. *Cell Death differentiation* (2008) 15(3):504–14. doi: 10.1038/sj.cdd.4402283
26. Hsu HS, Liu CC, Lin JH, Hsu TW, Hsu JW, Li AF, et al. Involvement of Collagen XVII in Pluripotency Gene Expression and Metabolic Reprogramming of Lung Cancer Stem Cells. *J Biomed Sci* (2020) 27(1):5. doi: 10.1186/s12929-019-0593-y
27. Hsu HS, Lin JH, Huang WC, Hsu TW, Su K, Chiou SH, et al. Chemoresistance of Lung Cancer Stemlike Cells Depends on Activation of Hsp27. *Cancer* (2011) 117(7):1516–28. doi: 10.1002/cncr.25599
28. Farmakovskaya M, Khromova N, Rybko V, Dugina V, Kopnin B, Kopnin P. E-Cadherin Repression Increases Amount of Cancer Stem Cells in Human A549 Lung Adenocarcinoma and Stimulates Tumor Growth. *Cell Cycle* (2016) 15(8):1084–92. doi: 10.1080/15384101.2016.1156268
29. Seo DC, Sung JM, Cho HJ, Yi H, Seo KH, Choi IS, et al. Gene Expression Profiling of Cancer Stem Cell in Human Lung Adenocarcinoma A549 Cells. *Mol Cancer* (2007) 6:75. doi: 10.1186/1476-4598-6-75
30. Vermeulen L, Sprick MR, Kemper K, Stassi G, Medema JP. Cancer Stem Cells–Old Concepts, New Insights. *Cell Death differentiation* (2008) 15 (6):947–58. doi: 10.1038/cdd.2008.20
31. Tian Y, Wang J, Qin C, Zhu G, Chen X, Chen Z, et al. Identifying 8-Mrnasi Based Signature for Predicting Survival in Patients With Head and Neck Squamous Cell Carcinoma *via* Machine Learning. *Front Genet* (2020) 11:566159. doi: 10.3389/fgene.2020.566159
32. Pei J, Wang Y, Li Y. Identification of Key Genes Controlling Breast Cancer Stem Cell Characteristics *via* Stemness Indices Analysis. *J Trans Med* (2020) 18(1):74. doi: 10.1186/s12967-020-02260-9
33. Zhang ML, Kem M, Mooradian MJ, Eliane JP, Huynh TG, Iafrate AJ, et al. Differential Expression of PD-L1 and IDO1 in Association With the Immune Microenvironment in Resected Lung Adenocarcinomas. *Modern pathology*. (2019) 32(4):511–23. doi: 10.1038/s41379-018-0160-1
34. Kadota K, Yeh YC, Villena-Vargas J, Cherkassky L, Drill EN, Sima CS, et al. Tumor Budding Correlates With the Protumor Immune Microenvironment and Is an Independent Prognostic Factor for Recurrence of Stage I Lung Adenocarcinoma. *Chest* (2015) 148(3):711–21. doi: 10.1378/chest.14-3005
35. Xu Q, Xu H, Chen S, Huang W. Immunological Value of Prognostic Signature Based on Cancer Stem Cell Characteristics in Hepatocellular Carcinoma. *Front Cell Dev Biol* (2021) 9:710207. doi: 10.3389/fcell.2021.710207
36. Aliebrahimi S, Kouhsari SM, Arab SS, Shadboorestan A, Ostad SN. Phytochemicals, Withaferin A and Carnosol, Overcome Pancreatic Cancer Stem Cells as C-Met Inhibitors. *Biomedicine pharmacotherapy* (2018) 106:1527–36. doi: 10.1016/j.biopha.2018.07.055
37. Maroun CR, Rowlands T. The Met Receptor Tyrosine Kinase: A Key Player in Oncogenesis and Drug Resistance. *Pharmacol Ther* (2014) 142(3):316–38. doi: 10.1016/j.pharmthera.2013.12.014
38. Yoshimura K, Inoue Y, Tsuchiya K, Karayama M, Yamada H, Iwashita Y, et al. Elucidation of the Relationships of MET Protein Expression and Gene Copy Number Status With PD-L1 Expression and the Immune Microenvironment in non-Small Cell Lung Cancer. *Lung Cancer* (2020) 141:21–31. doi: 10.1016/j.lungcan.2020.01.005
39. Chopin V, Lagadec C, Toillon RA, Le Bourhis X. Neurotrophin Signaling in Cancer Stem Cells. *Cell Mol Life Sci* (2016) 73(9):1859–70. doi: 10.1007/s00018-016-2156-7
40. Huang Q, Tan Q, Mao K, Yang G, Ma G, Luo P, et al. The Role of Adrenergic Receptors in Lung Cancer. *Am J Cancer Res* (2018) 8(11):2227–37.
41. Coelho M, Imperatori A, Chiaravalli AM, Franzi F, Castiglioni M, Rasini E, et al. Beta1- and Beta2-Adrenoceptors Expression Patterns in Human Non-Small Cell Lung Cancer: Relationship With Cancer Histology. *J Neuroimmune Pharmacol* (2019) 14(4):697–708. doi: 10.1007/s11481-019-09879-6
42. Schmall A, Al-Tamari HM, Herold S, Kampschulte M, Weigert A, Wietelmann A, et al. Macrophage and Cancer Cell Cross-Talk *via* CCR2 and CX3CR1 is a Fundamental Mechanism Driving Lung Cancer. *Am J Respir Crit Care Med* (2015) 191(4):437–47. doi: 10.1164/rccm.201406-1137OC
43. Wang S, Wang SY, Du F, Han Q, Wang EH, Luo EJ, et al. Knockdown of PAK1 Inhibits the Proliferation and Invasion of Non-Small Cell Lung Cancer Cells Through the ERK Pathway. *Appl immunohistochemistry Mol morphology*. (2020) 28(8):602–10. doi: 10.1097/PAI.0000000000000803

44. Zhu X, Guo X, Wu S, Wei L. ANGPTL4 Correlates With NSCLC Progression and Regulates Epithelial-Mesenchymal Transition *via* ERK Pathway. *Lung* (2016) 194(4):637–46. doi: 10.1007/s00408-016-9895-y

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership