TARGETING THE TUMOR MICROENVIRONMENT FOR A MORE EFFECTIVE AND EFFICIENT CANCER IMMUNOTHERAPY

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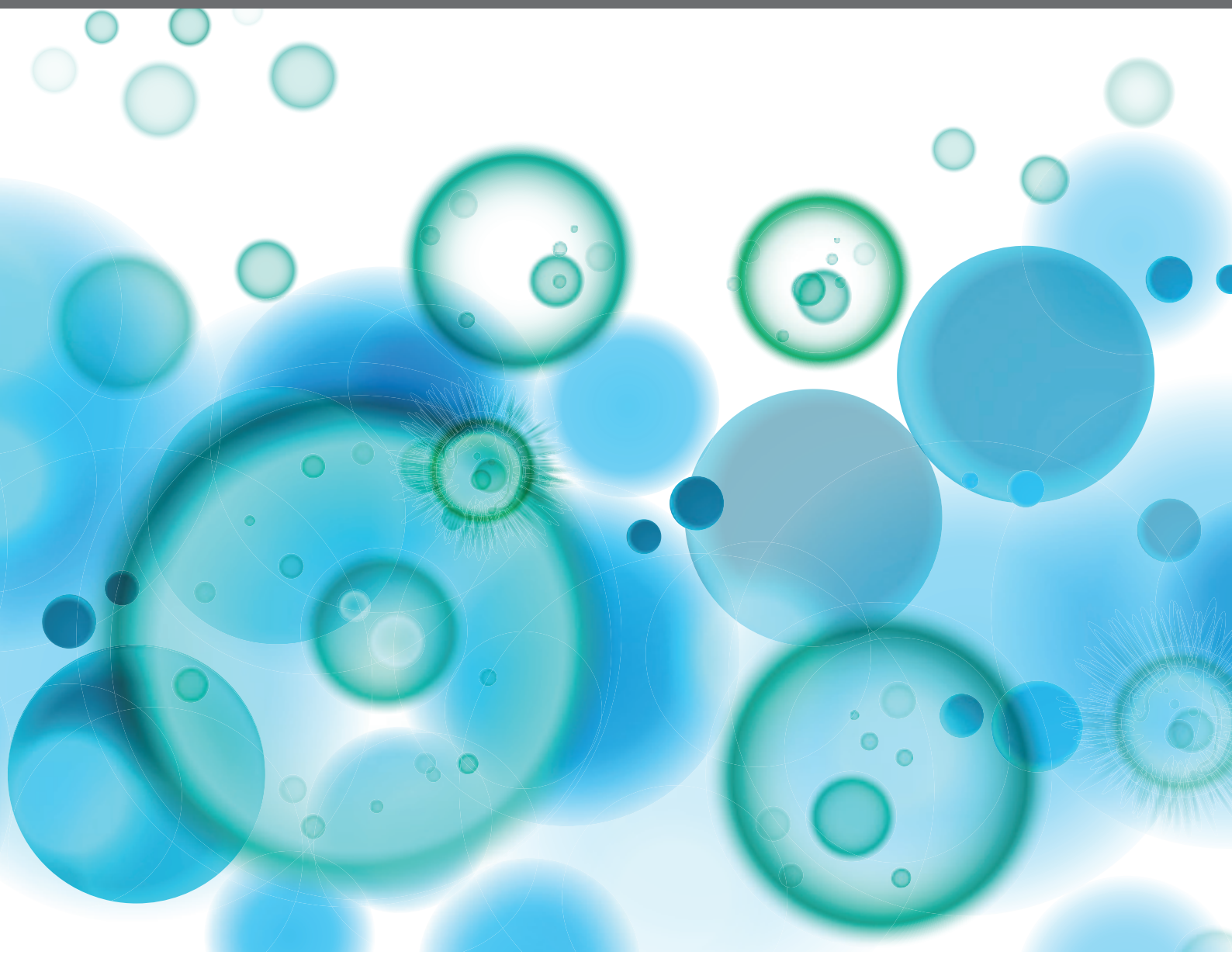

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TARGETING THE TUMOR MICROENVIRONMENT FOR A MORE EFFECTIVE AND EFFICIENT CANCER IMMUNOTHERAPY

Topic Editors:
Salem Chouaib, Institut Gustave Roussy, France
James Lorens, University of Bergen, Norway

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Table of Contents

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EDITORIAL
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Editorial: Targeting the Tumor Microenvironment for a More Effective and Efficient Cancer Immunotherapy

Salem Chouaib[1,2*] *and James Lorens*[3]

[1] *Department of Experimental Oncology, Thumbay Research Institute for Precision Medicine, Gulf Medical University, Ajman, United Arab Emirates,* [2] *INSERM UMR 1186, Gustave Roussy, Université Paris-Saclay, Villejuif, France,* [3] *Department of Biomedicine, Centre for Cancer Biomarkers (CCBIO) University of Bergen, Bergen, Norway*

Keywords: EMT - epithelial to mesenchymal transition, hypoxia, tumor microenvironment, anti-PD 1, T cells

Editorial on the Research Topic

Targeting the Tumor Microenvironment for a More Effective and Efficient Cancer Immunotherapy

Carcinomas are no longer considered a singular mass of tumor cells; but rather a complex and dynamic pseudo-organ, comprising transformed epithelial cells residing within a complex microenvironment with unique physiology, rich in different non-malignant cell types that interact physically and via paracrine signaling molecules. Immunologists were central to this fundamental re-evaluation of cancer by highlighting the continuous dialogue between immune cells and their cancer targets. Deeper understanding the role of the tumor microenvironment (TME) during cancer initiation and progression is critical both to further cancer biology and as a source of improved molecular diagnostics and therapeutics. The TME is an integral part of tumor physiology that nurtures the malignant process. Many reports indicate that a fundamental albeit deranged relationship between tumor and stromal cells is essential for tumor cell growth, progression, and development of life-threatening metastasis. Therefore, insight into this interaction and the underlying signaling, transcriptional, and metabolic pathways, can reveal valuable opportunities for therapeutic intervention during cancer progression.

The TME is composed of proliferating tumor cells, blood vessels, infiltrating inflammatory cells, and a variety of other associated stromal cells. Dynamic crosstalk between malignant cells and the tumor stroma in the TME determines the trajectory of tumor progression, its aggressiveness, heterogeneity, and response to cancer treatment. How the TME imposes challenges for cancer cells, including physical pressures, oxidative stress, nutrient deprivation, hypoxia, and immune surveillance will be discussed in this special issue.

From an immunological point of view, there is a paradoxical coexistence of tumor and tumor antigen-specific CD8 T cells in cancer patients. Cancer immune resistance arises from multiple negative immunoregulatory pathways that impede T cell-mediated tumor destruction. Tumor stroma components engage in an active and complex molecular cross-talk that compromises immunological recognition of tumors by killer immune cells. This immune suppressive shaping of the TME may be considered an initial immune checkpoint. Although immune checkpoint blockers (anti-PD-1, anti-PD-L1, anti-CTLA-4) on T cells provide improved survival in various metastatic cancer types, a high fraction of cancer patients fail these immunotherapeutic interventions. Strong evidence indicates that neoplastic cell responses to immunotherapy are not solely dependent on the qualitative and/or quantitative features of the T cells or the complexity of the genomic

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Catherine Sautes-Fridman, INSERM U1138 Centre de Recherche des Cordeliers, France

***Correspondence:**
Salem Chouaib
Salem.CHOUAIB@gustaveroussy.fr

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aberrations they harbor, but is also regulated by numerous dynamic properties of the TME. Among the microenvironment factors that play a dominant role in determining therapeutic responses to immunotherapy, hypoxia is central: It is a hallmark of most tumors with the potential for mediating metabolic and phenotypic changes (cell plasticity) as well as direct immune suppression. As a pervasive feature of the TME, hypoxia plays also a significant role in cancer progression and ultimately clinical outcome. One key cellular consequence of hypoxic stress is the dysregulation of DNA repair pathways, which contributes to the genomic instability and mutator phenotype observed in human cancers. Recently, cell plasticity has emerged as potential contributor to therapy evasion through regulation of cancer cell phenotypic and functional heterogeneity. It is now appreciated that microenvironmental stress during tumor development is frequently accompanied by cellular plasticity such as Epithelial-Mesenchymal transition (EMT) that facilitate adaption and selection of lethal cancer clones. Targeting carcinoma cell plasticity is in this regard an important strategy to better control the emergence of resistant variants and ensure a more effective cancer therapy.

With complex mechanisms of resistance limiting the efficacy of checkpoint inhibitor monotherapy, it is critical at present to develop combination approaches to allow more patients to benefit from immunotherapy. In this respect, immunotherapies are more effective when combined with agents that modulate the TME to overcome tumor tolerance and resistance, two key therapeutic challenges. A major barrier remains: How to shape the immunosuppressive TME to promote T cell effector function and overcome tumor immune resistance. Knowing the key suppressive and resistance mechanisms associated with the complexity of the TME will provide the means to break immune tolerance, develop new combinatorial therapeutic strategies and tailor efficient treatments. A better understanding of the crosstalk between signaling pathways and metabolic alterations that drive therapeutic resistance will provide the insight to develop novel therapeutic strategies. The TME is indeed an important target for anti-cancer therapy. Cancer patients will ultimately benefit from drug combinations based on an understanding of the tumor pseudo-organ and not just its individual components.

The aim of this special issue is to provide a comprehensive review of recent understanding of how TME influence on tumor immune resistance and suppression, with a particular focus on current therapeutic strategies to target molecular mechanisms that create and sustain the immune hostile tumor microenvironment. Broadening the clinical applicability of treatments in oncoimmunology requires an improved understanding of the mechanisms, in particular cellular plasticity, complexity, and hostility of the TME, that limit current cancer immunotherapy This reinvigorating of the anti-tumor immune response by targeting the TME can improve cancer treatment.

The papers written by experts in this special issue illustrate how far the field has advanced, but also remind us of the extent of its complexity. This issue offers a current perspective of important aspects of the TME in immune regulation and that impact cancer immunotherapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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METHODS
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Multitype Network-Guided Target Controllability in Phenotypically Characterized Osteosarcoma: Role of Tumor Microenvironment

*Ankush Sharma[1,2], Caterina Cinti[1] and Enrico Capobianco[2,3]**

[1] *Experimental Oncology Unit, UOS – Institute of Clinical Physiology, CNR, Siena, Italy,* [2] *Center for Computational Science, University of Miami, Miami, FL, United States,* [3] *Miller School of Medicine, University of Miami, Miami, FL, United States*

This study highlights the relevance of network-guided controllability analysis as a precision oncology tool. Target controllability through networks is potentially relevant to cancer research for the identification of therapeutic targets. With reference to a recent study on multiple phenotypes from 22 osteosarcoma (OS) cell lines characterized both *in vitro* and *in vivo*, we found that a variety of critical proteins in OS regulation circuits were in part phenotype specific and in part shared. To generalize our inference approach and match cancer phenotypic heterogeneity, we employed multitype networks and identified targets in correspondence with protein sub-complexes. Therefore, we established the relevance for diagnostic and therapeutic purposes of inspecting interactive targets, namely those enriched by significant connectivity patterns in protein sub-complexes. Emerging targets appeared with reference to the OS microenvironment, and relatively to small leucine-rich proteoglycan members and D-type cyclins, among other collagen, laminin, and keratin proteins. These described were evidences shared across all phenotypes; instead, specific evidences were provided by critical proteins including IGFBP7 and PDGFRA in the invasive phenotype, and FGFR3 and THBS1 in the colony forming phenotype.

Keywords: osteosarcoma cell lines, multitype networks, target controllability, protein network tomography, tumor microenvironment

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Salem Chouaib, Institut Gustave Roussy, France

Reviewed by:
Daniel Olive, Institut national de la santé et de la recherche médicale, France
Viktor Umansky, Deutsches Krebsforschungszentrum (DKFZ), Germany

***Correspondence:**
Enrico Capobianco
ecapobianco@med.miami.edu

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INTRODUCTION

In biological networks, control theory addresses questions such as (a) how we decompose the structure of a complex network into components to simplify their functional interpretability? (b) Can redundant nodes and links be reduced to guarantee better network performance? (c) What are the effects of disrupting network connectivity by acting over particular nodes?

It would be useful to find a so-called network skeleton or core serving efficiently general inference purposes, possibly with no loss of information. Such skeleton is expected to be significantly smaller than the network, while reproducing its characteristic properties. However, what is *a priori* the most informative or essential or reproducible sub-network? In most cases, the answer is empirical. As a result, when the network structure changes one can measure the effects by monitoring what can be identified as critical hotspots. In an attempt to select subsets of nodes and links, controllability may involve the search of a minimum dominating set (MDS) (1). Being a minimal set not unique,

this defines an NP-hard problem. Still, sets of the same size may differentiate by various node functional states, thus triggering a variety of connectivity paths and regulatory circuits.

Extending the application of such concepts to cancer networks is very tempting. Here, an assessment of controllability of influential nodes would be crucial to ensure that network integrity is sought against failures and attacks (2, 3). Key aspects in cancer are both monitoring the disease progression and evaluating the effects of therapies. However, exerting an effective control is complicated by the presence of a multitude of factors responsible of altering the normal physiological dynamics. When the latter are translated into gene or protein network dynamics, we would be interested in knowing what may change due to the insurgence of disease-related conditions. In general, two consequences may be observed: (a) intra-network state transitions, depending, for instance, on mutations affecting disease progression and (b) differential network configurations, elucidating the variations in connectivity patterns induced, for instance, by treatment effects.

Notably, a protein MDS was found enriched in disease, involved in regulatory functions and connected to protein complexes, thus legitimating a functional characterization in protein–protein interaction (PPI) networks (4). An existing categorization distinguishes between critical nodes (present in every minimal configuration), redundant nodes (never appearing in minimal sets), and intermittent nodes (appearing or not in minimal sets). Another recent study on large-scale PPI networks has classified proteins leading to disease mutations, viruses, and drug targets identification (5). Also, functional controllability was explored in epigenetically treated osteosarcoma (OS) cancer interactomes, and a module of sentinel nodes was identified as highly enriched in cancer hallmarks and marginally overlapping with both differentially expressed and mutated genes (6).

Here, we have considered experimental data susceptible of systems analysis. Specifically, the choice of OS is relevant from multiple viewpoints. First, it is a prevalent form of bone cancer with a relatively high incidence (second highest, overall) in young populations. In particular, metastatic OS shows less chances of survival (up to 30%).[1] Second, from a genomic perspective, genome-wide OS studies have reported correlation between diffuse dysregulated gene expression with genomic aberrations (7). Third, focused cancer research has been provided for this cancer, delivering a wealth of knowledge in support of clinical studies (see EuroBoNet[2]) (8). These collections of OS cell lines and xenografts have been analyzed at both genomic and epigenomic levels (9–12). Of even greater interest to our study, further extended phenotypic characterization results have been proposed by a study centered on 22 OS cell lines (13). Among the OS phenotypic features that were examined, there were *in vivo* tumorigenicity (Tp or tumorigenic phenoptype) and *in vitro* colony-forming ability (Cp or colony-forming phenotype), together with invasiveness (Ip or invasive phenotype) and proliferation capacity (Pp or proliferation phenotype).

These phenotypes reflect the OS heterogeneity that we here investigate through a network inference approach. In particular, a multitype network approach seems the most appropriate to deal with phenotypic characteristics underlying various transcriptional states and transcriptome–interactome regulation circuits involving various bioentities. The understanding of the regulation mechanisms is expected to drive the identification of novel OS therapeutic targets. However, there are currently no consistent results addressing the use and impact of networks for the identification of cancer targets. We propose, therefore, a novel direction, and **Figure 1** provides the main steps of our integrative inference approach.

MATERIALS AND METHODS

Controllability

Controllability of non-linear systems can be structurally approximated by canonical linear, time-invariant dynamics (14). Formally, the following representation holds: $dx(t)/dt = Ax(t) + Bu(t)$, with $x(t) = [x_1(t),\ldots\ldots, x_N(t)]$ capturing the state of a network of N nodes at time t; $u(t)$ an input vector of dim ension similar to A; A ($N \times N$) describing system wiring by interaction strength between components; B ($N \times M$, with $M \leq N$) identifying node controllability due to external controller. Such system is controllable if can be driven from any initial state to any desired final state in a finite time. A controllability matrix C (Kalman Matrix) is an ($N \times NM$) constant matrix that depends on system parameters and is defined as $C = [B, A, A^2B,\ldots, A^{N-1} B]$. Theory, following (15), says that a dynamical system is controllable if and only if it follows the Kalman's controllability rank condition, i.e., Rank (C) = N.

Spectral Decomposition

Controllability is associated with Spectral decomposition, another popular research direction in networks (see the following link for a few introductory concepts and a list of general references[3]). The primary aspect is that the steady-state configuration of a system or a network is proportional to its principal eigenvector (corresponding to the largest eigenvalue). In general, network eigenvalues are denoted by λ_i computed from the adjacency matrix, i.e., A $e(\lambda) = \lambda\, e(\lambda)$, and ordered from 1 to n in descending order, such that $\lambda_{MAX} = \lambda_1 \geq \ldots. \geq \lambda_n$ forms a complete orthonormal basis.

In particular, it is important to check whether $\lambda(1)$ corresponds to a localized state or to a delocalized state, which tells how the energy is distributed among spectral components. Notably, the modularity of the network is linked to such spectrum, and a property called participation ratio (PR) allows the quantification of the effective number of nodes significantly participating in a given eigenvector. When a concentration of such property occurs in just a few nodes, localization is observed. PR can be computed from the normalized eigenvector, as $e_i^N = e_i / e_i^*$, with the principal eigenvector as the denominator. Under normalization to unit in the L_2-norm, it holds: $PR = [\sum_{i=1,n} e_i^4]^{-1}$.

[1] http://www.cancer.org/cancer/osteosarcoma.

[2] http://eurobonet.pathobiology.eu/.

[3] https://en.wikipedia.org/wiki/Spectral_graph_theory.

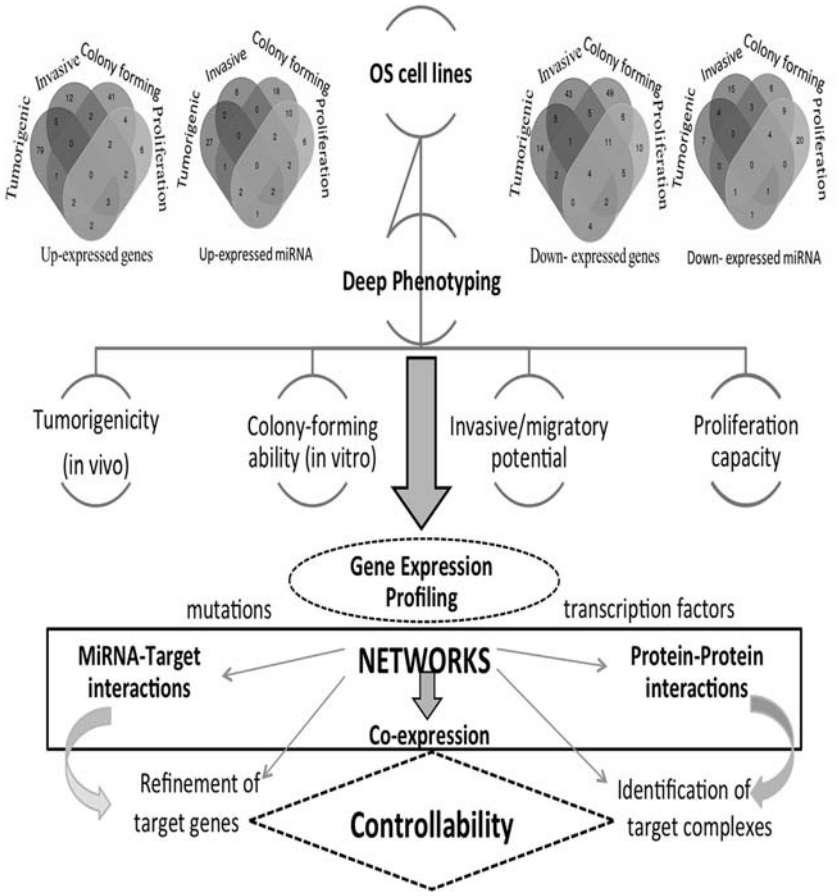


FIGURE 1 | Computational and analytical flowchart. Differentially expressed gene (DEG) profiles are reproduced from each osteosarcoma (OS) cell line and comprehensive comparative analyses are derived. Venn diagrams show DEGs and DE miRNAs for the different phenotypes here considered: tumorigenic, invasive, colony forming, and proliferation. Different types of networks are employed: gene co-expression, miRNA-target, and protein–protein interaction networks, including drugs. These are then functionally annotated, including pathways and protein complexes. Deciphering cancer regulation networks suggests the application of control concepts. These are hard to implement, but this challenge may be transformed into a sequence of tasks solved with the help of accurately selected fractions of nodes and corresponding links describing critical features. This goal corresponds to setting a target control problem, whose solution requires the search for a minimum number of driver nodes. In real cancer networks, it is natural to expect that only approximate solutions may hold. Through the identification of targets in cancer networks, we can establish the cancer relevance of functional controllability.

For the scopes of this work, it is of great relevance to compute the inverse participation ratio (IPR) (16, 17). This measure offers two limiting cases worth high consideration in target control situations. A value of $1/n$ indicates that the components are identically weighted, while a value of 1 indicates only one component is unitary and the rest as zero. In other terms, IPR indicates the reciprocal of the number of significantly contributing eigenvectors components. With regard to localization, in the limit of $n \rightarrow inf$, IPR is O(1) (or tends to 1), and thus the eigenvector is localized (possibly at few nodes), *vice versa* the eigenvector is delocalized if IPR $\rightarrow$ 0.

Spectral techniques may identify specific proteins relevant for structural and functional network properties [see (18) for protein network tomography, or also (19) for related aspects]. Extremal eigenvalues are related to dynamical properties of the networks (20, 21). The largest eigenvalue in all phenotypes lies below 2 and the largest eigenvalue observed for Tp network shows the highest variance, playing an important role in linear stability and synchronization (22). The eigenvalue plots are useful to show the best fit for scale-free networks, and such evidence is observed in all four phenotypes, indicating that a few of their vertices are structurally dominant (Figure S5 in Supplementary Material).

Cell Lines, Profiling, and Mutations

The examined OS cell lines are publicly available from GSE28425 (13). Also, 19 of 22 different OS cell lines were obtained from the resource EuroboNet. Recomputed differentially expressed genes (DEGs) could be grouped according to the characteristics of the cell lines (listed in Supplementary Material, **Table 1**). Data preprocessing from mRNA expression profiles was performed using the *Gene Expression* module v3.1.7 of *Illumina Bead studio* (v3.1.0.0). The *LUMI* package (R statistical framework) was used for variance stabilizing transformation and quantile normalization at the probe level. Intensity values were log-transformed and quantile-normalized for miRNA expression data. The fold change (FC) of the preprocessed microarray data, defined as ratio of the

TABLE 1 | Top-five differentially expressed genes (DEGs) (Top) and DE miRNAs (Bottom) in osteosarcoma (OS) phenotypes (C-I-P-T).

Reference phenotype	Gene symbol	log[fold change (FC)]	Shared phenotypes	Gene symbol	Log(FC)	Shared phenotypes
Tumorigenic Vs non-tumorigenic	*BGN*	3.492	I-T	*IL1A*	–2.221	P-T
	MGP	3.459	T	*EPB41L3*	–2.338	P-T
	DKK1	3.034	T	*NPPB*	–2.693	C-I-P-T
	LOX	2.873	T	*KRT17*	–2.752	C-I-P-T
	TM4SF1	2.74	T	*QPCT*	–3.081	I-T
Invasive Vs non-invasive	*DCN*	4.197	I-P-T	*KRT17*	–2.945	C-I-P-T
	COL1A2	2.963	C-I-P	*OClAD2*	–2.98	I-T
	S100A4	2.775	I	*IGFBP7*	–3.213	I
	S100A4	2.602	I	*COL4A1*	–3.37	C-I-P
	PDGFRA	2.375	I	*IER3*	–3.959	C-I-P
Colony forming Vs non-colony forming	*COL1A2*	2.895	C-I-P	*C9orf58*	–2.963	I-P
	HAPLN1	2.852	C-P	*LAMA5*	–3.015	C-I-P
	ALPL	2.832	C	*COL4A1*	–3.126	C-I-P
	KYNU	2.572	C-I-P	*ACTG2*	–3.384	C-I-P-T
	MAFB	2.431	C-P	*NPPB*	–3.389	C-I-P-T
Proliferation Vs non-proliferation	*COL1A2*	2.804	C-I-P	*KRT17*	–2.606	C-I-P-T
	MAFB	2.544	C-P	*COL4A1*	–2.643	C-I-P
	NDRG1	2.316	P-T	*LAMA5*	–2.962	C-I-P
	SNTB1	2.009	P	*ACTG2*	–2.982	C-I-P-T
	SPOCK	1.979	I-P-T	*NPPB*	–3.046	C-I-P-T
Reference phenotype	**miRNA symbol**	**Log(FC)**	**Shared phenotypes**	**miRNA symbol**	**Log(FC)**	**Shared phenotypes**
Tumorigenic Vs non-tumorigenic	*hsa-miR-199b-5p*	5.6	P-T	*hsa-miR-133b*	–2.1	T
	*hsa-miR-100**	3.66	I-T	*hsa-miR-449a*	–2.15	C-I-T
	hsa-miR-222	3.6	T	*hsa-miR-181a-2**	–2.38	T
	hsa-miR-136	3.34	T	*hsa-miR-142-3p*	–2.73	T
	hsa-miR-337-5p	3.06	T	*hsa-miR-15a*	–3.9	T
Invasive Vs non-invasive	*hsa-miR-193a-3p*	2.94	I	*hsa-miR-598*	–3.2	I
	*hsa-miR-100**	2.44	I-T	*hsa-miR-363*	–3.44	I
	hsa-miR-99a	2.41	I	*hsa-miR-34a*	–3.75	I
	hsa-miR-193a-5p	2.4	I	*hsa-miR-146a*	–4.29	C-I-P
	hsa-miR-449a	2.09	C-I-T	*hsa-miR-135b*	–5.7	I
Colony forming Vs non-colony forming	*hsa-miR-449a*	2.97	C-I-T	*hsa-miR-376c*	–2.67	C-I-P
	hsa-miR-545	2.77	C	*hsa-miR-146a*	–2.76	C-I-P
	*hsa-miR-505**	2.57	C	*hsa-miR-497*	–2.82	C
	hsa-miR-452	2.47	C-P	*hsa-miR-124*	–2.91	C
	hsa-miR-7	2.45	C	*hsa-miR-155*	–5.89	C
Proliferation Vs non-proliferation	*hsa-miR-199b-5p*	3.28	P-T	*hsa-miR-146a*	–3.49	C-I-P
	hsa-miR-452	2.67	C-P	*hsa-miR-377*	–3.51	P
	hsa-miR-34c-5p	2.63	P	*hsa-miR-155*	–3.67	P
	hsa-miR-152	2.34	P	*hsa-miR-376a*	–3.69	C-P
	hsa-miR-886-3p	2.25	P	*hsa-miR-376c*	–3.94	P

DEG profiles. (a) Tp state: BGN, encoding a member of the small leucine-rich proteoglycan (SLRP) family of proteins, related to bone growth, muscle development and regeneration, and collagen fibril assembly in multiple tissues, and regulating inflammation and innate immunity; MGP, inhibiting bone formation; DKK1, whose overexpression is associated with osteolytic bone lesions; LOX, encoding a member of the lysyl oxidase family of proteins with a role in tumor suppression, and crosslink collagen fibers in extracellular matrix (ECM), revealing a pre-metastatic niche in bones; TM4SF1, whose encoded protein is member of the tetraspanin family playing a role in the regulation of cell development, activation, growth, and motility; IL1A, an interleukin-1 cytokine involved in various immune responses, inflammatory processes, and hematopoiesis; EPB41L3, involved in multiple cancers. (b) Ip state: DCN, encoding a member of SLRP, mediating tumor suppression, autophagy, inflammation, and angiogenesis; COL1A2, encoding the pro-alpha 2 chain of type I collagen found in most connective tissues; S100A4, part of S100 proteins involved in the regulation of cell cycle progression and differentiation, and implicated in metastasis; PDGFRA, encoding a cell surface tyrosine kinase receptor for the platelet-derived growth factor family, with a possible role in tumor progression. (c) Cp state: ALPL, encoding a member of the alkaline phosphatase family of proteins, possibly linked to skeletal defects; LAMA5, part of Laminins, a family of ECM glycoproteins major non-collagenous constituent of basement membranes, and implicated in cell adhesion, differentiation, migration, and metastasis; ACTG2, involved in cell motility and cytoskeleton maintenance. (d) Pp state: NDRG1, member of the N-MYC downregulated gene family involved in stress responses, hormone responses, cell growth, and differentiation, whose encoded protein is necessary for p53-mediated caspase activation and apoptosis. DE miRNA profiles. hsa-miR-146a, which regulates inflammation and other innate immune system processes, is DE across phenotypes and is known to control cytokine signaling and toll-like receptors by binding to IL1 receptor associated kinase 1 (IRAK1); hsa-mir-199b-5p is highly upregulated in Tp and Pp. Also, these two phenotypes share the DE hsa-miR-100 located in chromosome 11, which contains cancer susceptibility loci and is associated with multiple cancers; hsa-miR-449a, which exerts influence post-transcriptionally in various cancers, presents opposite regulation sign, and recent OS studies showed that when down-expressed, it suppresses tumorigenicity (in vivo) and promotes cell apoptosis (in vitro) (23).

intensities between two groups of cell lines classified into different phenotypes (see **Table 1**), was log-transformed and computed with an empirical Bayes method from the packages *LIMMA* and *GEO2R* in *Bioconductor*[4] (24). The adjusted *p*-value from the *T* test was then determined; and for multiple test correction, the false discovery rate method (FDR) was used (25). A cutoff of 1.5 was used for selecting DEGs, i.e., $\log_2(FC) \geq 1.5$ or ≤ -1.5. The variations and missense mutations for DEGs in each phenotype of the OS cell lines were retrieved from the cancer Gene census (26), from exome sequencing data of patient diagnosed with OS (27), and using three OS cell lines (28). All mutation types included in cancer gene census were missense, coding silent, and of unknown phenotype; when confirmed somatic, they were layered on the DEGs in each OS phenotype. DEGs were then used for network reconstructions, each associated with the specifically identified phenotype.

Co-Expression Networks

The *Weighted Gene Co-expression Network Analysis* (WGCNA) package (29) was used to reconstruct weighted gene co-expression networks for the DEGs by OS phenotypes and compared with normal bone samples. The scale-free property (most nodes are weakly connected and dominated by a few highly connected hubs) for networks was preserved using optimal β parameter during network reconstruction (Figure S1 in Supplementary Material). WGCNA computes edge weights on any two connected genes on the basis of the so-called topology overlap measure. Edge weights with values between 0 and 1 measure the expression correlation between connected genes and shared neighbor genes (cut-off edge weight 0.05). The networks were visualized using force directed graph drawing (Cytoscape v3.3). Centrality measures were computed using *Netanalyzer* and *Centiscape*. Hub and essential genes were calculated using degree distribution, betweenness centrality (BC), maximal clique centrality, and bottleneck nodes. Topological properties are described in the glossary (see Supplementary Material).

Network Topology and Modularity

Centrality measures allow node or link ranking, and detection of intense traffic nodes and cross-linking network paths. Topological connectivity informs about the heterogeneity of networks (see Supplementary Material). Overlapping modules influencing community configurations were detected by *ModuLand via* local maxima search algorithms based on the Gradient Hill method (30). Modules were determined through an influence function calculated by *LinkLand* and *NodeLand*. The overall influence of the network is measured on each of its constituting nodes. Overlapping modules are identified on the basis of hills on the landscape, and each node of the network is assigned to the module with different strength.

MicroRNA-Target and PPI Networks

miRNA-gene target interaction for DE miRNAs (Agilent microarray data) was extracted from *miRTarBase*[5] (31) (this contains experimentally, validated miRNA-target interactions). The interactions data sources are 21 independent studies using reporter assays, western blots, and CLIP-Seq. We also extracted predictions from *Target Scan*[6] (32). It searches 6- to 8-mer sites matching the miRNA seed region, with the support of an unbiased confidence score called context++ based on 14 features for miRNA targeting efficacy. A global human proteome interaction map was collected from public databases containing non-redundant, loops exempt, experimentally validated undirected physical protein–protein binary interactions. The extracted sub-networks for each phenotype consisted of known interactions of proteins (up to first order) for DEGs.

Functional Annotations: GO, Pathways, Protein Complexes

GO annotations for DEGs were computed using *GEO2R*, using Bioconductor R packages for data analysis and transforms. The *BiNGO* plug-in was used for functional characterizations. Both FDR and Bonferroni corrections were used for multiple testing, the latter for molecular function annotation of the proteins containing variations. Note that pathway interaction cancer-specific data were retrieved from the Github repository[7] (33) (recently integrated in NDEx, the Network Data Exchange database[8]) (34). The protein complexes were retrieved from the CORUM database[9] (35), which manually annotated resources from mammalian organisms. Comprehensive annotations included protein complex functions, subunit composition, and cellular localization of complexes. Molecular functions are in Data S5 in Supplementary Material.

Previous Evidence

Both mRNA and miRNA profiles have been identified in Ref. (13). For Tp, 354 significant DEGs were found, together with two DE miRNAs (*miR-199b-5p* and *miR-100-3p*). Further results were obtained for Cp, with 35 DEGs and one miRNA (*mir-155-5p*), for Ip, with 206 DEGS and two miRNA (*miR-135b-5p* and *miR-a46a-5p*), and for Pp, with 300 DEGs and 11 miRNAs. Functional enrichment from the cell line panel was also provided by the authors. Noticing that regulatory circuits are partly shared and partly distinctly characterizing OS phenotypes, it is natural to consider such complexity from a systems level viewpoint. Of interest also the fact that four genes—*COL1A2*, *KYNU*, *ACTG2*, and *NPPB*—were pervasively classified as DEGs. However, none of them in general is specific to OS. Special attention deserves *RUNX2*, a member of the RUNX family of transcription factors (known master regulators of development) encoding a nuclear protein with a Runt sequence-specific DNA-binding domain. The protein is essential for osteoblastic differentiation and skeletal morphogenesis, for which novel drug targets have been recently identified (36).

[4]https://www.bioconductor.org/.

[5]http://mirtarbase.mbc.nctu.edu.tw/.

[6]http://www.targetscan.org/vert_71/.

[7]https://github.com/NCIP/pathway-interaction-database.

[8]http://www.ndexbio.org/.

[9]http://mips.helmholtz-muenchen.de/corum/.

RESULTS

Transcriptomic States: DEG and DE miRNA Profiling

Among the most altered genes in **Table 1**, ACTG2, NPPB, and KRT17 were significantly down-expressed in all phenotypes (**Table 1**; Data S1 in Supplementary Material). In particular, KISS1 is a gene suppressing melanoma (MEL) and breast cancer (BC) metastasis, and KRT17 shows up-expression that may be related to skin lesions and acts as a promoter of epithelial proliferation by regulating immune response. Tp, Ip, and Cp states shared molecular functions related to extracellular matrix (ECM) structural constituents containing collagen-related DEGs. Also the platelet-derived growth factor binding molecular function emerges. Phenotype-driven transcriptional states are summarized in **Table 1** (with annotations). With regard to DE miRNAs, most are phenotype specific and very few miRNAs are shared (**Table 1**). Of interest also the convolution between the upregulated *hsa-miR-138* and MYC through target genes CDK2, CTNNB1, NFKB1, E2F4, and ITGA6 implicated in cellular processes related to focal adhesion, *NFKB*- and *RB1*-signaling (37) (**Figure 2**). MYC oncogene is overexpressed in >70% of human cancers and transcriptionally regulating cell cycle, cell death, senescence, cell adhesion, angiogenesis, genome stability, microenvironment, and metabolism.

Interactomic States: Gene Co-Expression Networks

By using WCGNA, we reconstructed DEG-driven co-expression networks for all OS phenotypes. All co-expression networks appear in Figure S2 of the Supplementary Material. For instance, in Cp the high co-expression emphasizes functionally related gene sets. The network topologies reflect known properties, i.e., scale free and small world (see Figure S3 in Supplementary Material). Notably, redundant and diverse network configurations embed dynamics more difficult to control.

miRNA-Target Gene Interaction Networks

All the miRNA-target gene networks appear in Figure S3 of Supplementary Materials. We reconstructed the miRNA-target gene interactions in each phenotype by only considering DE interactors. Tp and Cp present relatively higher clustering coefficient (see the glossary in Supplementary Material). This indicates that 1st degree node neighbors (dnn) tend to interact with each other (see Table 1 in Supplementary Material). Core skeletons (see glossary in Supplementary Material) in networks were formed by high DEGs in all phenotypes, showing high community centrality (CC) values (Data S2 in Supplementary Material). Tp genes included: FBXO32 (a muscle atrophy F-Box protein); EMP1 (epithelial membrane protein-1) with a role in cell–cell interaction and cell proliferation control; CDK4, a cyclin-dependent kinase important for G1 phase progression. Then, CCND1, which regulates CDK kinases, emerges in the other three phenotypes with very high CC (see Data S1 and S2 in Supplementary Material). Also, the top 10% genes with high degree, BC and CC showed gene regulation by miRNAs. The high DE tumor suppressor *miRNA-449-A* inhibits proliferation and prevents metastasis, and regulates the co-expressed GAS1 (putative tumor suppressor) and CDK4. Multiple lowly expressed miRNAs regulated genes with fewer interactions: *hsa-miR548b* and *hsa-mir342* interacted with DE hubs in the Tp miRNA-gene target network.

Note that *miR-342-3p* interacts with FBXO32, NDRG1, CAMK2N1, and RGS4, involved in cellular activation and communication, immune system, kinases, etc. The essential genes CCND1, CDK6, and GFRA1 formed the Ip core skeleton network sharing a multitude of miRNA interactions. The highly overexpressed *hsa-miR-182*, frequently amplified in MEL and experimentally known to promote metastasis and migratory potential, co-regulated the co-expressed CDK4 and CCND2, with the down-expressed GFRA1 and with the over-expressed NPTX1 and PDGFRA (involved in developmental cellular processes). In Cp state, high DE miRNAs such as *miRNA-449-A* also showed interaction with the hub connectors CCND1 and TXNIP (encoding a thioredoxin-binding protein member of the alpha arrestin protein family that regulates redox signaling, and possibly a tumor suppressor). Also, *hsa-miR-630* interacts with the DE CTHRC1, a known positive regulator of osteoblastic bone formation. The DEGs IGFBP5, CLDN1, and ALDH1A3 were found regulated by *hsa-mir-1224-5p*, along with other miRNAs such as *hsa-miR-603*, sharing interaction with hub genes CCND1, KYNU, and WISP1.

In Pp state, the top 10% essential connector genes (GFRA1, TXNIP, CCND1, and CCND2) of the core skeleton shared many miRNA interactions. CCND1 and TXNIP genes were regulated by the *miR520* family (*miR-520c-3p*, *miR-520d-3p*, *miR-520a-3p*, *miR-520e*, *miR-186*), which reduces secretion of pro-inflammatory cytokines by NF-κB signaling inhibition. The other regulator *miR-186* is known to suppress cellular proliferation, and *miR-423-5p* is known for autophagy regulation in cancer cells. The top DE miRNA, over-expressed *hsa-miR-449a*, *hsa-miR-542-3p*, *hsa-miR199a-3p*, and down-expressed *hsa-miR-338-3p*, *mir142-3p*, *miR28-5p*, have strong role in proliferation in multiple cancers, including OS *via* their target genes. Also, *hsa-miR-182* is known to interact with DEGs (NDRG1, NPTX2, CCND2, RRAGA, and GFRA1), targets in cellular proliferation.

PPI Networks

Those associated with DEGs in each phenotype were extracted from non-redundant experimentally evidenced and curated sets of seed proteins in the human proteome. In Tp, proteins of the COL family (COL6A1, COL6A2, and COL6A3) appear, likewise Cathepsin (CTSB), interacting with PLAU and SLP1, and showing involvement in cellular processes related to collagen catabolic processes. In Cp, the majority of PPIs are involved also in cell migration and motility. In Ip, multicellular organismal development emerged. Finally, the biological processes involved in Pp interactions are related to ECM binding (complete annotations appear in Tables S2A–D in Supplementary Material). In each phenotype, the DEG-proteins showed few interactions and variation (Table S3 in Supplementary Material). As anticipated earlier, we also considered PPI networks expanded to their first order dnn (see Supplementary Material).

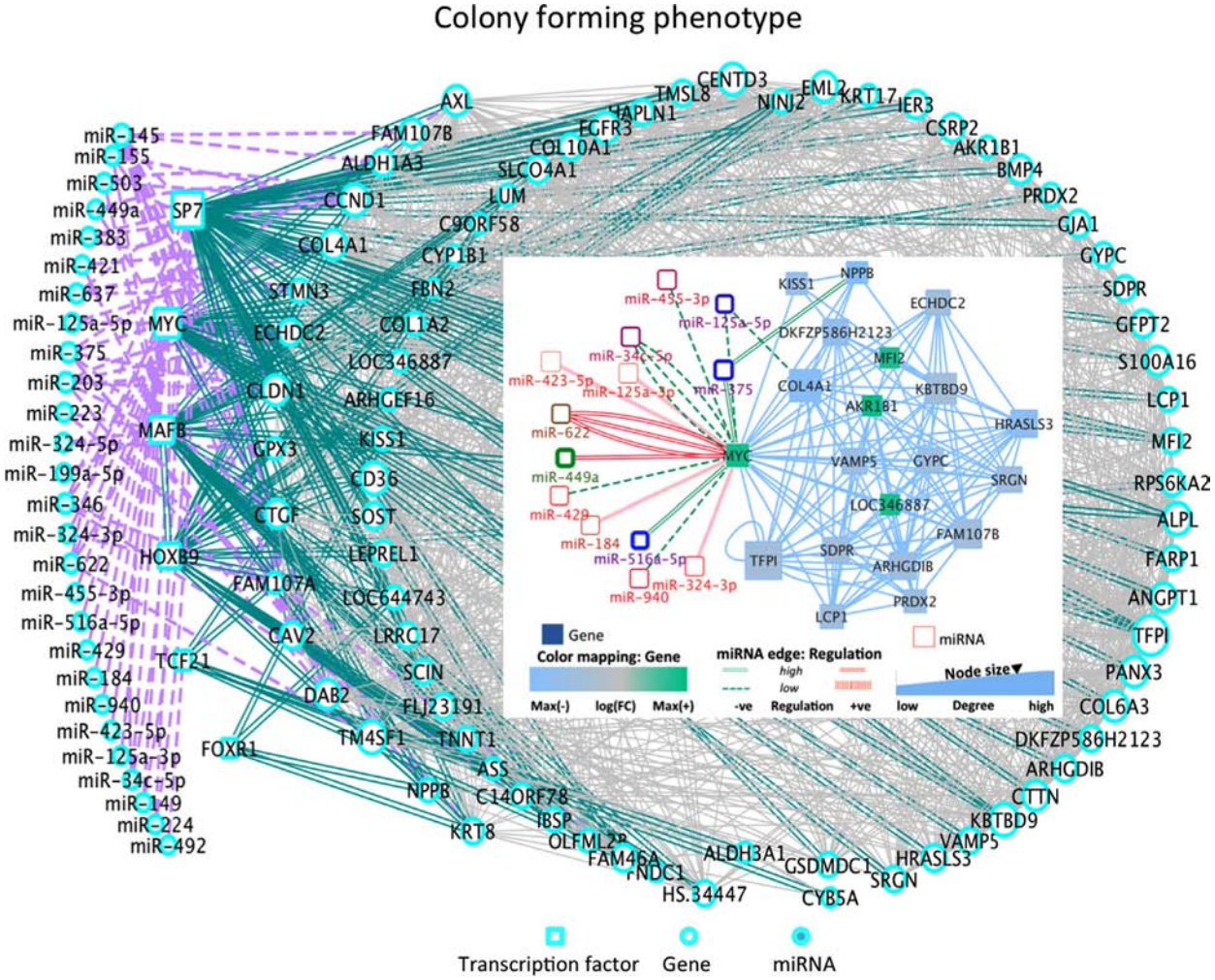


FIGURE 2 | DE miRNA-TF co-regulatory dynamics in Cp (inset: C-MYC sub-network). *Cp state:* the overexpressed *hsa-miR-545* can induce cell apoptosis and cell cycle arrest by targeting CCND1 and CDK4. *Hsa-miR-7* is involved in major cancer pathways. The over-expressed *MYC* is involved with highly over-expressed *hsa-miR-449a* and *hsa-miR-622*, and with down-expressed *hsa-miR-516a-5p* and *hsa-miR-375*. Also, the over-expressed *hsa-miR-224* interacts with SP7 and *hsa-miR-199a-5p* interacts with MAFB [role in producing osteoblasts and osteoclasts, and in their differentiation]. The down-expressed *hsa-miR-492* interacts with TF Pod1 (TCF21), a tumor suppressor frequently silencing through epigenetic mechanisms. Other states present further aspects of interest (see Figure S3 in Supplementary Material): *Tp state:* the down-expressed *hsa-miR181a-2* shows deregulation in human cancers, and the down-expressed *hsa-mir181a* is pro-apoptotic and suppresses invasion and proliferation in OS (38). The over-expressed *miR142-3p* suppresses tumor growth, invasion, migration, and proliferation in OS cells. A hub appears between TFs and the DEG NFIX interacting with 50 partners, including DE *hsa-miR-375*, *hsa-miR-149*, *hsa-miR-324-5p* (down-expressed) and *hsa-miR-765*, *miR*, *hsa-miR-423-5p*, *hsa-miR149*, *hsa-mir361-5p* (over-expressed). Note *hsa-miR-375* also regulates hub DEGs (NPPB, PHLDA1, EMP1, and IGFBP) functional in cancer processes. *Ip state*: the down-expressed *has-mir-363*, suppressing invasion, migration, and OS cell growth through direct targeting of MAP2K4 (39) and the over-expressed *miR-193a* are correlated with PLAU, which modulates signaling in DNA damage, Notch, NF-κB, Myc/Max. *Pp state*: *Hsa-miR-152* is over-expressed here and in osteoblasts. Both *hsa-miR376c* and *hsa-miR-377* showed high down-expression, potentially suggesting a role in OS proliferation. Inverse correlation in *Hsa-miR-376c* and its target TGFA is observed in OS tissues and cell lines. Decrease in TGFA and its downstream signaling molecule's expression due to over-expressed *mir-376c* is relevant in cellular proliferation and invasion in OS (40). Increased expression of *hsa-miR-377* with target CDK6 is already known to reduce cell proliferation and inhibit invasion in MG63 cell (41). No major TFs were DE in these cell lines.

PPI—miRNA Networks

The networks composed of interactions among DEG-related proteins and miRNA targets were reconstructed. The Tp state revealed limited heterogeneity, with a multitude of low DE miRNAs regulating proteins, namely the connector hub BCAS4 along with FBXO32, ADM, and CDK4 (Figure S4 in Supplementary Material). The down-expressed *hsa-mir-512-3p* regulated the over-expressed connector hub BCAS4, and NDRG1, a metastasis suppressor. The latter, along with ITGA11 and GAS1 proteins, plays a role in degradation of ECM and growth suppression and interacts with the highly DE *hsa-mir-449a*. Notably, the DE *hsa-miR-142-p* regulates SDC4, promoting LOX-dependent cross-linking of collagen, and providing bone health. The same miRNA then regulates IL1A, known to influence PLAU with regard to cancer invasion and metastasis. PLAU interacts with the highly over-expressed tumor suppressor *hsa-miR-193-3p*. Note that multiple

miRNAs from the 14q32 locus associated with increased OS risk were DE wide interactors. Namely, FBXO32 interacts with the over-expressed *hsa-miR-431*, the down-expressed *hsa-miR-144* and *hsa-miR-377*, and other lowly expressed miRNAs from other loci. Then, *hsa-miR-494*, *hsa-miR-665*, and *hsa-miR-765* regulate PPP2R2B whose protein exerts negative control on cell growth and division. Also, its promoter methylation determines resistance to treatment with mTOR inhibitors. Finally, it contains missense mutations in OS patients. Another interaction is between *hsa-miR-144* and PHLDA1, which has missense mutations and whose protein shows anti-apoptotic effects of insulin-like growth factor-1.

In Ip state, the connector hub protein PDGFRA is regulated by multiple miRNAs, including the over-expressed *hsa-miR-491-5p*, *hsa-miR-182, hsa-miR-298*, and the down-expressed *hsa-miR-140-5p* (42) (see **Figure 3**). The *hsa-mir-491* family is known to function in epithelial to mesenchymal transition and to influence cellular invasion and proliferation. The down-expressed *hsa-miR-298* interacts with the connector hub CCND1. COL4A1 and COL1A2 proteins, with unknown type mutations, are regulated by the DE *hsa-miR-767-5p*, showing functions related to oncogenic processes. *Hsa-miR-153* showed regulation of STMN2 protein having missense mutation (Table S3 in Supplementary Material). In Cp state, *hsa-miR-139-5p*, *hsa-miR144*, *hsa-miR217*, and *hsa-miR-615-3p* regulate the FBN1 protein containing missense mutation. The highly down-expressed *hsa miR-139-5p* shows anti-oncogenic and anti-metastatic effects, and is suggested to be a potent cancer biomarker (43). The down-expressed FARP1 protein (**Figure 4**) (critical node in PPI–miRNA network) interacts with the over-expressed *hsa-miR182*, which plays pivotal role in carcinogenesis. Importantly, FARP1 interacts with the lowly down-expressed *hsa-miR-874*, responsible for suppression of HDAC1 expression and enhanced Runx2 transcriptional activation during recovery of bone loss.

Finally, 17 DE miRNAs regulate thrombospondin 1 (THBS1), a connector hub in the Cp miRNA-PPI network, also regulated by *hsa-miR-139-5p* and *hsa-miR-144*, along with the highly over-expressed *hsa-miR-491-5P*, known to induce apoptosis and inhibition of AKT and MAPK, and leading to accumulation of the dephosphorylated BCL2L11 protein involved in anti- or pro-apoptotic regulation. Another interactor of THBS1 is COL4A1, a provincial hub interacting with numerous miRNAs and the high over-expressed *hsa-miR-542-5p*, promoting tumorigenesis and poor prognosis. In Pp state, provincial hubs appear (Figure S5 in Supplementary Material). CCND1 and CCND2 proteins interacting with MAFB show shared regulation by *hsa-miR-503*, a miRNA responsible for repression of cellular proliferation in fibroblasts (44). Multiple miRNA regulating each of these proteins were shared by also by GFRA1, the provincial hub TXNIP and then NDRG1. The highly over-expressed *has-mir-449a* and down-expressed *hsa-mir-512-3p* regulate TXNIP along with CCND1, GAS1, ITGA11, and NDRG1, and *hsa-mir-512-3p* increases the cellular proliferation and migration ability. The protein EEF1A1, containing missense mutation in OS patients, shows interaction with *hsa-miR-342-3p*, known to regulate variety of oncogenic processes, including cellular proliferation in different cancers. The OS phenotypes shared 32 cancer-related pathways (Table S4 in Supplementary Material) and comprised DEG-driven proteins either distinctly or jointly distributed.

Effects of Controllability on Networks

While **Figure 5** described the classification of nodes in multitype networks, critical nodes have the highest presence in Pp state (**Figure 6**). With gene–gene co-expression networks, fewer critical nodes are in Tp and Cp states compared with Ip and Pp states. With gene–miRNA interaction networks, Pp state reveals many critical nodes, whereas Ip state contained none. Most miRNAs were classified as type 1 redundant nodes in all cell lines (Data S3 in Supplementary Material).

The critical nodes in multilayer OS networks were differentiated. Critical links in gene–gene co-expression networks revealed critical nodes in Tp state (FBXO32 and FLJ10154) and Ip state (OCIAD2, SLC2A3, COL1A2, NNMT, and GAS1), showing interaction with other non-critical nodes, whereas Cp state showed interaction among critical nodes WISP1 and TNNT1, especially. Pp state showed rich interactions among critical nodes, say NINJ2 that interacts with MAFB, CCND2, and with EPB41L3; then TMEM200A interacting with IL1A (interactions with non-critical nodes appear). With gene–miRNA interaction networks, Tp state showed critical links containing critical nodes interacting with non-critical nodes, whereas Ip and Cp states contained miRNAs in critical links. miRNAs *miR-183*, *mir155*, *miR-590-3p*, *miR-499-3p*, *miR-497*, and *miR-637* present critical interactions in Ip state that regulate important genes, similar to Cp state where *mir-630* regulates CTHRC1 and *miR149* interacts with C8orf55. In Pp state, critical links include critical node IL1A interaction with non-critical MAGEA10; then, the non-critical DE DCN interacts with FAM20C, and *miR-630* shows regulation relative to CTHRC1.

With PPI-miRNA networks, Ip and Cp states are showed in **Figures 3** and **4**, respectively. Instead, the Tp state contained miRNAs (*hsa-miR-186* and more) interacting with non-critical genes along with critical genes, such as FHL2, COL6A2, and NDRG1. In Pp state, only the critical node CKB showed interaction with kRT81 and the DE *miR-375* regulates highly DE NPPB genes (critical interactions). The miRNAs *miR-153*, *mir-342-3p*, and *miR-139-5p* regulated STMN2, EEF1A2, and DTX3, respectively. NDRG1, critical multilayer network node involved in stress responses, cell growth, differentiation, and metabolic pathway, is also critical for Tp and Pp states in first order PPIN. FBXO32 is critical in Tp gene–gene and gene–miRNA networks. Multilayer OS critical nodes, such as TGM (Tp state), KRT8 and COL4A1 (Ip state), KRT8 (Cp state), and CKB and COL4A1 (Pp state) (**Figure 6**) are also identified as critical nodes in corresponding PPIN first order networks (Data S3 in Supplementary Material), but with interactions lower than average degree. Redundant nodes in first-order PPIN across all phenotypes were peripheral. Serpin1, KRT18, and GAS1 (critical node in Ip state) are among the many hub nodes in different layers of biological networks and are regulated by a multitude of DE miRNAs.

Protein Complexes

Critical nodes in multilayer networks participate in the selected protein complexes (Figure S5 and Data S4 in Supplementary Material). Notably, these refer to interactions with the tumor microenvironment (TME) of relevance for cancer progression toward metastasis. TME is known to contain distinct cell types, part of ECM-related macromolecules. We found that 48% of critical nodes in Tp and Pp PPI constituted complexes, while Ip and Cp ones reached 55.9 and 56.8% (**Figure 6**). Specifically, interacting critical nodes were identified in Ip protein sub-complexes: (i) LAMA5 encoding a laminin alpha chain (laminin is a family of ECM glycoproteins), implicated in cell adhesion, differentiation,

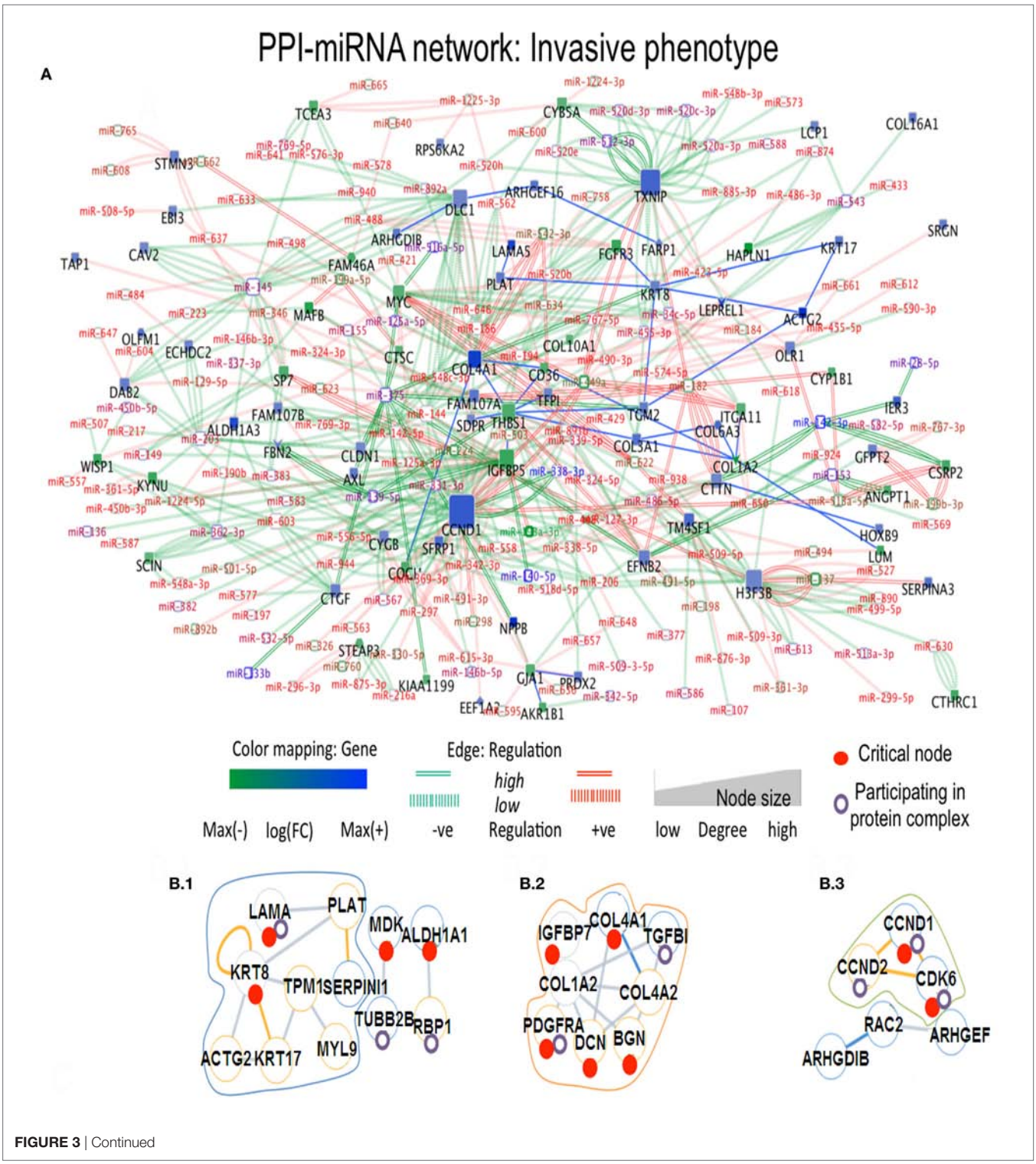


FIGURE 3 | Continued

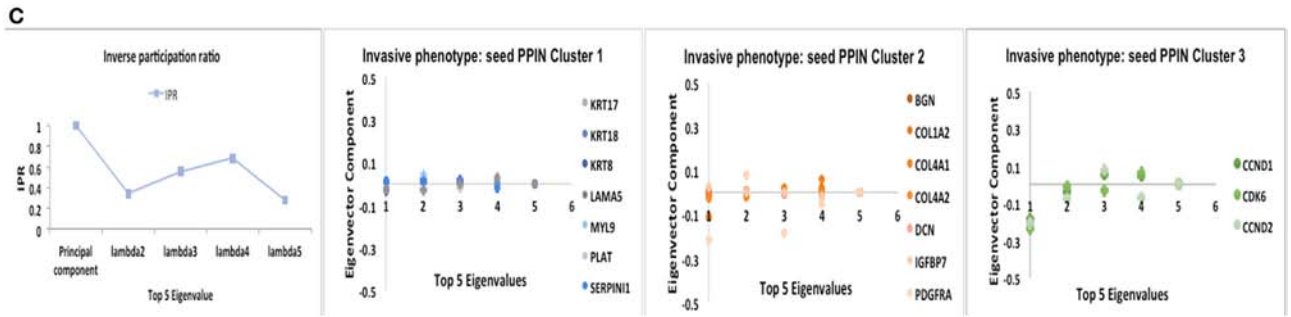


FIGURE 3 | Composite targets in Ip. **(A)** Network configuration. **(B)** Identified sub-complexes. **(C)** TOP eigenvector component values corresponding to interacting seed proteins for the top-5 eigenvalues. The first eigenvector values refer to principal eigenvalues. Red dots denote critical nodes in first order PPIN networks and violet circles denote proteins participating in complexes. *Notes*: *miR-140-5p* regulates a critical node, ALDH1A1 and is classified as a critical link; critical nodes KRT8, COL4A2, COL4A1, and PLAT interact with other non-critical nodes.

migration, and metastasis; (ii) KRT8, member of the type II keratin family, and contributing to cellular structural integrity and cellular differentiation. (iii) DCN, encoding a protein of the small leucine-rich proteoglycan (SLRP) family (collagen fibril assembly) that binds to multiple cell surface receptors, influences tumor suppression by stimulating autophagy and inflammation and inhibiting angiogenesis and tumorigenesis (45–47); (iv) BGN, encoding a SLRP protein, also regulating inflammation and innate immunity; (v) COL4A1, a subunit of the type IV collagen playing a role in angiogenesis; (vi) IGFBP7, coding for an insulin growth factor binding protein (cell adhesion, cellular senescence, and autophagy); (vii) PDGFRA, encoding a cell surface tyrosine kinase receptor (tumor progression); and (viii) CCND1 (and CCND2), cell cycle regulatory proteins or D-type cyclins promoting cell cycle progression from G1 to S phase by binding to and activating the cyclin-dependent kinases CDK4 and CDK6. By aberrantly contributing to proliferation of cancer cells in a wide variety of human cancers, these kinases represent biomarkers and pharmacological targets in view of anticancer therapeutics (48, 49).

In the Cp network, distinct critical nodes are identified in (i) KRT17 (keratin), regulating epithelial cell growth (tissue repair) and stimulating Akt/mTOR pathway; (ii) FGFR3, encoding a member of the fibroblast growth factor receptor family, and interacting with fibroblast growth factors, and ultimately influencing mitogenesis and differentiation; (iii) THBS1, which encodes an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions, active in platelet aggregation, angiogenesis, and tumorigenesis. The PPP2CA protein, a known tumor suppressor, is a pervasive critical node, also at first order PPIN level. The cAMP-dependent protein kinase catalytic subunit alpha complex containing critical nodes is shared between Tp, Cp, and Pp states, whereas the CD44 antigen-related complex is shared between Tp, Ip, and Pp states. Numerous proteins complexes containing critical nodes specifically characterize the Tp state: ERBB1 (EGFR), MMP14, and PLAUR; the Ip state: IKKB and RASA; the Cp State: GATAD2B, ACTB, ACTG1, NDUFA8, PPP2R2A, and SOS1; and the Pp state: CKB, RHOA, and AP2B1. In particular, GATAD2B and ACTB form the LARC complex. No interactions among proteins having missense mutations in OS were found in protein complexes.

Eigen-Decomposition Results

The IPR measure (see Materials and Methods) for lowest non-zero eigenvalues in PPI-miRNA network were twofold higher for Tp (IPR = 1.7) as compared with Pp (IPR = 0.7). The IPR for the lowest non-zero eigenvalue in Ip and Cp networks was 1.4 and 1.19, respectively. The lowest non-zero eigenvalues that were observed for Pp network indicate presence of strong communities (i.e., nodes with fewer connections between groups than within groups and behaving nearly as disconnected components and resulting in non-zero eigenvalues). The eigenvectors are also associated with the lowest non-zero eigenvalues, still with higher IPR (Figure S5 in Supplementary Material).

The eigenvector scatterplots of **Figure 3** with the five largest eigenvalues, and referred to the seed interacting proteins and critical nodes in Ip PPI complexes, showed variable bar length, i.e., eigenvector component values not concentrated in a single state but distributed among multiple energy states. Higher values appear for eigenvector component referred to the ALDH1A1 and the RBP1 proteins, interacting with DEG proteins (encircled in blue, C row) in the fifth largest eigenvalue χ_5. The Tp and Pp plots for eigenvector components (Figure S4A in Supplementary Material; **Figure 4B**) demonstrate similar pattern for principal eigenvalue. The critical nodes identified in PPI-miRNA networks show extremal values (high negative or positive) for some of the interacting proteins in Tp; this appears in the eigenvector plots for lowest non-zero eigenvalues (Figure S5 in Supplementary Material). In Ip, the critical nodes CCND1, CCND2, and CDK6 participating in the B.3 complex showed eigenvector components approaching 0.2 for each node, suggesting delocalization. The other connected cluster containing critical node PDGFRA participating in the B.2 complex, along with critical node TGFBI, showed eigenvector component localized around zero. In Cp, the connected component contains critical nodes TGM2 and LAMA5 linked to another critical node THBS1 participating in many important complexes (Data S4 in Supplementary Material), and showing very localized eigenvector component. The connected component of seed proteins in Tp also includes the FHL

family of proteins participating in complexes, and the critical protein TGM2, which interacts with ACTG2 while participating in different complexes. Pp contained only the LAMA5 protein involved in complexes.

Figures 3 and **4** refer to examples of protein complexes considered as possible candidate targets and retrieved from miRNA-PPIN configurations. The eigenvalues plotted with the IPR, which quantifies the number of states for a particle, and the eigenvector components (nodes, proteins) localization, or delocalization help to emphasize the target potential. High localization is equivalent to IPR telling that the distribution is concentrated on a few nodes/proteins. Lack of concentration indicates that a set of interacting

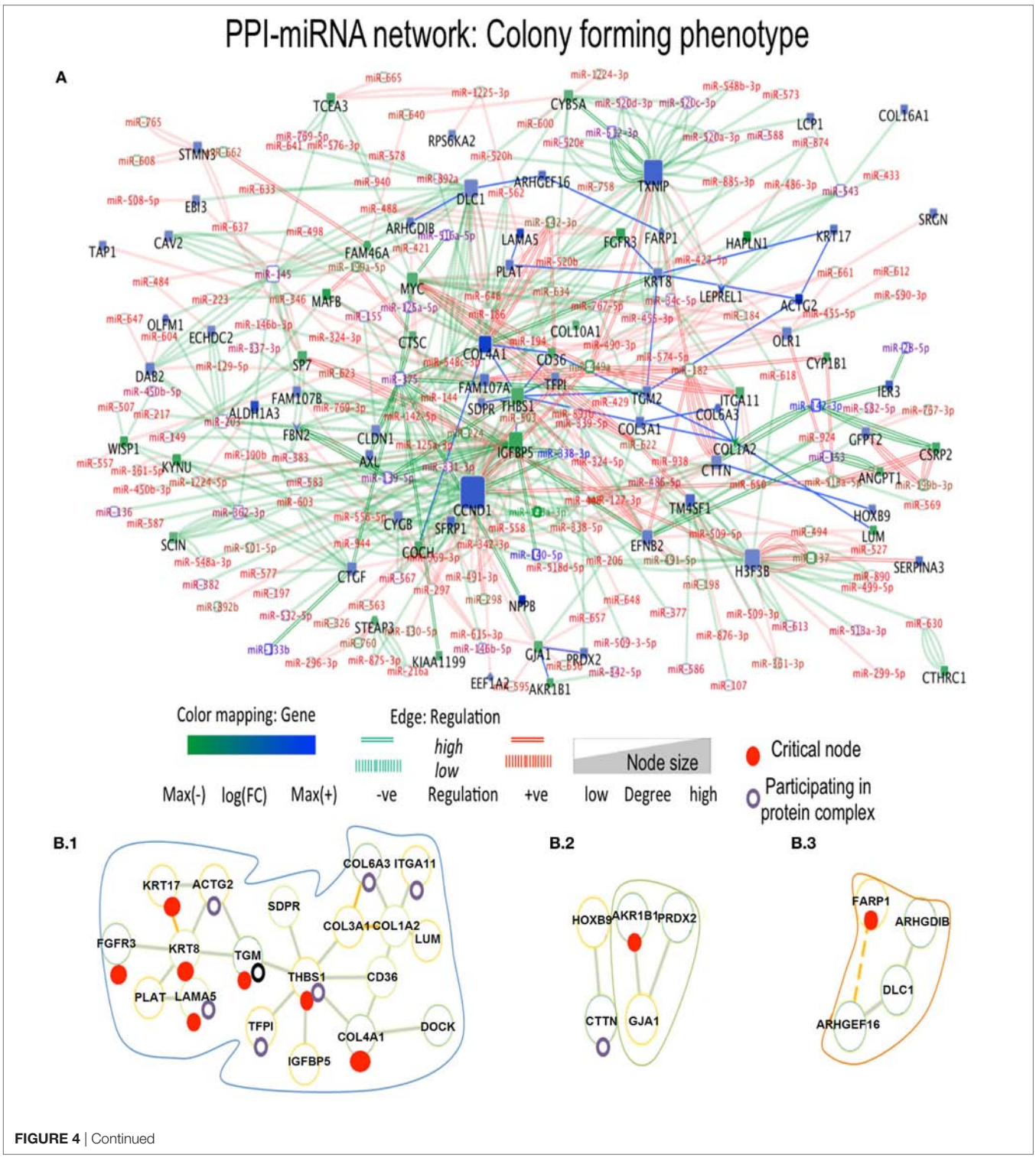


FIGURE 4 | Continued

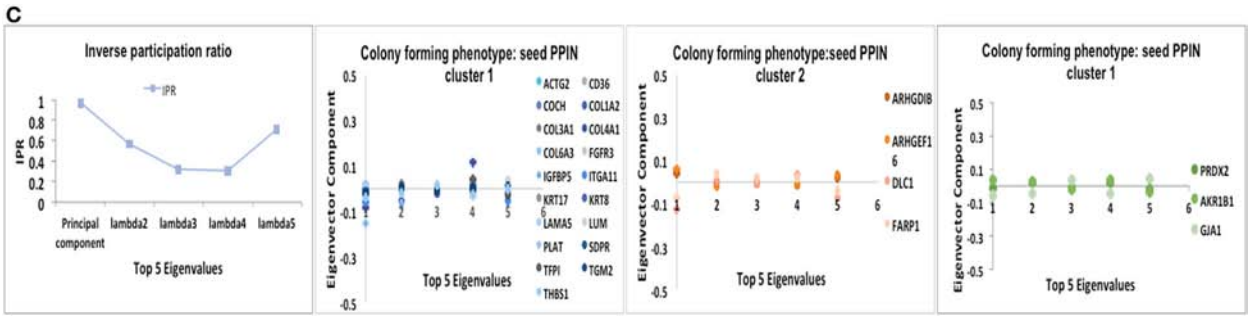


FIGURE 4 | Composite targets in Cp. **(A)** Network configuration. **(B)** Identified sub-complexes. **(C)** TOP Eigenvector component values corresponding to interacting seed proteins for the top-5 eigenvalues. The first eigenvector values depend on principal eigenvalues. Red dots denote critical nodes in first order PPIN networks and violet circles denotes proteins participating in complexes. *Notes*: miRNAs constituted the majority of critical interactions along with critical nodes DLC1, ACTG2, and FARP1 showing interaction with other non-critical nodes, and critical node KRT8 interacts with another DE critical node ACTG2. FARP1 showed missense mutation and involvement in pathways related to RhoA regulation.

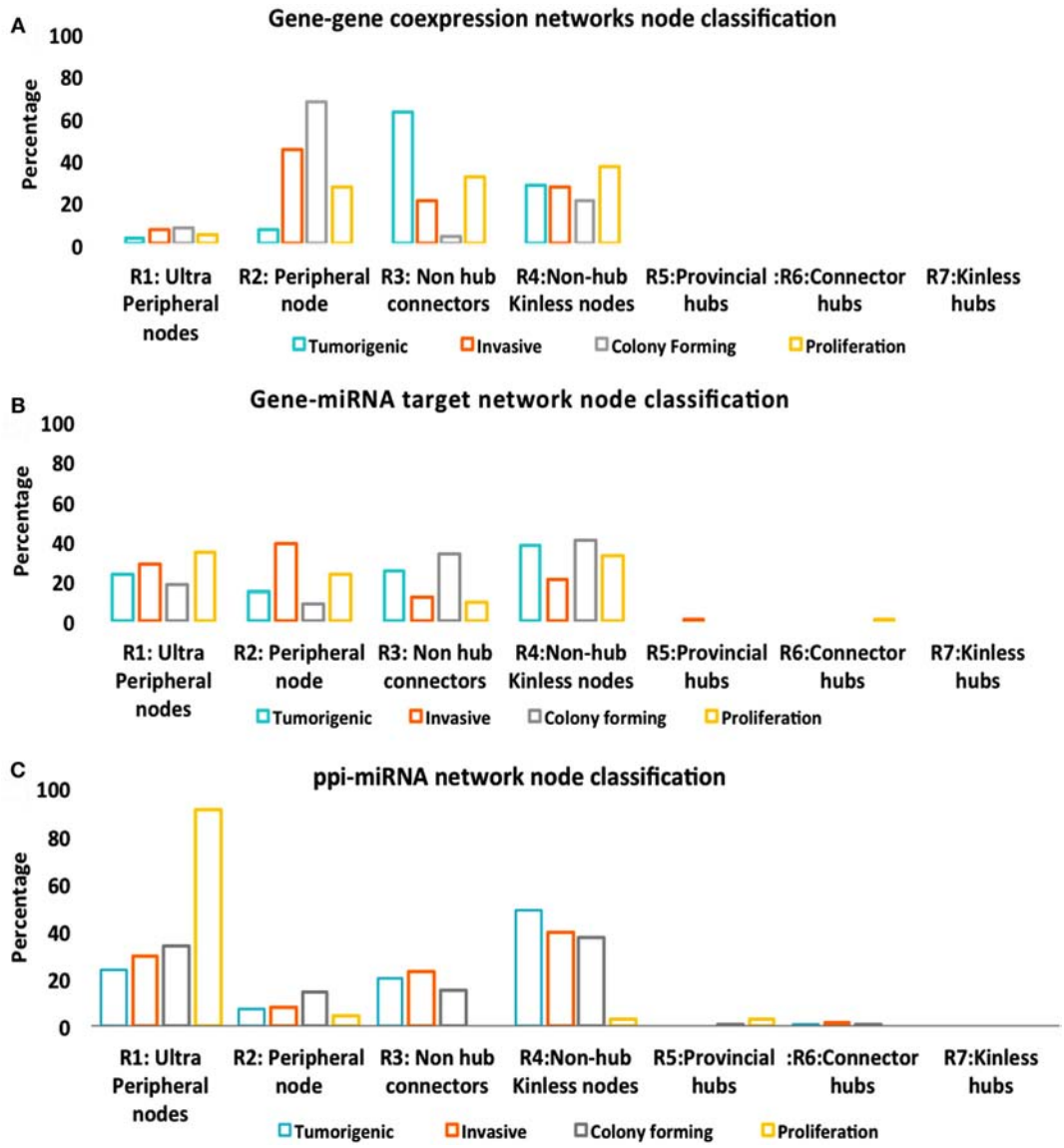
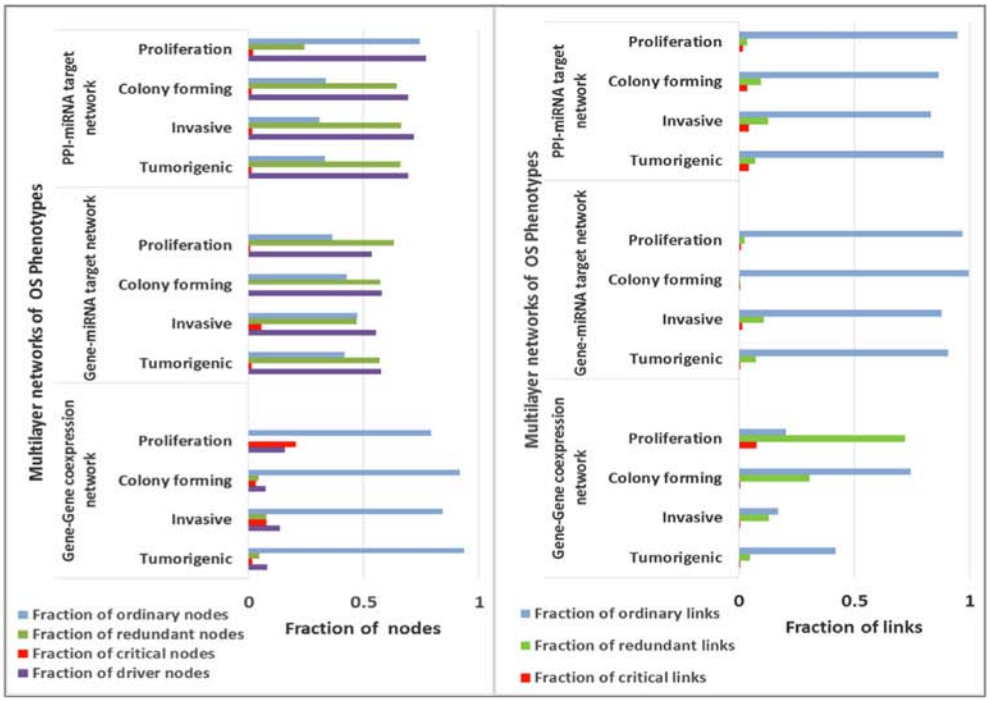


FIGURE 5 | Node classification. **(A)** Gene–gene co-expression networks. **(B)** Gene–miRNA targets. **(C)** Protein–protein interaction (PPI)-miRNA target network.



Critical nodes in multilayer networks

Tumorigenic	*Invasive*	*Colony forming*	*Proliferative*
TGM2	KRT8	KYNU	CKB
NDRG1	SLC2A3	KRT8	NDRG1
	COL4A1		EPB41L3
	ALDH1A1		CCND1
	PLAT		EFNB3
			S100A16
			IL1A
			TXNIP
			MAFB
			COL4A1
Gene-gene	Gene - miRNA	PPI - miRNA	All layers

Number of Nodes	Tumorigenic	Invasive	Colony forming	Proliferative
Critical nodes in 1st order network	113	102	161	79
N. nodes present in complexes	55	58	90	38

FIGURE 6 | Controllability analysis. *Top panel*: gene–gene co-expression networks, miRNA-gene target networks and protein–protein interaction (PPI)-miRNA interaction networks showing occurrence of critical, ordinary, and redundant nodes. *Mid panel*: Critical nodes in multi-layered networks mapped to first order networks. *Bottom panel*: critical nodes computed in PPI first order networks and number of critical nodes in protein complexes that are manually curated and experimentally validated in CORUM database. Further statistics on classification of nodes in various networks is provided in **Figure 5**.

proteins, participating to a sub-complex, may better identify a potential composite target. Specifically, pieces of evidence for a couple of phenotypes are proposed (other pieces of evidences are in Figure S4A in Supplementary Material; **Figure 4B**). The local context of a node in terms of interconnectivity patterns is relevant, therefore, to identify the potential of the candidate target beyond the individual node, thus identifying a composite target that can elucidate the functional relevance of the node itself based

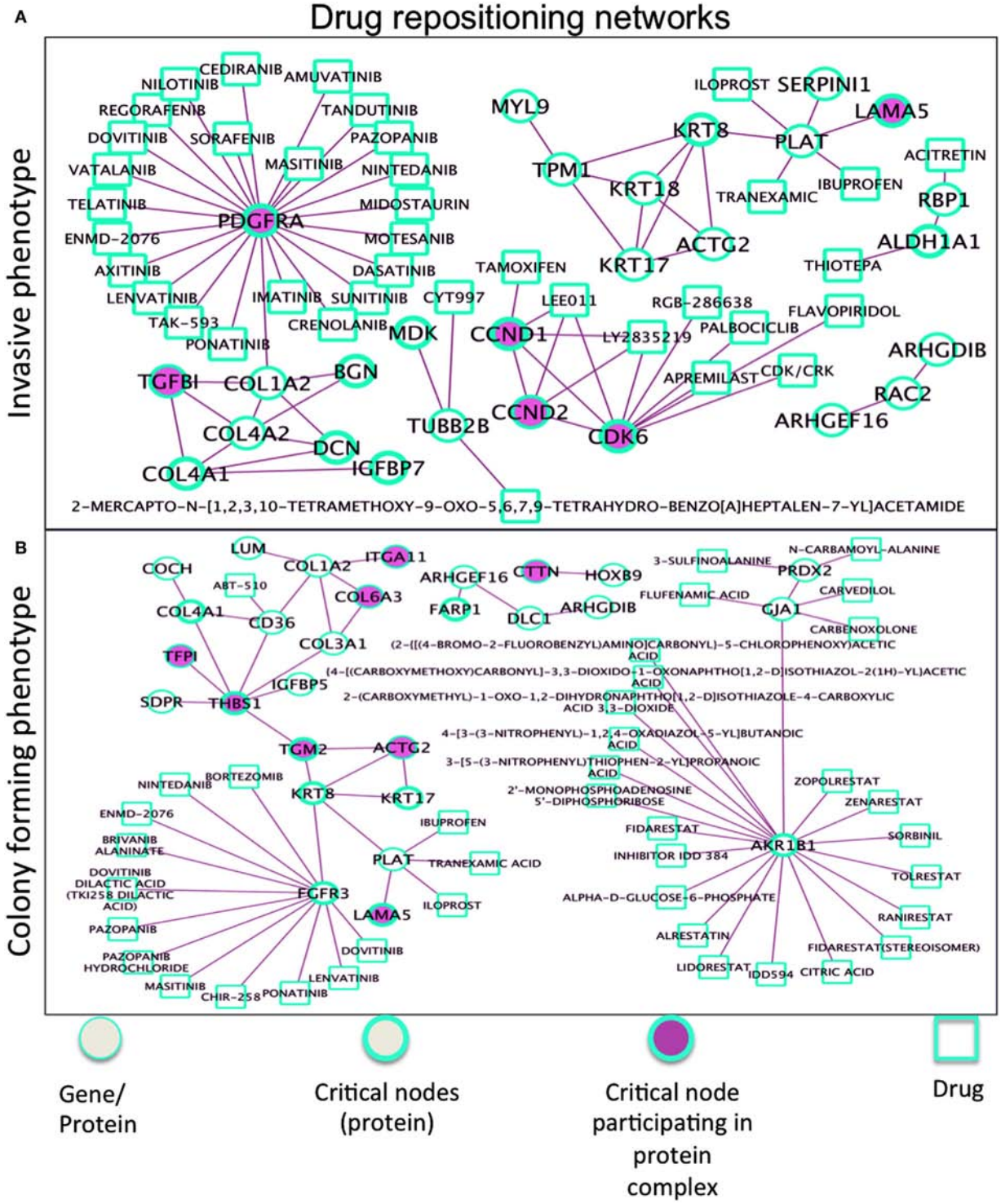


FIGURE 7 | Drug repositioning networks for **(A)** Ip and **(B)** Cp. The R/Bioconductor package rDGIdb is used, as an R wrapper to query the drug–gene interaction database (DGIdb). As a result, PDGFRA has interactors, such as imatinib, dasatinib, sunitinib, sorafenib, pazopanib, and nilotinib, none specific and all inhibiting different kinases [i.e., imatinib also KIT and AB1 (with dasatinib used for imatinib resistance), sunitinib also VEGF and FLT3 (like crenolanib too), sorafenib also RAF etc], which might reveal advantageous. Two other networks in Figure S6 in Supplementary Material. In Tp, critical nodes CTSB and PLAU widely interact with drugs. MAOA interacts with antidepressant drugs, associated with decrease in bone mineral density and increasing risk of fracture. The Pp network proteins participating in complexes showed limited interactions with drugs, Pyridoxal Phosphate interacts with KYNU (collagen) relevant to osteosarcoma. The list of interactions is available in Data S6 in Supplementary Material.

on the other interacting nodes. The presence of identified critical nodes in target sub-complexes brings additional value, as this means improved wide-spectrum controllability.

Drug Interactions, Repositioning, and Repurposing

Drug repositioning involves discovery of new roles for drugs, especially those with high failure rate and long-term development. Our phenotype-driven networks embedding critical nodes may gain further relevance when associated drugs are considered (**Figure 7**). The comprehensive resource here used is drug–gene interaction database (50, 51), with drug–gene interaction data from 15 different resources (52, 53). In the Ip drug-target network, the well-known Tamoxifen shows interaction with the CCND1 protein participating in complexes with CCND2 and CDK6 and also share interactions with other drugs, namely LEE011 (ribociclib), and LY2835219 (abemaciclib), both CDK4/6 inhibitors. Considering then the target PDGFRA (overexpressed in Ip), a drug compound of interest is lenvatinib (multiple kinase inhibitor), then regorafenib (multikinase inhibitor targeting angiogenesis, stromal/microenvironment and oncogenesis), and also nintedanib (small molecule tyrosine-kinase inhibitor, targeting VEGFR and FGFR). Inhibition of PDGF receptor signaling (with antibodies or DNA aptamers) has proven useful for treating cancer patients, leading to the development of different types of antagonists of its signaling, such as binders targeting the receptors and preventing their activation or promoting their degradation, and low molecular inhibitors of the receptor kinases. In the Cp network (bottom panel), AKR1B1 (member of the aldo/keto reductase superfamily, which consists of more than 40 known enzymes and proteins) showed interaction with many drugs, likewise FGFR3 (member of the fibroblast growth factor receptor family) emerged to be second reactive protein interacting with other cancer treatment drugs. Also, THBS1 appears (an adhesive glycoprotein mediating cell-to-cell and cell-to-matrix interactions, and involved in platelet aggregation, angiogenesis, and tumorigenesis), but with no drug interactors.

DISCUSSION

Despite inspiring much of the initial network literature, reverse engineering revealed limitations for dynamical biological systems. These need extended sensitivity tests for assessing parameter inferability (54). Two recent changes occurred: model systems have started to include enormous data volumes (big data), leading network inference approaches to unprecedented sophistication (multilayer networks). Generalizations such as reciprocal engineering (the interactome scaffold connecting pieces of experimental evidence and determining the target pathways), and forward engineering (pathway modulation used to analyze downstream phenotypes) (55). More importantly, controllability has emerged as a paradigmatic example of research direction with almost ubiquitous applications.

Multiple phenotypically differentiated OS cell lines may clarify target relationships. Our inference approach is centered on networks. One aim was to exert control on targets, single and composite ones, with the latter benchmarked to protein complexes. Pooling together heterogeneous evidenced data creates the premises for the analysis of systemic regulation dynamics of difficult replicability or interpretability. Deciphering such complexity requires multi-type networks. Because nodes and links represent genes, miRNAs, proteins, transcription factors, etc., the corresponding associative dynamics have relevance depending on their integrability. As a result, the identified OS targets were characterized by critical proteins, individually relevant or interacting in sub-complexes. Examples were offered by SLRP proteins and D-type cyclins, but distinct effects were also emerging from IGFBP7 and PDGFRA, critical proteins in invasive conditions, and from FGFR3 and THBS1, appearing in colony-forming phenotype. Collagen, laminin, and keratin proteins were shared across phenotypes.

It is clearly relevant the emergence of TME due to these identified targets. We stress the fact that the evidenced targets are connected, which suggests that multidrug targeted approaches may be particularly indicated. Such multiplicity of targets across OS phenotypes increases the overall complexity, but naturally reflects the role played by TME in this disease, and also justifies the ongoing phase I/II trials as important steps for more critical assessment of TME in OS pathogenesis (56).

We have then observed a few other specific aspects: (A) from the same reference system of pan-cancer cell lines, results depend on the computational tools used for the analysis. For instance, profiling the data discriminates among many measurements and their bio-annotations, all subjected to various degree of stringency to establish significance. But profiling is not sufficient, and calls for further inference shifting from the analysis of signatures of individual bioentities to the analysis of modules of connected bioentities; (B) shared and distinct features emerging at phenotype levels may vary quite substantially, while receiving influence from the adopted measurement system, and the best way to put forth causative instead of confounding effects is to evaluate pieces of evidence at a systems level and to exploit the embedded metrics to leverage their possible linkages; (C) networks are naturally differentiated, depending on data characteristics (OS phenotypes, in our study), but also on the object of investigation, targets in our case. Starting from the topological properties, we achieved accurate analyses through controllability and spectral concepts, so far widely unexplored, but with potential toward target discovery.

In dynamical systems, steady-state network configurations are usually considered to be proportional to the principal eigenvector corresponding to the largest eigenvalue. The residual eigenvectors refer to non-steady-state conditions, addressing system disequilibrium. Network modularity reflects the eigenvector properties, and allows measurement, for instance, through the PR, which quantifies the effective number of network nodes representing significant eigenvector components. In scale-free networks, such components tend to be localized in a few well-connected nodes. Correspondingly, the IPR indicates the reciprocal of the number of eigenvector components offering a significant contribution, thus measuring the localization degree of a particular eigenvector. A recent application of network controllability for a large-scale study aimed at identifying disease genes and drug targets (5). Differently classified nodes allowed to assess distinct functional and regulatory roles. Controllability pinpointed hotspots

("fragile nodes") informative about state transitions from health to disease. Critical controllability was examined both structurally (PPIN) and functionally (transcriptome) in large-scale integrated systems, associating critical nodes and drug targets (57). We reconciled these characteristics by proposing novel strategies to identify a variety of targets within OS phenotypic heterogeneity. Especially, exerting control on composite targets might lead to improved drug repositioning or repurposing[10] with cost-effectiveness advantages for cancer therapy.

ETHICS STATEMENT

This study has not involved patients, being based on publicly available data from experimental studies on a panel of cell lines.

AUTHOR CONTRIBUTIONS

AS: performed method computations and data analyses; drafted manuscript parts. CC: reviewed the experimental evidences and the biological findings. EC: conceived and designed the methodological pipeline and wrote the manuscript. All authors approved the manuscript in its final form.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00918/full#supplementary-material.

SUPPLEMENTARY DATA S1 | Sheet 1: Comparison of log (FC) of miRNAs analyzed by Namløs et al. (10) and log(FC) computed in each OS phenotype. *Sheet 2*: Differential expression of miRNAs in (A) tumorigenic, (B) invasive, (C) colony forming, and (D) proliferation. *Sheet 3*: Centrality statistics of gene–gene co-expression and miRNA-target genes. Hubs in both networks. *Sheet 4*: Various centrality measures of DEGs in Tp (tumorigenic Vs non-tumorigenic cell lines). *Sheet 5*: Various centrality measures of DEGs in Ip (invasive Vs non-invasive cell lines). *Sheet 6*: Various centrality measures of DEGs in Cp (colony forming Vs non-colony forming cell lines). *Sheet 7*: Various centrality measures of DEGs in Pp (proliferation Vs non-proliferating cell lines).

SUPPLEMENTARY DATA S2 | Sheet 1: Centrality statistics of gene–gene co-expression networks. Hubs in gene–gene co-expression networks. *Sheet 2*: Controllability status of nodes: 0 = critical, 1 = redundant, 2 = ordinary in type I and type II along with various centrality measures for nodes in tumorigenic DE miRNA-DE target gene interaction network. *Sheet 3*: Controllability status of nodes: 0 = critical, 1 = redundant, 2 = ordinary in type I and type II along with various centrality measures for nodes in invasive DE miRNA-DE target gene interaction network *Sheet 4*: Controllability status of nodes: 0 = critical, 1 = redundant, 2 = ordinary in type I and type II along with various centrality measures for nodes in colony forming DE miRNA-DE target gene interaction network. *Sheet 5*: Controllability status of nodes: 0 = critical, 1 = redundant, 2 = ordinary in type I and type II along with various centrality measures for nodes in proliferation DE miRNA-DE target gene interaction network.

SUPPLEMENTARY DATA S3 | Sheet 1: Classification type I nodes in multitype networks for all OS phenotypes. *Sheet 2*: Fraction of nodes and links in multitype networks for all OS phenotypes. *Sheet 3*: (A) Critical nodes computed in gene–gene co-expression, miRNA-target gene interaction, PPI-miRNA and PPI first order interaction networks. (B) Number of critical nodes in first order and number of critical nodes present in protein complexes from the CORUM database. *Sheet 4*: Information related to critical nodes identified in PPI first order network having differential expression in other OS phenotypes. *Sheet 5*: Classification of type I links in multitype networks for all OS phenotypes.

SUPPLEMENTARY DATA S4 | Sheet 1: Details of seed proteins corresponding to DE genes participating in experimentally determined protein complexes stored in CORUM database. *Sheet 2*: Details of proteins (gene symbol, Uniprot id) participation in complexes experimentally determined in different organisms (systems) stored in CORUM database. *Sheet 3*: Detailed information on the proteins present in protein complexes containing critical nodes shared and specific to OS phenotype-first order PPIN. *Sheet 4*: Information of complete list of complexes present in CORUM. Gray shaded cells contain complexes with involvement of c-MYC protein.

SUPPLEMENTARY DATA S5 | Sheet 1: Molecular functions of top-10 overlapping core proteins of identified modules of tumorigenic PPI first order network for DEGs in OS phenotypes. *Sheet 2*: Molecular functions of top-10 overlapping core proteins of identified modules of invasive PPI first order network for DEGs in OS phenotypes. *Sheet 3*: Molecular functions of top-10 overlapping core proteins of identified modules of colony forming phenotype PPI first order network for DEGs in OS phenotypes. *Sheet 4*: Molecular functions of top-10 overlapping core proteins of identified modules of proliferation phenotype PPI first order network for DEGs in OS phenotypes. *Sheet 5*: Details in protein-pathways interactions shared between and specific to OS phenotypes.

SUPPLEMENTARY DATA S6 | Sheet 1: Drug—gene interactions for DEGs retrieved from the dGIDB database using RdGIdb package for R with details on number of experimental verification for (A) tumorigenic, (B) invasive, (C) colony forming, (D) proliferation.

[10]https://ncats.nih.gov/preclinical/repurpose.

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CD4+ and Perivascular Foxp3+ T Cells in Glioma Correlate with Angiogenesis and Tumor Progression

Luyan Mu[1,2], Changlin Yang[3], Qiang Gao[4], Yu Long[2,3], Haitao Ge[2], Gabriel DeLeon[3†], Linchun Jin[2,3], Yifan (Emily) Chang[3†], Elias J. Sayour[3], Jingjing Ji[5], Jie Jiang[6], Paul S. Kubilis[3], Jiping Qi[6], Yunhe Gu[6], Jiabin Wang[2], Yuwen Song[1], Duane A. Mitchell[3], Zhiguo Lin[2] and Jianping Huang[3]**

[1] Department of Neurosurgery, The Fourth Affiliated Hospital, Harbin Medical University, Harbin, China, [2] The Fourth Section of Department of Neurosurgery, The First Affiliated Hospital, Harbin Medical University, Harbin, China, [3] Lillian S. Wells Department of Neurosurgery, University of Florida, Gainesville, FL, United States, [4] Department of Geriatrics, The Second Affiliated Hospital of Harbin Medical University, Harbin, China, [5] Department of Pathology, The Second Affiliated Hospital of Harbin Medical University, Harbin, China, [6] Department of Pathology, The First Affiliated Hospital, Harbin Medical University, Harbin, China

Background: Angiogenesis and immune cell infiltration are key features of gliomas and their manipulation of the microenvironment, but their prognostic significance remains indeterminate. We evaluate the interconnection between tumor-infiltrating lymphocyte (TIL) and tumor blood-vasculatures in the context of glioma progression.

Methods: Paired tumor tissues of 44 patients from three tumor-recurrent groups: diffuse astrocytomas (DA) recurred as DA, DA recurred as glioblastomas (GBM), and GBM recurred as GBM were evaluated by genetic analysis, immunohistochemistry for tumor blood vessel density, TIL subsets, and clinical outcomes. These cells were geographically divided into perivascular and intratumoral TILs. Associations were examined between these TILs, CD34+ tumor blood vessels, and clinical outcomes. To determine key changes in TIL subsets, microarray data of 15-paired tumors from patients who failed antiangiogenic therapy- bevacizumab, and 16-paired tumors from chemo-naïve recurrent GBM were also evaluated and compared.

Results: Upon recurrence in primary gliomas, similar kinetic changes were found between tumor blood vessels and each TIL subset in all groups, but only CD4+ including Foxp3+ TILs, positively correlated with the density of tumor blood vessels. CD4 was the predominant T cell population based on the expression of gene-transcripts in primary GBMs, and increased activated CD4+ T cells were revealed in Bevacizumab-resistant recurrent tumors (not in chemo-naïve recurrent tumors). Among these TILs, 2/3 of them were found in the perivascular niche; Foxp3+ T cells in these niches not only correlated with the tumor vessels but were also an independent predictor of shortened recurrence-free survival (RFS) (HR = 4.199, 95% CI 1.522–11.584, $p = 0.006$).

Conclusion: The minimal intratumoral T cell infiltration and low detection of CD8 transcripts expression in primary GBMs can potentially limit antitumor response. CD4+ and perivascular Foxp3+ TILs associate with tumor angiogenesis and tumor progression in glioma patients. Our results suggest that combining antiangiogenic agents with immunotherapeutic approaches may help improve the antitumor efficacy for patients with malignant gliomas.

Keywords: gliomas, angiogenesis, tumor-infiltrating lymphocytes, progression, recurrence

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Amod Ashok Sarnaik, Moffitt Cancer Center, United States

***Correspondence:**
Zhiguo Lin
linzhiguo@hotmail.com;
Jianping Huang
jianping.huang@neurosurgery.ufl.edu

***Present address:**
Gabriel De Leon, Department of Surgery, Duke University Medical Center, Division of Applied Therapeutics, Durham, North Carolina, United States;
Yifan (Emily) Chang, Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY, United States

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INTRODUCTION

Tumors recur in the resection margin in nearly 90% of CNS malignancies after primary surgery and adjuvant therapies (1, 2). Understanding which significant alterations occur in the tumor immune microenvironment during progression is critical for designing effective immunotherapeutic strategies. Tumor angiogenesis and immune cell infiltrations are crucial tumor-driven processes in tumors (3, 4); however, mechanistic insights regarding their interplay, as well as their prognostic significance in glioma recurrence remain indeterminate.

Astrocytic gliomas arise from astrocytes and are the most common type of glioma, representing 64% of human CNS malignant tumors (5). Nearly all the patients with primary gliomas will face tumor recurrence thus necessitating a better understanding of glioma progression. Recently, the angiogenesis inhibitor Bevacizumab has been shown to prolong recurrence-free survival (RFS), although no increase in overall survival (OS) was seen, in patients with newly diagnosed or recurrent GBM (6–8). This drug inhibits the formation of tumor blood vessels induced by vascular endothelial growth factor A (VEGF-A); increases T cell infiltrations (9, 10); reverses expression of inhibitory molecules associated with T cell exhaustion (11); and may directly mediate antitumor effect (12). These results imply that angiogenesis plays an important role in GBM progression, but the failure of these agents to improve patients' OS also suggest that negative feedback pathways (such as hypoxia) may be spontaneously activated resulting in increased tumor cell invasion (13, 14).

Tumors can orchestrate complex biological networks via angiogenesis and recruitment of regulatory immune cell subsets. The VEGF/receptor axis has been shown to have strong immune regulatory properties (3, 9–11). Under hypoxia condition, tumor-infiltrating lymphocytes (TILs) were found to express VEGF-A (15). Evidence indicates that the tumor blood vessel/tumor endothelium can be a substantial barrier for TIL to extravasate into the intratumoral space stymieing their ability to mount a strong antitumor response (16). Distinctly separating TILs into perivascular and intratumoral populations will provide better insight for us to determine the interaction between tumor angiogenesis and T cell infiltration.

Although several studies have previously evaluated lymphocyte infiltrations in glioma patients (17–22), those studies have focused on non-paired patient samples, which could potentially introduce significant variations. Since gliomas are heterogeneous, district genetic or phenotypic characteristics have been observed in cells from the same tumor (23, 24), which may be conserved in recurrence. Distinctions between primary and recurrent tumors in the same patient may enable a better understanding of transition/transformation during tumor progression. In this study, we studied 44 paired tumor samples (before and after of progression) in three categories of recurrence from astrocytic glioma patients and probed the interconnection between differential geographic T cell subsets and tumor blood vessels. Additionally, we also analyzed 15-paired GBMs samples from patients who had failed bevacizumab therapy and determined distinct TIL changes within these tumors. Our data suggest that CD4+ and perivascular Foxp3+ TILs impact angiogenesis and tumor recurrence in patients with gliomas.

MATERIALS AND METHODS

Patient Population

This study included 44 patients who underwent surgery for primary and recurrent glioma by MRI imaging at the same hospital from 2005 to 2014 (Table S2 in Supplementary Material). The average period between diagnosis and surgeries was 5.4 days (range 2–10 days). The inclusion criteria included the following: greater than 18 years of age at the time of primary diagnosis, a histopathological diagnosis of diffuse astrocytomas (DA) or GBM, and the availability of formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks. The patients were separated into three groups: (1) the DA–DA (DA, diffuse astrocytoma) group (DAs that recur as DAs, 15 pairs); (2) the GBM–GBM group (GBMs that recur as GBMs, 15 pairs); and (3) the DA–GBM group (DAs that recur as GBMs, 14 pairs). None of the 44 patients received treatments before their primary surgery. Subsequently, patients received standard radiotherapy (60 Gy), while some received concomitant–adjuvant chemotherapy (temozolomide/other). The recurrence-free survival (RFS) was defined as the time from the date of the primary surgery to the first MRI-confirmed tumor recurrence. All recurrent patients underwent recurrent surgery. OS was defined as the interval between primary surgery and death or last follow-up. Survival after recurrence (SR) was defined from the recurrent surgery to the date of death or the last follow-up. The mean follow-up period was 1,355.1 days (range 289–3,520 days), during which 36 patients died, and 8 patients remained alive. No patients were lost to follow-up. Tumor size was calculated based on enhanced MR or CT images (on the layer with the maximum amount of tumor) as follows: long diameter (centimeter) × wide diameter (centimeter) × thickness (centimeter) × 0.5 (25). All specimens were selected after histopathological review to ensure that we would have sufficient tissue for analysis. Treatment information was obtained from the time of primary surgery, including any adjuvant treatment. The latest updated OS information was obtained on February 13, 2016. For patients who were still alive, the date of the last follow-up was used in the data analysis. All subjects gave written informed consent in accordance with the Declaration of Helsinki and research protocols were reviewed and approved by the Committee on Human Research at the Harbin Medical University, China.

Microarray Analysis of Gene Expression

To generate high-quality RNA from paired FFPE samples, a Sensation Plus™ FFPE Amplification and WT Labeling Kit was used to extract and amplify total RNA that was derived from whole tumor FFPE samples. The total RNA obtained from each sample was quantified using a NanoDrop ND-1000, and we selected three of the highest-quality paired samples from each group. The samples were used for labeling and submitted for hybridization array scanning using an Affymetrix Gene Chip® Human Transcriptome Array 2.0. The microarray was processed,

and the data were analyzed by Beijing Biolancet Technology, Co., Ltd. Nine pairs of samples were processed by Affymetrix HTA2.0 kit. After sequencing and QC, raw microarray data (CEL) were analyzed by Affymetrix Expression Console 1.4 software. HTA-2_0 library and annotation files were downloaded from Expression Console's build-in database. Raw data were further normalized by the RMA-algorithm (Table S3 in Supplementary Material). Differentially expressed genes between samples were identified using fold-change filtering to identify distinct gene expression profiles between samples. For gene enrichment analysis, paired recurrent and primary gliomas samples' microarray data were normalized by the log2 algorithm of genes mean value. Then, Gene Set Enrichment Analysis (GSEA) (26, 27) was applied to analyze the normalized data by groups. Enrichment Score, Normalized Enrichment Score, False Discovery Rate, and Nominal *p*-Value were reported by GSEA. Interpreting GSEA Results of the above four key statistics can be found in http://software.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html.

Histological Analysis, Immunohistochemistry, and Immunofluorescence

A systematic neuro-pathological review was performed based on the 2007 World Health Organization classification guidelines for CNS tumors (28). Paired samples of slides were reviewed to determine their histological classifications. Immunohistochemical staining was performed for CD3 (clone LN10, dilution 1:200, Quanhui, China), CD4 (clone UMAB64, dilution 1:50; ZSGB-BIO, China), CD8 (clone SP16, dilution 1:100; ZSGB-BIO, China), Foxp3 (clone mAbcam 450, dilution 1:50; Abcam), and CD34 (clone EP88, dilution 1:150; ZSGB-BIO, China). The secondary antibodies came as an immunohistochemical kit (KIT-5930, Maxim, China) and were incubated for 40 min at room temperature. Each staining batch included a negative control that was processed without the primary antibody, a biological negative control that consisted of normal brain tissues (the donor had died of myocardial infarction), and a positive control (tonsil tissues).

The IHC analyses were performed using a quantitative approach under a light microscope (Leica SP2, Leica Optical Co. Ltd., Germany). Subpopulations of TILs expressing CD3, CD4, Foxp3, or CD8 were divided into intratumoral fractions, which contained no vessels in each high powered field (HPF) (40× objective and 10× eyepiece), and perivascular fraction, which contained more than one vessel in each HPF (29, 30). The mean counts of CD34+ vascular circles were obtained from 10 consecutive HPF by three pathologists blinded to outcome data. Similarly, for TIL frequencies, the results were determined from a mean of 10 consecutive HPFs of typical tumor regions. The average scores counted by three experienced pathologists blinded to the clinical background were recorded as the final result. When there was a large difference in scores between observers, the score was re-evaluated to reach an agreement. Results from CD3+, CD4+, FoxP3+, CD8+ cell, and CD34+ vascular circle counts in each patient were used in the statistical analysis.

The following antibodies were used for two-color immunofluorescence: rabbit anti-CD34 and mouse anti-CD3 monoclonal antibodies (as described above). The primary antibodies were incubated overnight at 4°C. The sections were then incubated with goat anti-mouse fluorescein-isothiocyanate-conjugated secondary antibodies and goat anti-rabbit rhodamine-conjugated secondary antibodies for 2 h at room temperature. The nuclei were stained with DAPI. Finally, the sections were visualized using a confocal microscope (as described above).

Statistical Analysis

The raw data were tabulated using Microsoft Excel (Microsoft Corp.) and analyzed using nonparametric tests and matched-paired-tests (Wilcoxon tests or Mann–Whitney *U* tests). Chi-square tests were used to identify differences in chemotherapy and sex. Spearman's rank order correlation analyses were performed to detect significant associations with positive marker expression. A Kaplan–Meier survival analysis was used to evaluate differences in RFS, SR, and OS. To adjust for potential confounders, Cox proportional hazards models were used to evaluate hazard ratios (HRs) for recurrence or death according to the number of identified TIL subpopulations and clinical features. All statistical analyses were performed using SPSS 13.0 (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). All tests used to determine the level of significance were two-sided. A (two-tailed) *p*-value threshold of 0.05 was considered to indicate statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

RESULTS

Similar Kinetic Changes between Tumor-Associated Blood Vessels and TIL Subsets upon Recurrence

To learn more about overall genetic changes before and after recurrence across the three recurrent groups (DA–DA, DA–GBM, and GBM-GBM), three pairs of FFPE glioma samples from each group were analyzed by Microarray. A distinct pattern of gene expression was observed before and after each recurrence (Figure S1A in Supplementary Material). Among these altered genes upon recurrence, VEGF-A was found increased by 10.2-fold after tumor recurrence in the DA–GBM recurrent group (Table S1 in Supplementary Material). Distinct clinical outcomes were also revealed in the three groups; the longest RFS was the DA to GBM recurrence (Table S2 and Figure S1B in Supplementary Material). Based on these results, blood vessels were measured using a vascular endothelial cell marker, CD34. Tumor-infiltrating T cells, such as CD3+, CD4+, and CD8+ T cells were determined by immunohistochemistry (**Figure 1A**; Figure S2A–C in Supplementary Material). Compared to other two groups, the primary tumors in the GBM-GBM group showed relatively higher on the average density of CD34+ circles and T cells, while the DA–GBM group displayed a wider-range of T cell infiltrations (**Figures 1B–E**). Similar kinetics of CD34+ circles and infiltrating T cells were observed when these parameters were compared before and after tumor recurrence/progression; compared to recurrent GBM,

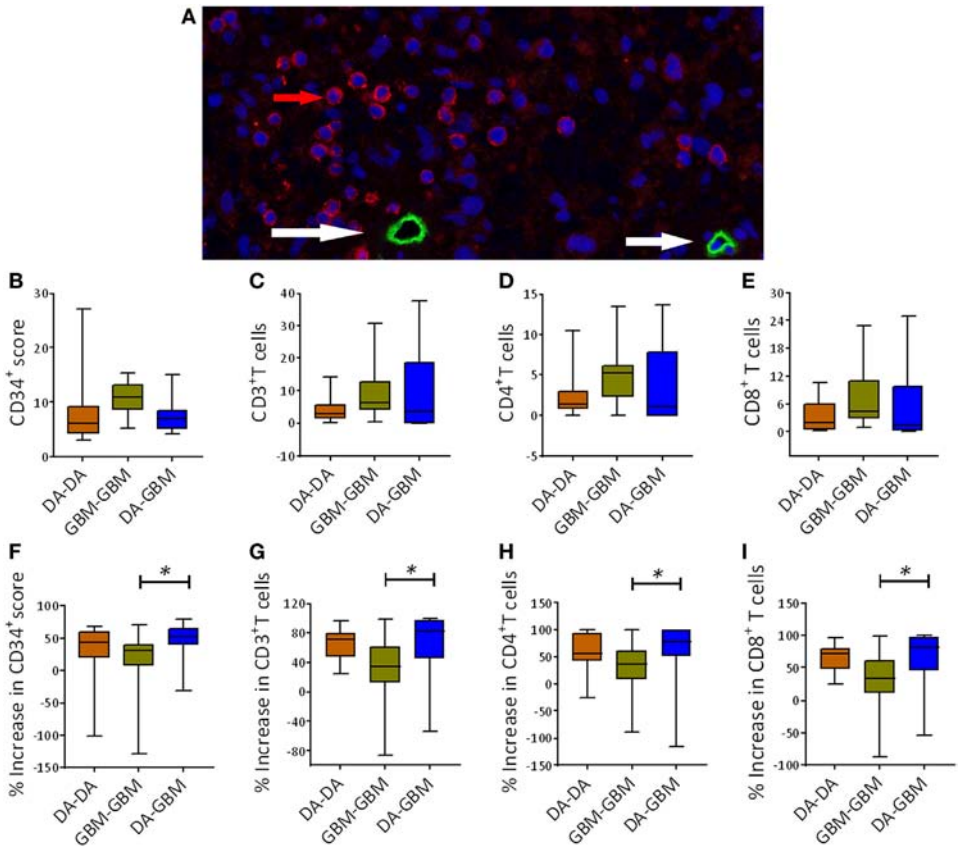


FIGURE 1 | A similar kinetic movement of tumor angiogenesis and T cell infiltrations upon tumor recurrence [e.g., diffuse astrocytomas (DA)–DA, 15 pairs, DA–GBM, 15 pairs and GBM-GBM, 14 pairs]. **(A)** A representative fluorescent immunohistochemistry staining of tumor blood vessels measured by CD34 staining (green, red arrow) and tumor-infiltrating CD3+ T cells (red, white arrow) in a surgical resected primary GBM sample. **(B–E)** The baseline counts of CD34+ circles, CD3+, CD4+, and CD8+ cells in tumors. Counts were carried out for average numbers [10 consecutive high powered fields (HPFs)] of these marker expressing cells in primary tumors among three groups. **(F)** Enhancement of CD34+ circles after the recurrence. A calculation was carried out by comparing the increase in CD34+ circles between recurrent and primary tumors. **(G–I)** A similar measurement was also carried out for the average numbers of infiltrating CD3+, CD4+, and CD8+ T cells in these patients' tumors, respectively. The significance between two groups was measured using Mann–Whitney *U* test, $^*p < 0.05$.

significantly increased CD34+ circles and T cell subsets were found in secondary GBM (**Figures 1F–I**).

CD4+ T Cells Are Associated with Tumor Blood Vessels

To test which infiltrating T cell subset associated most closely with tumor angiogenesis, we next did a Spearman's rank correlation analysis between the density of tumor vessels and T cell infiltrating subsets. CD4+ T cells correlated strongly with CD34+ circles in both primary and recurrent tumors across all three groups; notwithstanding primary tumors of DA–GBM, there was no association with CD8+ T cells (**Figures 2A–C**).

CD4 Transcript Expression Is Abundant in Primary GBMs, and Activated CD4+ T Cells Are Enriched in Bevacizumab Resistant Tumors

We found that CD4 transcript was predominantly expressed in primary GBM (>29-fold higher than CD8 alpha and beta chains), based on the RNA-seq analysis solely culled from TCGA database (TCGA Research Network: http://cancergenome.nih.gov/, **Figure 3A**). No similar results were observed for some few other cancer types, such as kidney renal clear cell, testicular germ cell tumor using TCGA Pan-Cancer datasets (30 different cancer types) (**Figure 3B**). To confirm our observation that infiltrating CD4+ T cells may be associated with tumor angiogenesis, microarray data of paired bevacizumab resistant tumors from previously published reports were analyzed and paired chemo-naïve tumors were used as a comparison control (31, 32). Only signals of activated CD4+ T cells, not other cell populations, were significantly increased in tumors after a recurrence of bevacizumab-treated patients (**Figure 3C**); no such trend found in the chemo-naïve recurrent patient tumors (**Figure 3D**).

Increasing Levels of Perivascular TILs after Glioma Recurrence

T cell tumor trafficking and tumor angiogenesis have been shown to be linked biological phenomena (3). We found both factors had an upwards trend upon recurrence; we next separated

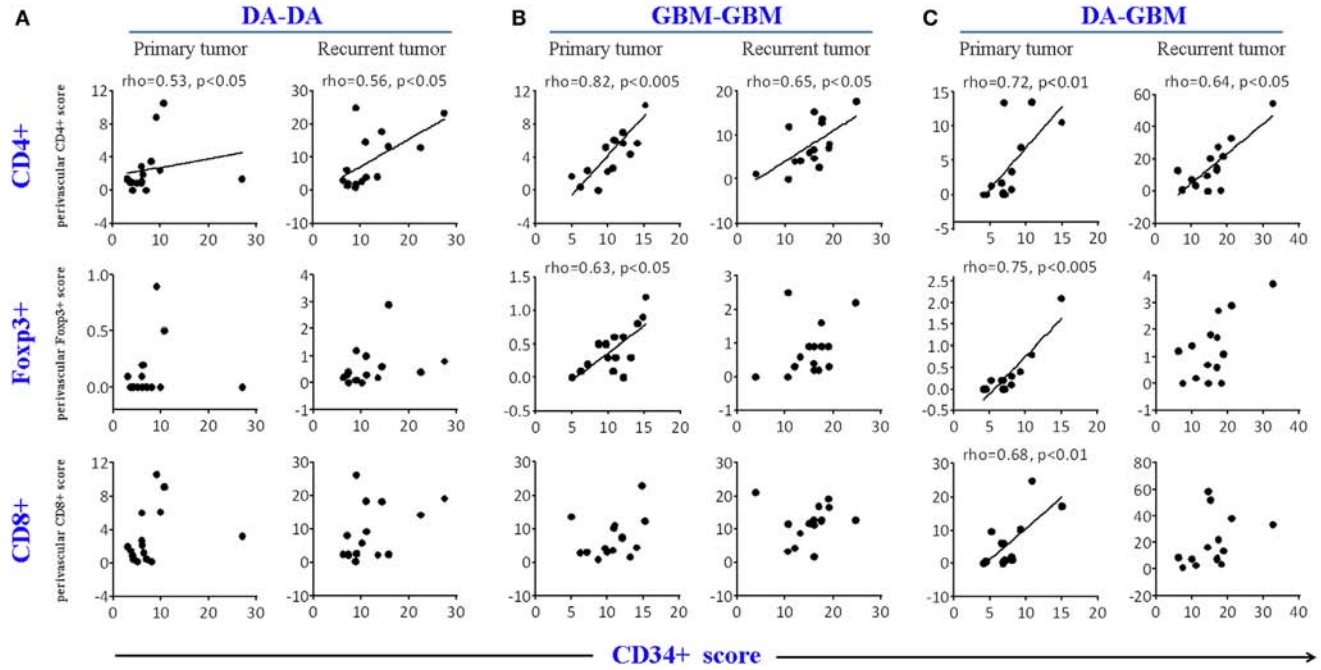


FIGURE 2 | A correlation was found between the density of CD34 circles and CD4+, but not for CD8+ T cells, in primary and recurrent tumors. The correlation CD4+ or CD8+ T cell counts with CD34 circles [average cell or vascular circle counts were obtained from 10 consecutive high powered fields (HPFs)] in primary or recurrent tumors in diffuse astrocytomas (DA)–DA group **(A)**, GBM–GBM group **(B)**, and DA–GBM group **(C)**. Spearman's rank order correlation analyses were performed and graphs with a correlation (Spearman's rho ≥ 0.5, $p < 0.05$) are indicated.

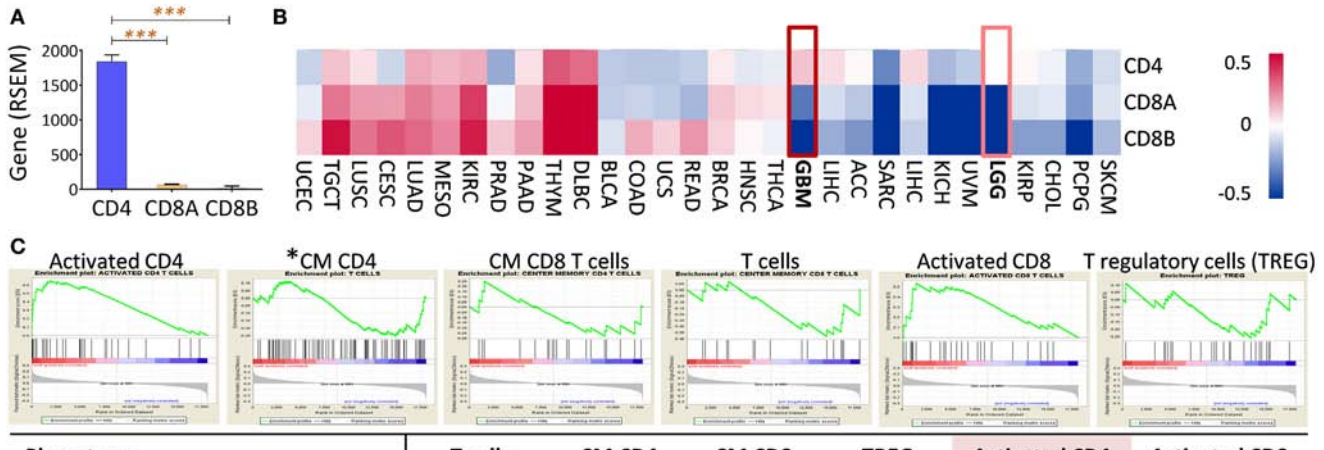
tumor-infiltrating T cells into perivascular and intratumoral CD3+, CD4+, CD8+, and Foxp3+ T cell subsets (Figure S2A–C in Supplementary Material). We demonstrated that there were more perivascular than intratumoral TILs in primary and recurrent tumors. In primary tumors, only one-third of the TILs were located in the intratumoral space, and two-thirds of the TILs were in the perivascular niche; these findings reproduce previously published reports (30). When comparing the cell numbers before and after recurrence, the perivascular T cells were enriched after tumor progression across all three groups; the intratumoral T cells, however, were only increased in recurrent tumors of the DA–GBM group (**Figures 4A–F**).

Perivascular Foxp3+ Tumor-Infiltrating T Cells Associate with Angiogenesis and Is an Independent Factor That Predicts Glioma Progression/Recurrence

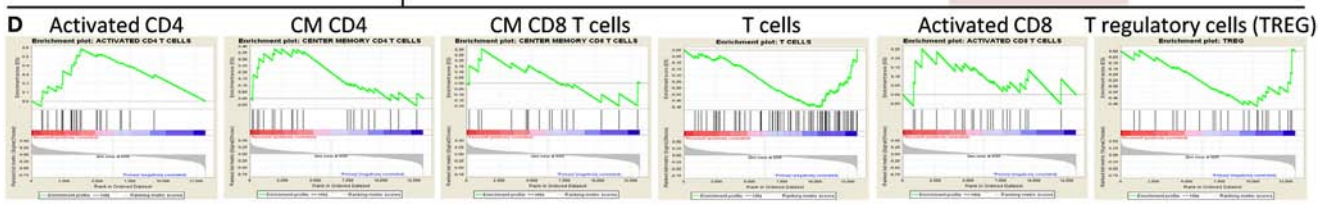
The association between tumor-infiltrating CD4+ T cells and tumor angiogenesis led us to further test the association between angiogenesis and a subset of immune inhibitory CD4+ T cells or regulatory T cells (Tregs). We found that perivascular Foxp3+ (**Figures 5A–B** upper panel), and CD4+ T cells (**Figures 5A–B**, lower panel) in the primary tumors correlated with tumor blood vessels. No similar trend was found for the CD8+ T cells in both geographic locations (data not shown). We sought to confirm the observation by using gene enrichment for Treg signature genes using paired samples. The results indicate that tumor recurrence significantly increases CD4 (similar to the results from bevacizumab resistant tumors) and Tregs signals in these tumors, whereas other cell subsets, i.e., NK, Th17, show contrasting results (**Figure 5C** and data not shown). To evaluate the correlation between differential geographic T cell subsets and clinical outcomes (RFS, OS), Kaplan–Meier survival curves were plotted using these tested parameters against RFS and OS. We found that only perivascular CD4+ T cells and Tregs were associated with shorter RFS ($p = 0.007$ for CD34, $p = 0.01$ for CD4+ T cells and $p = 0.001$ for Foxp3+ T cells). No association was observed for these factors with respect to OS (**Figures 5D–E**). Additionally, there was no association found for perivascular or intratumoral CD8+ T cells with respect to RFS and OS (Figure S3 in Supplementary Material). Next, to assess if key T cell subsets independently predict glioma progression, Cox multivariate regression analyses were performed (by including factors, such as age, sex, chemo/radiotherapy, CD34+ circles and perivascular/intratumoral infiltrating T cell subsets). The results concluded that only perivascular Foxp3+ T cells were found to be an independent predictor of shortened RFS (HR = 4.199, 95% CI 1.522–11.584, $p = 0.006$) when all the patients were included.

DISCUSSION

Several clinical and experimental studies have evaluated the correlation between TILs and survival in patients with gliomas.



Phenotype	T cells	CM CD4	CM CD8	TREG	Activated CD4	Activated CD8
Enrichment Score	-0.25	-0.28	-0.38	-0.26	0.65	0.53
Normalized Enrichment Score	-1.06	-0.85	-1.11	-0.86	2.20	1.70
Nominal p-value	0.34	0.67	0.32	0.72	0.000	0.012
**FDR q-value	0.73	0.85	0.69	0.92	0.000	0.023



Phenotype	T cells	CM CD4	CM CD8	TREG	Activated CD4	Activated CD8
Enrichment Score	-0.45	0.38	0.30	-0.48	0.59	0.25
Normalized Enrichment Score	-1.34	1.17	0.82	-1.32	1.17	0.62
Nominal p-value	0.20	0.24	0.70	0.20	0.39	0.83
FDR q-value	1.00	0.54	0.85	0.72	0.71	0.86

*CM: central memory; ** FDR q-value: False Discovery Rate (FDR) adjusted p-value or q-value.

FIGURE 3 | CD4 full-length transcript expression is abundant in primary GBM and enriched in bevacizumab-resistant tumors. **(A)** CD4 was a predominant gene transcript product (measured by RNA-seq by Expectation-Maximization, RSEM) of T cells in primary GBM compared with transcripts of CD8 alpha (CD8A) and beta (CD8B). **(B)** Gene profile of CD4, CD8A, and CD8B in other tumors, including GBM. RNAseq data of TCGA pan-cancer gene expression were used, and CD4, CD8A, and CD8B gene expressions from primary tumors were clustered as Heatmap using Subio Platform after logarithmic transformation and normalization. **(C)** Activated CD4+ T cells (highlighted) were significantly enriched in recurrent tumors after Bevacizumab treatment had failed in GBM patients. Paired surgical tumor samples before and after Bevacizumab treatment derived from 15 patients were analyzed by microarray (32). **(D)** No CD4 enrichment was in paired GBMs without any treatment. Microarray data from 16 pairs of tumor samples from recurrent GBM without any therapy were analyzed (33). The raw data were enriched between baseline and recurrent tumors using Gene Set Enrichment Analysis (GSEA) software (Broad Institute). The classification of the cell populations was based on the T cell-related metagenes (26).

Some reports indicate a positive correlation between the abundance of CD3+ or CD8+ TILs and patients survival (19, 20, 34, 35), whereas others report conflicting observations (36). For Tregs, no consensus has been reached for patients with gliomas (22, 35, 37). Nearly all the reports were based on analysis from un-paired patient populations. In this study, we collected 44 paired patient tumor samples and compared changes in gene expression before and after tumor recurrence. Compared to the other two groups, the most significant change in gene expression after recurrence was seen in the DA–GBM group (secondary GBM). Intriguingly, this group had the longest RFS, wider ranges of T cell infiltration in primary tumors, the greatest change in tumor blood vessel and TIL subsets post-recurrence, and the strongest correlation with the tumor vascularization, suggesting that disease progression in this group is unique. The kinetics of CD34+ vascular circles and the abundance of T cell subsets after recurrence showed an identical trend which led us to hypothesize that an interconnection exists between neovascularization and TILs. Indeed, we found a positive correlation between CD4+ TILs and the CD34+ circles, but not for CD8+ or CD3+ T cells (CD3+ T cell data not shown). To figure out why only CD4+ T cells correlated, we analyzed RNA-seq data of 155 primary

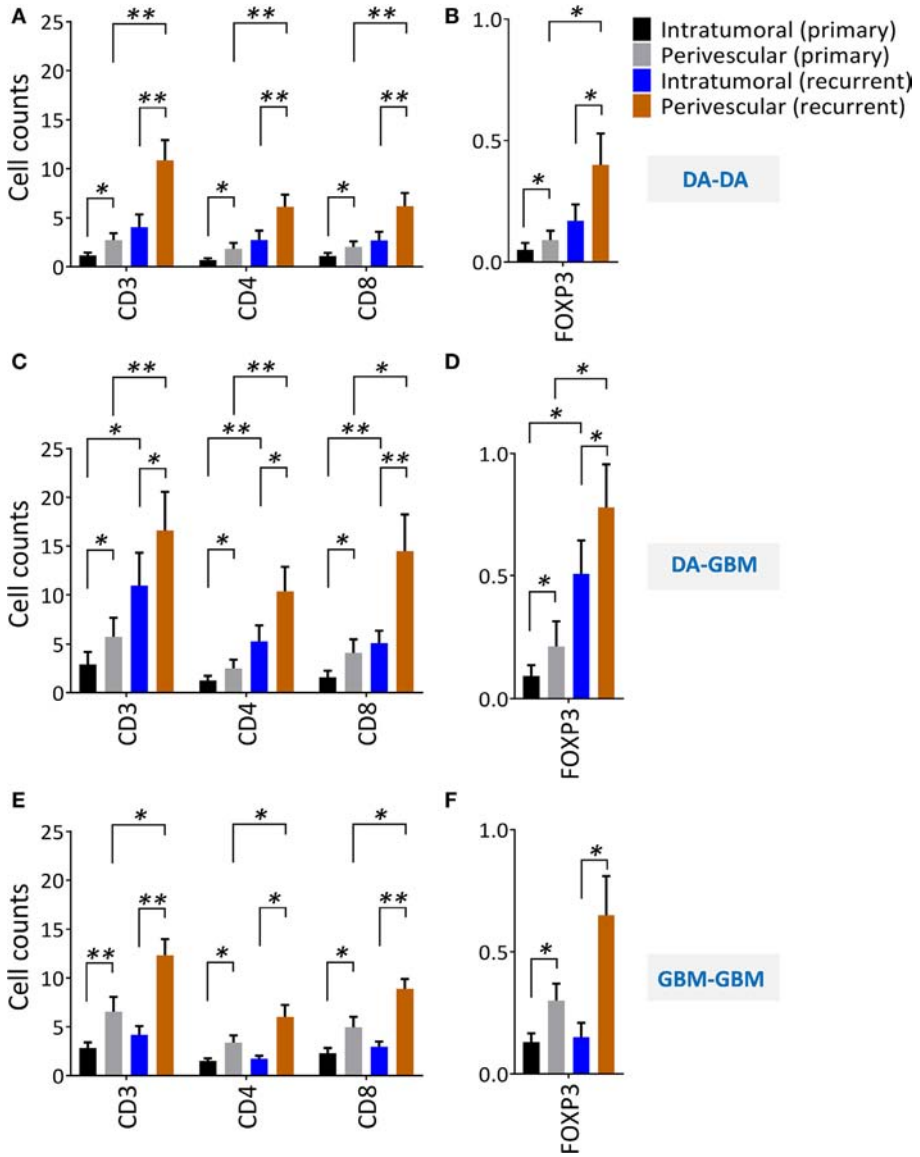


FIGURE 4 | A significant increase of perivascular tumor-infiltrating lymphocytes after tumor recurrence. Perivascular and intratumoral infiltrating CD3+, CD4+, CD8+, and Foxp3+ T cells before and after recurrence were evaluated from randomly selected consecutive 10 high powered fields (HPFs). **(A,B)** diffuse astrocytomas (DA)–DA, **(C,D)** DA–GBM, and **(E,F)** GBM–GBM. Same scales are plotted for all the recurrent groups. The significances were determined respectively using paired *t*-test.

GBMs culled from TCGA. In these tumors, CD4 expression was approximately 29-fold higher than CD8 transcripts (CD8A and B). This difference is not expected to be due to an error from sample preparation and analysis because the same analysis of other cancer types demonstrated higher expression of CD8 transcripts then CD4. The observation led us to speculate that the CD8+ T cells seeing in tumor tissues by IHC may be inadequate for the RNA-seq detection. Additionally, results from our recent study indicate that selective apoptosis of CD8+ T cells occurs in GBMs (38). Next, we utilized public data from Bevacizumab-treated paired patient samples; 15 patients had microarray data for their tumors before and after the bevacizumab treatment (31, 32). The results demonstrated that genes of activated CD4+ T cells were significantly increased in tumors after the recurrence, whereas other cell populations presented less or no change. These results confirmed our observation that CD4+ T cells are preferentially linked with tumor angiogenesis. Moreover, the gene profiles obtained by RNA-seq analysis of isolated CD4+ and CD8+ T cells also suggests that the activated CD4+ T cells express more pro-angiogenic molecules such as a CD40 ligand and Aquaporin 3 (39, 40). The evidence from previous reports supports our hypothesis. In an ischemia animal model, CD4-deficient mice have impaired capability to undergo angiogenesis (41). Although we were

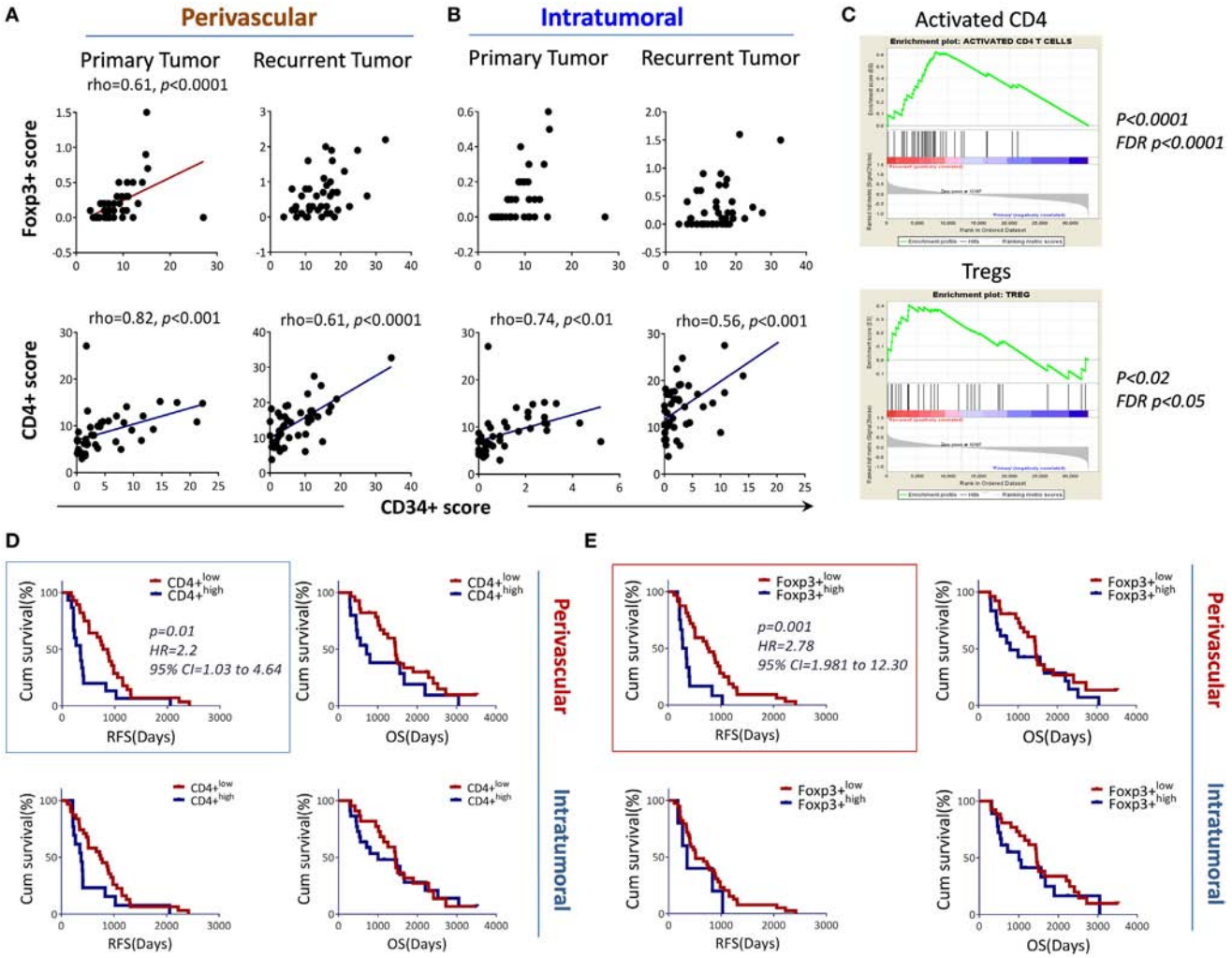


FIGURE 5 | Perivascular Foxp3+ T cells associated with angiogenesis and tumor progression. **(A,B)** Perivascular Foxp3+ (upper panel) or CD4+ T cells (lower panel) were positively associated with CD34+ circles, respectively. **(C)** Regulatory T cells (Tregs) signature genes enriched by the tumor recurrence. The microarray data described in Figure S1 in Supplementary Material were input into Gene Set Enrichment Analysis (GSEA) for an analysis of activated CD4 T cells and Tregs. **(D)** Association of perivascular or intratumoral CD4+ T cells with RFS (left) and overall survival (OS) (right). **(E)** Association of intratumoral or perivascular Foxp3+ T cells with RFS and OS. The association between the markers and RFS, OS is shown using Kaplan–Meier survival curves, the median value was used for stratifying the low and high in each recurrent group. The log-rank test was used to compare the differences.

unable to be sure that FoxP3+ cells were CD4+ T cells by IHC staining (due to technical difficulties), we predict these cells to be Tregs based on: (1) morphology; (2) geographic location (i.e., perivascular association); (3) tumor immunosuppressive microenvironment limiting passage of activated T cells; and (4) gene enrichment studies demonstrating enhanced activated CD4+ cells and Tregs upon tumor recurrence. Ongoing studies in animal models have linked glioma angiogenesis and Treg involvement; combining VEGF and CD25 blockades restores the IFN-γ production by T cells previously suppressed by gliomas and significantly prolongs the OS in mice compared with single drug treatment (Long et al., manuscript in preparation). These data suggest that CD4+ T cells, including Tregs, may be involved in glioma angiogenesis.

T cell infiltration into the Virchow–Robin space is distinct from T cell infiltration into intratumoral spaces (42). Moreover, intratumoral TILs appear to associate with more favorable clinical outcomes (43). In gliomas, however, more investigations are necessary to draw definitive conclusions (30, 44). Our results showed that only one-third of all TILs are located in the intratumoral space while two-thirds of them (presumably CD4+ T cells, including Tregs) surrounding the tumor vessels. We found that Tregs highly correlated with the density of blood vessels in primary tumors, but only in the perivascular zone. Importantly, these perivascular Tregs cells were identified as an independent risk factor for tumor recurrence.

Recent genetic classification schemas have advanced our understanding of molecular characteristics and factors that

impact tumor progression and survival (45–47). The status of IDH, *TERT* promoter mutations, and the deletion of chromosome 1p/19q was found to significantly impact patient clinical outcome (45, 48, 49). Beyond the scope of this report, we also examined our patients' IDH mutation status; among the 44 patients, 22 tumors harbored the mutation, while the other 22 were IDH wild type (mostly from the GBM-GBM group). We uncovered that IDH mutations impact on the tumor immune landscape, and affect survival outcomes (Mu et al., manuscript under review). We included all patient data in **Figure 5**, and when patients in the GBM-GBM groups were removed, the main conclusion of Foxp3+ T cells as the independent risk factor for tumor recurrence remained true. Presumably, these Foxp3+ T cells are CD4 positive since it was the predominant T cell expressed transcript observed in GBM, and we also have found that CD8+ T cells are apoptotic in GBM (50). Thus, these CD4+ Foxp3+ T cells not only play the key role in pro-immunosuppression but also possess the pro-angiogenic function of the CD4+ T cells. The dual effects of these cells in primary tumors make them a strong player in the promotion of tumor progression in juxtaposition with the extremely low expression of CD8 transcripts in primary GBMs, which can be a major obstacle in tumor treatment.

In summary, only one-third of TILs were found in the intratumoral space with minimal expression of CD8 transcripts in primary tumors, thus limiting the overall strength of the antitumor response. The predominant population of CD4+ T cells may promote tumor angiogenesis, and in conjunction with perivascular CD4+ Tregs predispose tumor recurrence/progression in patients with gliomas.

AUTHOR CONTRIBUTIONS

Conception and design: LM, ZL, and JH. Development of methodology: LM, CY, QG, YL, HG, YC, LJ, JQ, JJi, JJiang, YG, JW, and YS. Data analysis: LM, CY, PK, and JH. Analysis and interpretation of data: LM, CY, ES, ZL, and JH. Writing, review, and revision of the manuscript: LM, YC, GL, ES, DM, ZL, and JH. Study supervision: JH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01451/full#supplementary-material.

FIGURE S1 | Global gene profile changes and clinical outcomes for patients with DA to DA (DA-DA); DA to GBM (DA-GBM) and GBM to GBM (GBM-GBM) recurrence.

FIGURE S2 | Representative images of CD34+ circles and intratumoral and perivascular CD3+, CD4+, CD8+, and Foxp3+ TIL cell subsets in 3 recurrent groups.

FIGURE S3 | No association between CD8+ T cells with RFP in primary tumors.

TABLE S1 | Upregulated genes after DA-GBM.

TABLE S2 | Patients' demographic and clinical characteristics.

TABLE S3 | Microarray analysis of paired surgical resected tumors (3 pairs DA to DA, 3 pairs GBM to GBM and 3 pairs DA to GBM).

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REVIEW
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Multifaceted Effects of Extracellular Adenosine Triphosphate and Adenosine in the Tumor–Host Interaction and Therapeutic Perspectives

Paola de Andrade Mello[1], Robson Coutinho-Silva[2] and Luiz Eduardo Baggio Savio[2]**

[1] Division of Gastroenterology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States, [2] Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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***Correspondence:**
Robson Coutinho-Silva rcsilva@biof.ufrj.br;
Luiz Eduardo Baggio Savio savio@biof.ufrj.br, edubsavio@gmail.com

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Cancer is still one of the world's most pressing health-care challenges, leading to a high number of deaths worldwide. Immunotherapy is a new developing therapy that boosts patient's immune system to fight cancer by modifying tumor–immune cells interaction in the tumor microenvironment (TME). Extracellular adenosine triphosphate (eATP) and adenosine (Ado) are signaling molecules released in the TME that act as modulators of both immune and tumor cell responses. Extracellular adenosine triphosphate and Ado activate purinergic type 2 (P2) and type 1 (P1) receptors, respectively, triggering the so-called purinergic signaling. The concentration of eATP and Ado within the TME is tightly controlled by several cell-surface ectonucleotidases, such as CD39 and CD73, the major ecto-enzymes expressed in cancer cells, immune cells, stromal cells, and vasculature, being CD73 also expressed on tumor-associated fibroblasts. Once accumulated in the TME, eATP boosts antitumor immune response, while Ado attenuates or suppresses immunity against the tumor. In addition, both molecules can mediate growth stimulation or inhibition of the tumor, depending on the specific receptor activated. Therefore, purinergic signaling is able to modulate both tumor and immune cells behavior and, consequently, the tumor–host interaction and disease progression. In this review, we discuss the role of purinergic signaling in the host–tumor interaction detailing the multifaceted effects of eATP and Ado in the inflammatory TME. Moreover, we present recent findings into the application of purinergic-targeting therapy as a potential novel option to boost antitumor immune responses in cancer.

Keywords: purinergic signaling, P2X7 receptor, CD39, CD73, tumor microenvironment, immunotherapy

INTRODUCTION

Cancer is still one of the world's most pressing health-care challenges, leading to death in an estimated number of 600,920 patients per year in the United States (1). However, recent advances in cancer immunotherapy have transformed the treatment of several patients, extending and improving their lives (2, 3). Immunotherapy is a new developing therapy that boosts patient's immune system

to fight cancer, by modifying tumor–immune cells interaction in the tumor microenvironment (TME) (4). According to the cancer immunoediting concept, the interaction between cancer and immune cells occurs in three essential phases: elimination, equilibrium, and escape—from cancer immune surveillance to immune escape (5–7). In the elimination and equilibrium phase innate and adaptive immune system—mainly NK and T cells—mount an effective immune response against the highly immunogenic tumors, and allow the less immunogenic ones escape (8–16). This immunologic pressure selects and favors tumor variants resistant to the immune system to proliferate (immunoevasion) (9, 17). During this process, both cancer and inflammatory cells release several soluble factors such as cytokines, chemokines, growth factors, matrix-degrading enzymes, and nucleotides that facilitate tumor immune escape and allow tumor growth, angiogenesis, invasion, and metastasis (18–22). Therefore, targeting multiple molecules that avoid immunoevasion and boost antitumor immune responses are the leading paths to successfully treat a whole range of tumor types (3).

Among the nucleotides released in the TME, extracellular adenosine triphosphate (eATP) and adenosine (Ado) are potent modulators of both immune and tumor cell response (23, 24). eATP and Ado exert their effects acting through P2 and P1 purinergic receptors, respectively, triggering the so-called purinergic signaling (25, 26). Purinergic signaling has long been involved with inflammation and cancer having a pivotal role in modulating cell migration, proliferation, and cell death (27, 28). P2 and P1 receptors are expressed by nearly all cell types (immune and non-immune cells) and differently trigger cell signaling according to their subtypes (29–31). The P2 receptor is subdivided into two separate subfamilies, P2X (P2X1–7) ionotropic ion channels receptors and P2Y (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11–P2Y14) G-protein-coupled receptors (25, 26), whereas the P1 receptor family (A_1, A_{2A}, A_{2B}, and A_3) only comprised by G-protein-coupled receptors subtype (32). These different purinergic receptors express distinct agonist affinity and specificity, therefore influencing both tumor and immune cells behavior according to the levels of eATP/Ado in TME (33–35).

Levels of eATP and Ado are tightly controlled by several ectonucleotidases. Among them, CD39 and CD73 are the most important ecto-enzymes expressed in cancer cells, regulatory immune cells and vasculature responsible for modulating purinergic signaling within the TME (36, 37). CD39 is a member of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family that comprised of eight members (E-NTPDase1–8), each one with a distinct cellular location and catalytic properties (36, 37). E-NTPDase1 (CD39), E-NTPDase2, E-NTPDase3, and E-NTPDase8 are plasma membrane-bound enzymes that degrade with different affinities adenosine triphosphate (ATP) and ADP to AMP (24, 36, 37). AMP is in turn converted to Ado by CD73, which is an ecto-5′-nucleotidase cell-surface enzyme (37). This sequential activity of CD39/CD73 is the main pathway for the eATP scavenging and generation of Ado in the tumor interstitium (24, 36).

Once accumulated in the TME, eATP and Ado act as signaling molecules triggering different and opposite effects on both host and tumor cells. While eATP boosts antitumor immune response and Ado attenuates or suppresses immunity on the host side (38–45), both molecules can mediate growth stimulation or inhibition on the tumor cells, depending on the specific receptor activated (46–52). Regardless, the final effect on tumor growth—either beneficial or detrimental—will depend on the eATP/Ado levels, the panel of P2 and P1 receptors subtypes and CD39/CD73 expression by immune, tumor, and stromal cells in the TME (22).

Therefore, despite its complexity and dual behavior, modulation of purinergic signaling by targeting eATP/Ado pathways appears to be a promising strategy to modify cancer and immune cells cross talk in the TME (24, 36, 53). In this review, we will discuss the role of purinergic signaling into the host–tumor interaction detailing the multifaceted effects of eATP and Ado in the inflammatory TME. Furthermore, we will highlight the application of combining purinergic-targeting therapies with other anticancer treatments as a potential new strategy to overcome immune escape, potentiate antitumor immune response, and, consequently, restrain tumor growth.

eATP IN THE TME

Measurement of eATP levels in different biological context reveals that healthy tissues present very low levels (10–100 nM) of this nucleotide in the pericellular space, while in sites of tissue damage, inflammation, hypoxia, ischemia, TME or metastases it can reach high levels (hundreds of micromoles per liter) (24, 54–56). ATP is abundantly released in the extracellular space due to cell death, cell stress, and activation of pannexin/connexin channels on immune and endothelial cells (54, 57, 58). In these settings, increased levels of eATP are sensed as a "danger signal" by the innate immune cells resulting in their recruitment to the damaged-tissue site (42, 57, 59–61). Particularly in the TME, eATP acting through P2 receptors boosts the antitumor immunity at the same time that stimulates endothelial and tumor cells (27, 36, 42, 48, 60).

eATP Effect on the Host Side

Activation of P2 receptors by eATP shapes various innate and adaptive immune responses (30). The P2X and P2Y receptors expression (either constitutive or upregulated in pathological conditions) varies according to the cell type and therefore dictates immune cell function, such as metabolism, adhesion, activation, migration, maturation, release of inflammatory mediators, cytotoxicity, and cell death, as extensively reviewed in Ref. (30, 36, 62). In the innate immunity, activation of $P2Y_2$ and P2X7 receptors leads to stimulation of myeloid cells and promotes chemotaxis of macrophages and neutrophils (38, 63–65). At the same time, engagement of $P2Y_2$ and P2X7 receptors induces dendritic cells (DCs) activation and chemotaxis (66). Indeed, stimulation of $P2Y_{11}$ receptor inhibits IL-12 and boosts IL-10 release by DCs (67) whereas it activates granulocytes (68). In the adaptive immunity, engagement of various P2X receptors, such as P2X1, P2X4, P2X5, and P2X7, results in T-cell activation (39, 69–71). Among them, P2X7 has been linked to stimulation of $CD4^+$ and $CD8^+$ effector T cells (40, 69, 72) as well as NKT cells (73), induction of Treg apoptosis (41, 74, 75), and inhibition of Tr1 cell differentiation (76). In addition, ATP acting *via* the P2X7 receptor is crucial to

the generation of inflammatory Th17 lymphocytes by contributing for the generation of a microenvironment with high levels of IL-1β, IL-6, and IL-17 (77, 78).

In the context of TME, recent studies have highlighted the importance of eATP acting through the P2X7 receptor in the chemotherapy-elicited anticancer immune response, also known as immunogenic cell death (ICD) (42, 60). Accordingly, ATP derived from dying tumor cells stimulates P2X7 receptors in DCs, thus activating the NLRP3/ASC/caspase-1 inflammasome and driving the secretion of interleukin-1β (IL-1β). IL-1β is then required for the adequate polarization of IFNγ-producing $CD8^+$ T cells, which is critical for the efficacy of chemotherapy (42, 60).

Despite its role in ICD, eATP-P2X7 signaling has also been related to the control of tumor growth. Recent studies have shown that host P2X7 expression limits tumor growth and metastasis spread by supporting an antitumor immune response (47, 79). Host P2X7 seems to boosts cytokine release, chemotaxis, and tumor infiltration by inflammatory cells. Accordingly, P2X7 host genetic deletion in mouse (P2X7-KO) impaired immune response against melanoma (B16) and colon carcinoma cells (CT26), leading to accelerate tumor growth in comparison to P2X7-WT hosts. Moreover, transplantation of P2X7-WT bone marrow to P2X7-KO mice reduced tumor growth at a rate similar to the P2X7-WT group (47).

Even though eATP acting through P2X7 receptor seems to be an important signaling to stimulate immune cell response against the tumor, a critical role for the ATP/P2X7 receptor axis in modulating myeloid-derived suppressor cells (MDSCs) functions in the TME has also been described (23). Accordingly, P2X7 receptor activation stimulates the release of reactive oxygen species, arginase-1, and transforming growth factor-β 1 (TGF-β1) from monocyte MDSCs present in the TME, contributing to MDSC immunosuppressive effect. Therefore, considering these contradictory effects the use of both antagonist/agonist of the P2X7 receptor has been investigated as a promising novel strategy for anticancer therapy and will be discussed with more details below.

eATP Effect on the Tumor Side

Practically all types of cancer cells express P2X and P2Y receptors that efficiently sense changes in ATP concentration in the TME and modulate different cellular functions such as proliferation, differentiation, and apoptosis (24, 28). Cancer cells may be more sensitive to the cytotoxic or to the trophic effect of e ATP according to the expression of their P2 receptor subtypes as well reviewed in Ref. (28).

Among the P2Y receptors, stimulation of $P2Y_2$ and $P2Y_{11}$ receptors leads to cell proliferation and migration of human hepatocellular carcinoma (HCC) cells (49, 80). $P2Y_2$ receptor activation is also highly involved with tumor invasiveness and metastatic diffusion in prostate and breast cancer (81–87). On the other hand, eATP-$P2Y_2$ receptor signaling inhibited nasopharyngeal carcinoma and human colon carcinoma growth (50, 88). $P2Y_1$ receptor activation induces apoptosis and inhibits human intestinal epithelial carcinoma, prostate cancer, and melanoma cell proliferation (89–91).

In the P2X receptors family, a role for P2X3, P2X5, and P2X7 in carcinogenesis has already been depicted, with a major focus on the P2X7 receptor. P2X3 receptor overexpression seems to be crucial for HCC cell survival and basal proliferation as well as proliferation in response to changes in ATP concentrations in the TME (92). Moreover, high P2X3 receptor expression is associated with poor prognosis in patients with HCC. P2X5 overexpression was also demonstrated in human basal cell and squamous carcinomas, but differently, it was expressed exclusively on cells undergoing proliferation and differentiation, suggesting a different role in tumor growth (93).

P2X7 is far the most P2X receptor subtype studied in cancer. Unlike the other P2 receptors, P2X7 is unique for its capacity to form a nonselective pore on the plasma membrane upon stimulation with high levels of eATP, leading to cell death (94, 95). Its role in carcinogenesis remains a controversy, but now it is known that P2X7 receptor triggers cell death or growth according to its level of activation and cell type stimulated (94, 96–98). As mentioned earlier, P2X7 receptor overstimulation with a high level of exogenous eATP triggers tumor cell death, while its tonic stimulation with endogenous eATP often induces cancer cell survival and proliferation (28, 99, 100). Whereas the former leads to a marked mitochondrial catastrophe, the latter stabilizes the mitochondrial network, increases mitochondrial potential, oxidative phosphorylation, and aerobic glycolysis, culminating in a large increase in the overall intracellular ATP content and gain in proliferative advantage by P2X7-expressing cells (99). P2X7 receptor activation also triggers NFATc1, Erk, PI3K/Akt, and HIF-1α intracellular pathways (101–103), being the PI3K/Akt pathway linked to the P2X7-dependent tumor cell growth, invasiveness, metastatic spreading, and angiogenesis (101, 104). Also supporting a role for P2X7 receptor in tumor growth is the fact that many types of cancer such as leukemia (98, 105, 106), melanoma (107), neuroblastoma (108), pancreatic adenocarcinoma (109), esophageal carcinoma (110), breast (111), prostate (112), thyroid (113), and head and neck cancer (114) showed an increased expression of P2X7 receptor. Moreover, *in vivo* experiments demonstrated that blocking P2X7 receptor activation by either silencing or a pharmacological manipulation decreased tumor progression and inhibited metastatic diffusion (100, 115). Therefore, it seems reasonable to say that P2X7 receptor is an important target in cancer therapy not only for its role in the immune system but also for its impact on tumor growth. An overview of eATP effect on tumor and host side is illustrated in **Figure 1**.

eADENOSINE IN THE TME

High levels of extracellular adenosine (eAdo) were also demonstrated in the TME. While Ado levels in healthy tissue are around the nanomolar range, it can reach the micromolar range in the tumor core (36, 51, 116, 117). In the later context, many factors can contribute to Ado production, but hypoxia seems to be the main driver for the eAdo accumulation (118). In this setting, eAdo is mainly generated at the expenses of the eATP metabolism *via* the sequential enzymatic activity of CD39 and CD73 (119–122). CD39 catalyzes the first enzymatic reaction by breaking down ATP and ADP into AMP, whereas CD73 hydrolyzes AMP into Ado. CD73 irreversibly converts AMP to Ado being considered the rate-limiting enzyme for Ado formation (37, 122).

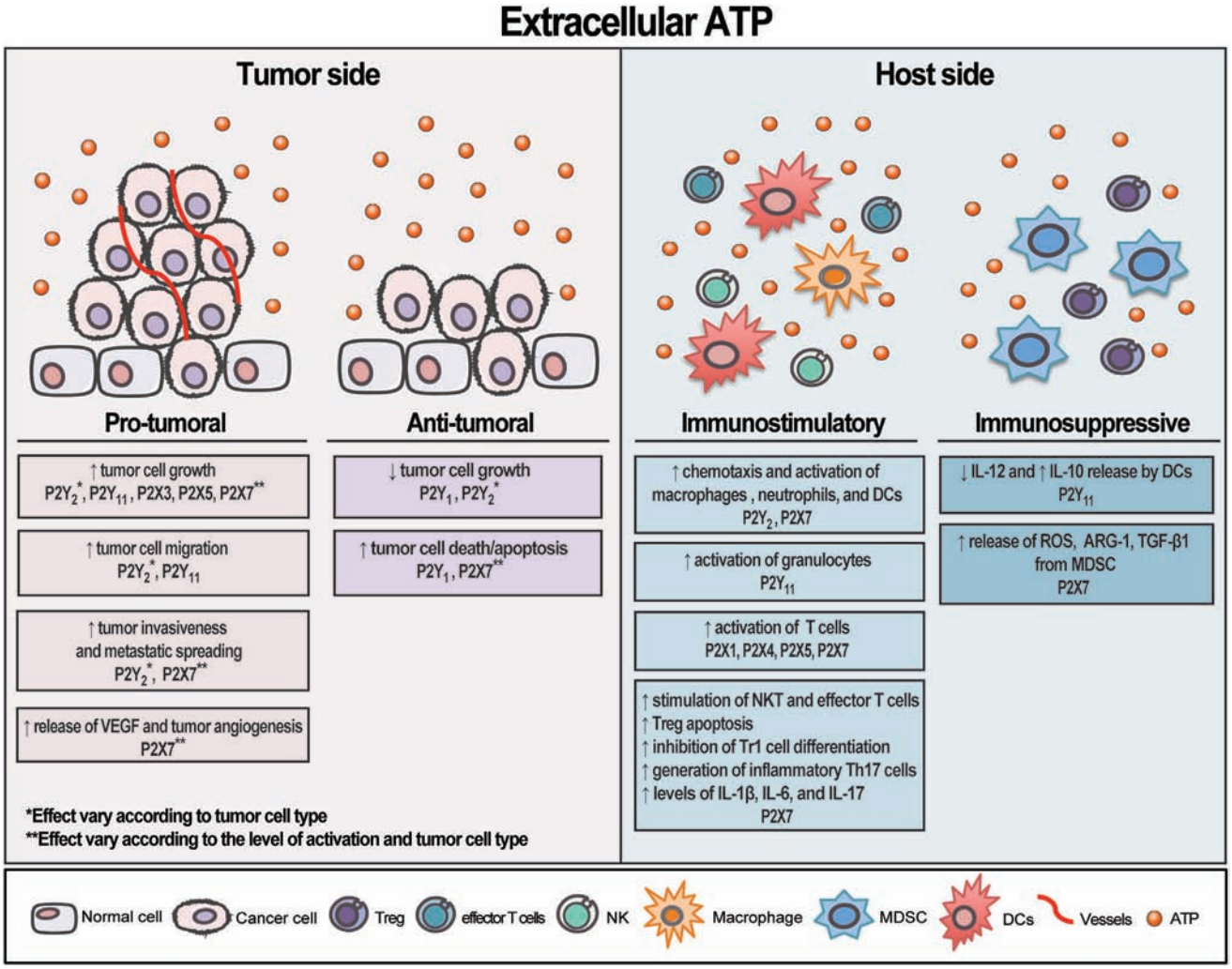

FIGURE 1 | Schematic illustration showing extracellular adenosine triphosphate (eATP) contrasting effects on tumor and host side. eATP can trigger different and opposite effects on both tumor and host cells depending on the cell type and receptor activated. The final result—either stimulating or restraining tumor growth—will depend on the eATP levels, the panel of P2 receptor subtypes and CD39/CD73 expression by tumor and immune cells present in the tumor microenvironment. Overall, eATP is a potent pro-inflammatory mediator, mostly boosting immune cells response.

Many cells have the capacity to generate eAdo in the TME, such as tumor cells (43, 120, 123–126), Tregs (120, 127, 128), Th17 (129, 130), MDSCs (44, 131, 132), endothelial cells (127, 133, 134), cancer-associated fibroblast (135, 136), and mesenchymal stromal/stem cells (MSCs) (45, 137). Exosomes derived from $CD39^+CD73^+$ tumor cells (138), Tregs (139), or MSCs (45) can also contribute to eAdo production. Once in the pericellular space, Ado can exert a local signaling effect through the activation of the P1 purinergic receptors, be metabolized to inosine or recaptured by the cell *via* nucleoside transporters (140).

Likewise eATP, eAdo acts as an endogenous immunomodulatory molecule, but unlike the former, it mostly mediates immunosuppressive effects (30). Particularly in the tumor interstitium, eAdo acting through P1 receptors downregulates cell-mediated immunity at the same time that stimulates tumor cells and promotes angiogenesis (45, 133, 136, 137).

eAdo Effect on the Host Side

Extracellular adenosine exerts immunosuppressive activities in various immune subsets, interfering with antitumor immune responses (36). Innate and adaptive immune cells react to Ado stimulation according to the expression/density of the four P1 receptor subtypes, namely A_1, A_{2A}, A_{2B}, and A_3 (30, 32). These receptors sense different levels of Ado and are classified as high-affinity (A_1, A_{2A}, and A_3) and low-affinity receptors (A_{2B}) (32). A_1 and A_3 are Gi-coupled receptors that inhibit adenylate cyclase and cyclic AMP production, while A_{2A} and A_{2B} are Gs-coupled receptors that stimulate cAMP synthesis and downstream signaling pathways (32, 141).

Activation of A_{2A} and A_{2B} receptors protect tissues against excessive immune reaction and therefore play a major role in Ado immunosuppressive effects (142–146). Stimulation of A_{2A} receptor is related to the inhibition of DC activation (147), Th1/Th2 cytokine production (148, 149), T cells proliferation and

activation (148, 149), and NK cells activation, maturation, and cytotoxicity (125, 150), as well as enhancement of the suppressive function of Tregs, Tr1 cells, and macrophages (151–153). In addition, A_{2A} receptor activation prevents the LPS-induced increase in ectonucleotidase activities during inflammation (154, 155).

Activation of the A_{2B} receptor has a major effect on Tregs and MDSCs, stimulating Treg proliferation or differentiation from naïve T cells, production of IL-10 (156) and enhancing the suppressive function of MDSCs (44). A_{2B} signaling is also linked to vascular endothelial growth factor (VEGF) secretion and tumor angiogenesis (44, 157). Engagement of A_{2A} and A_{2B} receptors inhibits neutrophils activation (158) and immune cells adhesion to endothelial cells (127). On the other hand, activation of A_1 and A_3 receptors promotes neutrophils chemotaxis and stimulates pro-inflammatory activities (158).

In general, Ado accumulation in the TME and its immunosuppressive effect *via* A_{2A} and A_{2B} receptors is a critical regulatory mechanism implemented by the tumors to evade the immune-mediated cancer cells destruction, allowing tumor growth and impairing cancer immunosurveillance (159). In this way, new strategies targeting Ado production and signaling have emerged as a promising approach in cancer immunotherapy and will be discussed in more details below.

eAdo Effect on the Tumor Side

Differently from its effect on the host side, where Ado is well known for its strong immunosuppressive activities, on the tumor side Ado can either stimulate or inhibit tumor growth, depending on the cell type and receptor expressed by the tumor bulk (160). Likewise, pro- and antitumoral effects coming from A_1, A_{2A}, A_{2B}, and A_3 activation have been described (160). A_1 receptor activation is related to stimulation of MDA-MB-468 breast carcinoma cells proliferation (161) and melanoma cells chemotaxis (162). On the other hand, it may inhibit LoVo colon (163), TM4 Sertoli-like (164), MOLT-4 leukemia, T47D, HS578T, and MCF-7 breast, and glioblastoma cancer cells proliferation (160). Ado-A_1 signaling has also been reported to protect endometrial carcinoma invasion and metastasis, by promoting cortical actin polymerization, increasing cell–cell adhesion thus preserving epithelial integrity (165). In the same manner, activation of A_{2A} and A_{2B} receptors leads to controversial scenarios depending on the cell type studied. A_{2A} stimulation results in increased MCF-7 breast cancer proliferation (166), whereas it promotes A375 melanoma cell death (167). Activation of A_{2B} receptor inhibits ER-positive MDA-MB-231 breast cancer cell proliferation, while it boosts oral squamous cell carcinoma progression (168, 169). Stimulation of A_{2B} receptor also leads to reduced cell–cell contact and increased cell scattering in breast, lung, and pancreatic cancer cell lines, suggesting a role for this receptor in tumor invasion and metastatic spreading (170). These conflicting results might reflect differences in the experimental settings where distinct tumor cell lines were exposed to diverse agonist/antagonist drugs with different specificity and selectivity. Moreover, the use of specific agonist might not reflect the real effect triggered by Ado in the context of the tumor bulk given the complexity and heterogeneity of cells, Ado receptors, and downstream signaling that interact to produce the final cellular response.

A_3 is by far the most studied Ado receptor in cancer and conflicting results have also been reported for this receptor. A_3 receptor is expressed by many tumor cell lines, such as HL60 and K562 human leukemia (171, 172), Jurkat lymphoma (173), U937 monocytic–macrophagic human cell lines (174, 175), Nb2 rat lymphoma (176), A375 human melanoma (177), PGT-betamouse pineal gland tumor cells (178), human glioblastoma (179, 180), and human prostatic cancer cells (181). Moreover, A_3 overexpression (either protein or mRNA levels) has been reported in human melanoma, colon, breast, small-cell lung, thyroid, pancreatic, and HCC vs adjacent normal tissue, supporting the notion that A_3 receptor levels may reflect the status of tumor progression (182–184). In accordance with this statement, A_3 activation increases HT29, DLD-1 and Caco-2 colon cancer cell proliferation (160). However, A_3 stimulation also results in antitumoral effects, inhibiting proliferation of Nb2-11C and YAC-1 lymphoma, K562 and HL60 leukemia, B16-F10 and A375 melanoma, LN-Cap and PC3 prostate carcinoma, MIA-PaCa pancreatic carcinoma, breast and Lewis lung carcinoma cells (176, 185–189). Contrasting responses were also reported for A_3 stimulation on metastatic spreading, leading to either increased (HT29 colon carcinoma) or decreased (prostatic cancer) cell migration (179, 181). Despite these dual effects, the A_3 receptor has been pointed as a potential target for tumor growth inhibition (182, 190). A phase I/II clinical trial using an A_3 agonist for the treatment of advanced unresectable HCC has been performed and despite preliminary data, favorable results were demonstrated in patients (191).

Rather than acting through P1 receptors, eAdo can also promote tumor cell death *via* its continuous uptake into the cell (52). Our group demonstrated that Ado formed from eATP degradation is the main factor responsible for apoptosis induction in human cervical cancer cells. Accordingly, eAdo transported into the cell through the nucleoside transporters leads to AMPK activation, p53 increase, PARP cleavage, and autophagy induction, culminating in cell death (52). Similar results were also reported in human gastric cancer cells (192), malignant pleural mesothelioma cell (193), mouse neuroblastoma cells (194), astrocytoma cells (195), and human epithelial cancer cells originating from breast, ileum, colon, and ovary (89, 196), bringing a distinct insight into the Ado effect on the tumor side. An overview of eAdo effect on tumor and host side is illustrated in **Figure 2**.

PURINERGIC SIGNALING AS POTENTIAL TARGET FOR CANCER THERAPY

As depicted alongside this review, purinergic signaling has a major role in controlling tumor growth, survival, and progression, not only by acting on tumor cells but also by modulating the immune system and the interaction of tumor and immune cells in the TME (24). Therefore, many potential targets involving ATP and Ado signaling has emerged as attractive candidates for cancer therapy. In this topic, we will discuss recent findings in this field highlighting P2X7, CD39, CD73, and A_{2A} receptor targeting therapy to restrain tumor progression *in vivo* models and in patients.

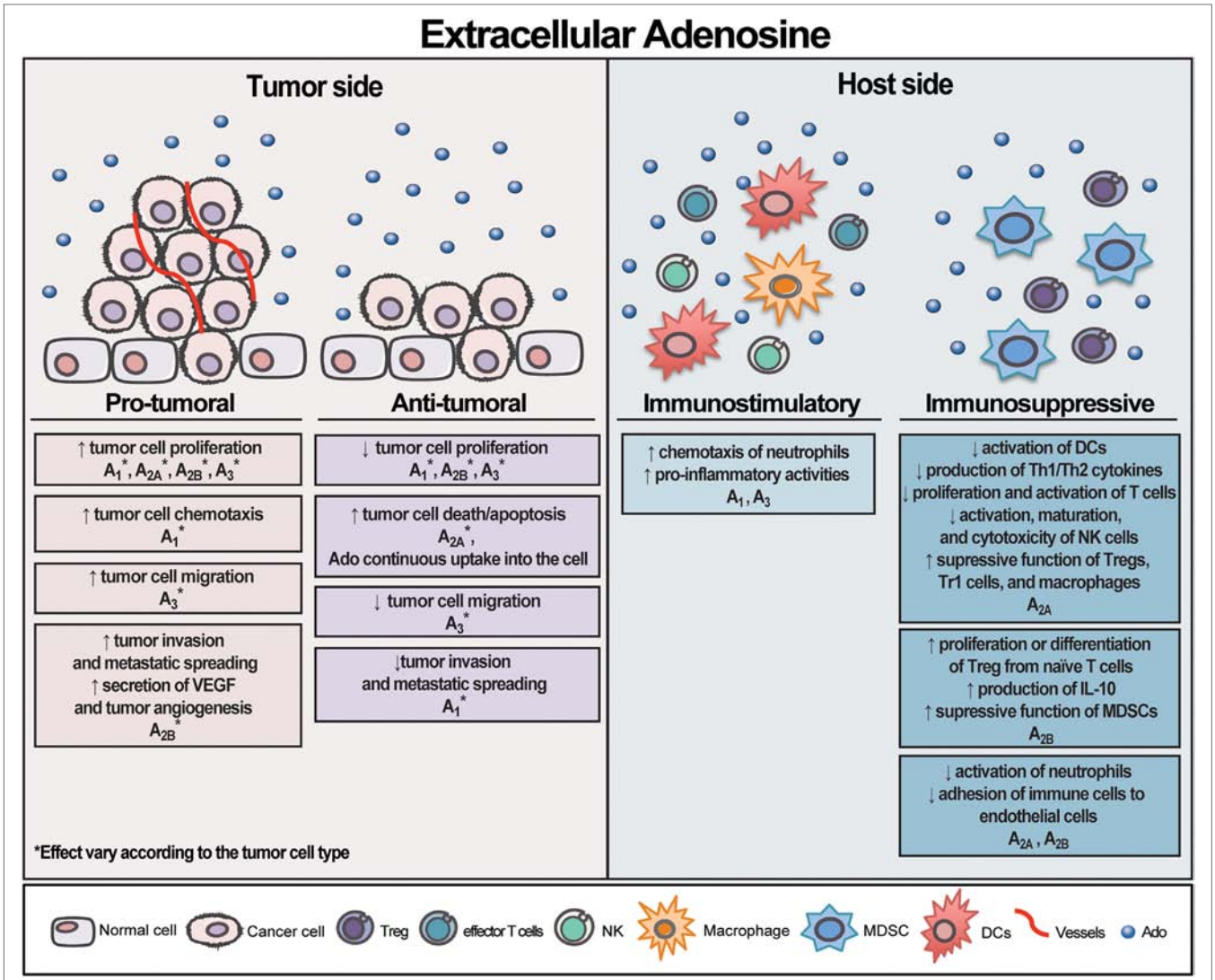

FIGURE 2 | Schematic illustration exhibiting extracellular adenosine (eAdo) opposing effects on tumor and host side. Likewise extracellular adenosine triphosphate (eATP), eAdo can exert distinct and contrasting effects on both tumor and host cells depending on the cell type and receptor activated. eAdo can also promote tumor cell death *via* its continuous uptake into the cell. As depicted by eATP, the sum of eAdo levels, the group of P1 receptor subtypes, and CD39/CD73 expression by tumor and immune cells in the tumor microenvironment will dictate the final effect on tumor growth. Overall, eAdo is a potent immunosuppressive nucleoside, mostly inhibiting immune cell responses.

Targeting P2X7 Receptor in Cancer Therapy

As discussed earlier, the P2X7 receptor has contrasting effects when activated on the tumor or the host cells, potentiating or inhibiting tumor growth—depending on the level of stimulation—while boosting inflammation, respectively. Evidence supporting P2X7 growth-promoting activity has increased recently, and it appears to result from a large number of effects, i.e., inducing the release of immunosuppressive molecules by MDSCs and promoting VEGF release, angiogenesis, and tumor cell proliferation (23, 100). On the other hand, P2X7 receptor seems to restrain tumor growth by promoting DC/cancer cell interaction, cytokine release, chemotaxis, and infiltration of immune cells in the TME (53). Therefore, both strategies either stimulating or blocking P2X7 receptor have been studied to hinder cancer growth (46, 197).

P2X7 receptor overstimulation by using high levels of eATP was the first attempt to increase tumor cell death through its known apoptotic/necrotic function. Administration of very high levels of ATP (25 and 50 mM) effectively reduced the growth of hormone-refractory prostate cancer and melanoma tumors *in vivo*, respectively (198, 199). However, these studies were performed in nude athymic mice, therefore excluding a role for the immune system on this antitumor effect. eATP acting exclusively through P2X7 receptor also inhibited colon carcinoma and melanoma tumor growth in C57BL/6 wild-type mice, by perturbing the balance between two signaling axes—P2X7-PI3K/AKT and P2X7-AMPK-PRAS40-mTOR—and promoting tumor cell

death through autophagy (48). Again, this result was focused on the stimulation of the tumor P2X7 receptor, and no mention to the host counterpart was reported. Regardless of these promising results, three clinical trials fail to demonstrate a beneficial impact by using exogenous ATP to treat cancer in patients, being an improvement of the quality of life the only positive effect demonstrated (200–202). Besides eATP, the use of P2X7 receptor agonists, such as BzATP and ATPγS, has also been employed to delay tumor growth, but once more, only the effect on the P2X7 receptor tumor side was evaluated (203, 204). Accordingly, BzATP inhibited the formation of DMBA/TPA-induced skin papillomas and carcinomas in wild-type FVB mice (203), while ATPγS decreased the tumor growth and metastasis of mouse mammary carcinoma cells in wild-type C57BL/6 mice (204).

P2X7 receptor activation through the eATP released from the irradiation and chemotherapy has also an important role in controlling tumor response to those treatments (205–207). In glioblastoma, P2X7 receptor expression by tumor cells dictated patient response to radiotherapy (208). Accordingly, high levels of P2X7 receptor are associated with good prognosis and increased glioma radiosensitivity. Moreover, P2X7 silencing prevents tumor response to radiation in an *in vivo* model of glioblastoma, reinforcing that functional P2X7 expression is crucial for an efficient radiotherapy response (208). Likewise, eATP acting *via* P2X7 receptor on DCs is determinant for the chemotherapy-induced ICD, stimulating host-specific immune responses (206, 207). We recently showed the importance of P2X7 receptor overactivation in colon cancer cells to potentiate chemotherapy cytotoxicity (209). According to our data, hyperthermia—by influencing plasma membrane fluidity—boosted P2X7 functional responses to eATP, leading to maximal tumor cell death, mainly in association with chemotherapy drugs. Therefore, P2X7 hyperactivation by hyperthermia might be used as an adjunct therapy in the treatment of cancer.

Tumor P2X7 receptor expression and activation and its impact on cancer proliferation have long been investigated. However, two recent studies also demonstrated a critical role for the host P2X7 receptor in stimulating the antitumoral immune response and restraining the tumor growth (47, 79). Correspondingly, animals with host genetic deletion of P2X7 were not able to mount an effective host inflammatory response, reporting reduced cell infiltration at the tumor bed, accelerated tumor growth, and metastatic spreading in comparison to the wild-type group.

Although the overstimulation of P2X7 receptor with agonists appears to be the most logical strategy to decrease tumor proliferation, by inducing both tumor cell death and antitumor immune response, recent studies have been demonstrated that blocking P2X7 receptor activation is more efficacious in preventing tumor growth, mainly in those cancers in which P2X7 receptor is overexpressed (28, 46, 47, 100). Administration of P2X7 inhibitors and antagonists has been shown to decrease cancer cell growth or spreading in animal models of colon (100), breast (115) and ovarian carcinoma (210), neuroblastoma (101), melanoma (47, 100), and glioma (211).

Several inhibitors and antagonists have been used to block P2X7 receptor in tumor cells, including oxidized-ATP (100, 212), BBG (210), AZ10606120 (47, 100, 101), A740003 (47, 101), A438079 (115), and also P2X7 blocking antibodies (115). A recent phase I clinical trial using anti-P2X7 antibody to treat basal cell carcinoma demonstrated exciting results and showed that 65% of patients respond to the treatment and had a significant reduction on the lesion area (213). The authors support the use of antibodies against P2X7 receptor as a safe and well tolerable treatment for BBC.

An important point to be considered is that the use of P2X7 receptor antagonists have been shown to demonstrate strong anticancer effects in immune-competent mice expressing P2X7 in both tumor and host side (47, 100), suggesting that blocking P2X7 on the tumor side is critical to the final antitumor action, despite the mild immunosuppressive effect due to inhibition of the P2X7 on the host side (53). Regardless, more studies investigating the P2X7 receptor function in host/tumor interactions, and their impact on tumor growth will indicate the feasibility of using P2X7 as a new target in cancer therapy.

Blocking CD39 Activity—First Step to Inhibit Ado Formation and Restore Antitumor Immune Response

The conversion of eATP to Ado, either in physiological or pathological conditions, is mainly coordinated by the sequential activity of CD39 and CD73. In the TME, those enzymes will affect tumor growth according to their ability to produce Ado and therefore trigger an immunosuppressive signaling (24, 37).

Increased expression of CD39 has been widely reported in several tumors, such as medulloblastoma (214), sarcoma (215), HCC (216), pancreatic cancer (217), colorectal cancer (218, 219), gastric cancer (216), and endometrial cancer (220); as well as in infiltrating immune cells (216, 221–224) and tumor endothelial cells (216, 225), influencing tumor growth, metastasis and angiogenesis. As an example, expression of CD39 by Tregs plays a permissive role in a mouse model of hepatic metastasis by inhibiting NK cell antitumor immunity and contributing to tumor immune escape (226).

Therefore, strategies to block CD39 activity and Ado generation has become a new approach to avoid Ado immunosuppressive effects and restores the antitumor responses (36). So far, few approaches targeting CD39 by using pharmacological inhibitors, genetic deletion or antibodies have been rendered promising results (215, 224, 226, 227). As reported in the literature, blocking CD39 activity by using the inhibitor ARL67156 partially overcomes T cell hyporesponsiveness in a subset of patient samples with follicular lymphoma (224). In the same line, CD39 blockage with both inhibitor (ARL67156) and antibody (OREG-103/BY40) increased T cells and NK cell-mediate cytotoxicity against SK-MEL-5 melanoma cells (228). In an *in vivo* model, injection of POM1, a pharmacological CD39 inhibitor, was able to limit B16-F10 melanoma and MCA 38 colonic tumor growth at the same rate as demonstrated in animals CD39$^{-/-}$ (226). Indeed, CD39 deletion inhibited metastatic melanoma and colonic growth in the liver as well as decreased tumor angiogenesis (226). Similarly, CD39 deletion abrogated B16-F10 melanoma and LLC lung carcinoma tumor growth, angiogenesis, and pulmonary metastases in mice (227). In another study, treatment with a specific

anti-CD39 antibody significantly improved survival in a lethal metastatic patient-derived sarcoma model (215).

Altogether, these studies indicate that blocking Ado formation through targeting CD39 is a promising strategy in cancer therapy not only for boosting the antitumor immune response (immunotherapy) but also for blocking tumor angiogenesis (antiangiogenic therapy). However, future studies involving the use of anti-CD39 antibodies will provide supportive insights into the potential clinical application of CD39-targeting therapy in oncology (36).

Inhibiting CD73 Activity—Second Step to Block Ado Formation and Improve Antitumor Immune Response

CD73 is a 5′ ectonucleotidase enzyme that degrades extracellular AMP—derived from the ATP metabolism—to Ado (37). As mentioned earlier, the sequential enzymatic activity of CD39 and CD73 is the main pathway for the generation of Ado in the tumor interstitium. In this context, CD73-derived Ado exerts many immunosuppressive effects to attenuate antitumor immunity (122). Likewise CD39, CD73 is expressed by cancer cells, regulatory immune cells, and the vasculature, therefore affecting tumor growth, metastasis and angiogenesis (36).

Elevated CD73 expression has been reported in several types of human cancers such as glioma (229–231), head and neck (128), melanoma (232), thyroid (233), breast (234–238), pancreas (239), colon (219, 240), bladder (241, 242), ovarian (243), prostate (244), and leukemia (126), being positively correlated with poor prognosis. In addition to tumor-derived CD73, host CD73 also negatively regulates tumor immunity (245). Accordingly, both hematopoietic and nonhematopoietic expression of CD73 is important to promote tumor immune escape. For example, Tregs-derived CD73 contributed to their immunosuppressive effects (245), while enzymatic activity of CD73 on tumor-associated endothelial cells restricted T cells homing to tumors (127). Altogether, these data suggest that both tumor and host CD73 cooperatively protect tumors from the immune system response, favoring cancer growth and spreading. Supporting this assumption, studies performed with CD73-deficient mice showed that animals lacking CD73 have an increased antitumor immunity and are resistant to carcinogenesis (245–247). Therefore, targeting CD73 appears to be a useful therapeutic tool to treat cancer.

Many approaches using small molecules inhibitors such as ACPC and antibodies against CD73 have shown important antitumor and antimetastatic effects in various preclinical models of melanoma (127, 245, 246, 248), fibrosarcoma (247), breast (125, 134, 235, 249, 250), prostate (247), and ovarian cancer (123). Those effects are mainly attributed to the immune-stimulating activity of CD73 blockage on host and tumor cells. However, a role for CD73 in controlling cancer cell proliferation independently of the immune system was also reported (251). Accordingly, CD73 gene-silencing in human tumor cells promoted cell-cycle arrest and apoptosis, decreasing cell growth rate in a xenograft tumor model.

Targeting CD73 has also been shown to suppress tumor angiogenesis (133, 134). Anti-CD73 therapy with monoclonal antibody significantly reduced tumor VEGF levels and abolished tumor angiogenesis in a mouse model of breast cancer (134). Accordingly, tumor-derived CD73 triggered VEGF production by tumor cells, while endothelial-derived CD73 promoted the formation and migration of capillary-like structures by endothelial cells, demonstrating that CD73 expression on tumor and host cells contribute to tumor angiogenesis.

A phase I clinical trial study is currently undergoing to test safety, tolerability, and antitumor activity of anti-CD73 mAb, MEDI9447, in cancer patients (NCT02503774) (**Table 1**). MEDI9447 is a selective, potent, and non-competitive inhibitor of CD73 that blocks both membrane-bound and soluble states of this enzyme (252). Preclinical data using mouse syngenic CT26 colon carcinoma tumor model showed that MEDI9447 inhibited tumor growth by promoting changes in both myeloid and lymphoid infiltrating leukocytes within the tumor interstitium (253). Among these changes, increasing number of $CD8^+$ effector T cells and activated macrophages in the TME

TABLE 1 | Clinical trials currently underway that are testing the potential use of anti-CD73 mAb and A_{2A} antagonists alone or in combination with other immunotherapies to treat cancer.

Phase	Propose of study	Intervention	Condition	ID
I	Evaluate the safety, tolerability, pharmacokinetics, immunogenicity, and antitumor activity	Monotherapy: anti-CD73 mAb (MEDI9447) or Combination: anti-CD73 mAb (MEDI9447) and anti-PD-L1 mAb (MEDI4736)	Advanced solid tumors	NCT02503774
I/Ib	Determine the safety, tolerability, feasibility, and preliminary efficacy	Monotherapy: adenosine (Ado) A_{2A} receptor antagonist (PBF-509) or Combination: Ado A_{2A} receptor antagonist (PBF-509) and anti-PD-1 mAb (PDR001)	Non-small cell lung cancer	NCT02403193
I/Ib	Study the safety, tolerability, and antitumor activity	Monotherapy: Ado A_{2A} receptor antagonist (CPI-444) or Combination: Ado A_{2A} receptor antagonist (CPI-444) and anti–PD-L1 mAb (atezolizumab)	Non-small cell lung cancer Malignant melanoma Renal cell cancer Triple negative breast cancer Colorectal cancer Bladder cancer Prostate cancer	NCT02655822

has been reported. In addition, mice treated with a combination of anti-CD73 and anti-programmed cell death protein (PD)-1 antibodies showed increased tumor rejection and survival rates when compared with mice treated with an individual antibody. Synergistic effects by combining CD73 blockade with other currently available anticancer agents, including anthracycline (254), radiation (160), anti-cytotoxic T-lymphocyte antigen (CTLA)-4 antibodies (255, 256), and anti-PD-1 antibodies (255) have also been reported and highlight the potential clinical application of CD73 target therapies in combination with other anticancer modalities to improve antitumor immune response as well as tumor death.

Blocking A_{2A} Receptor—Alternative Approach to Restrain Ado Immunosuppressive Effect and Boost the Antitumor Immunity

Targeting the Ado receptor A_{2A} is also an alternative approach to block the Ado immunosuppressive effect and boost the antitumor immunity (36). As depicted earlier, A_{2A} receptor plays an important role in triggering Ado immunosuppressive activities in many immune subsets. Therefore, blocking Ado A_{2A} receptor with antagonist appears to be an attracting strategy, besides CD39 and CD73 inhibition, to increase innate and adaptive

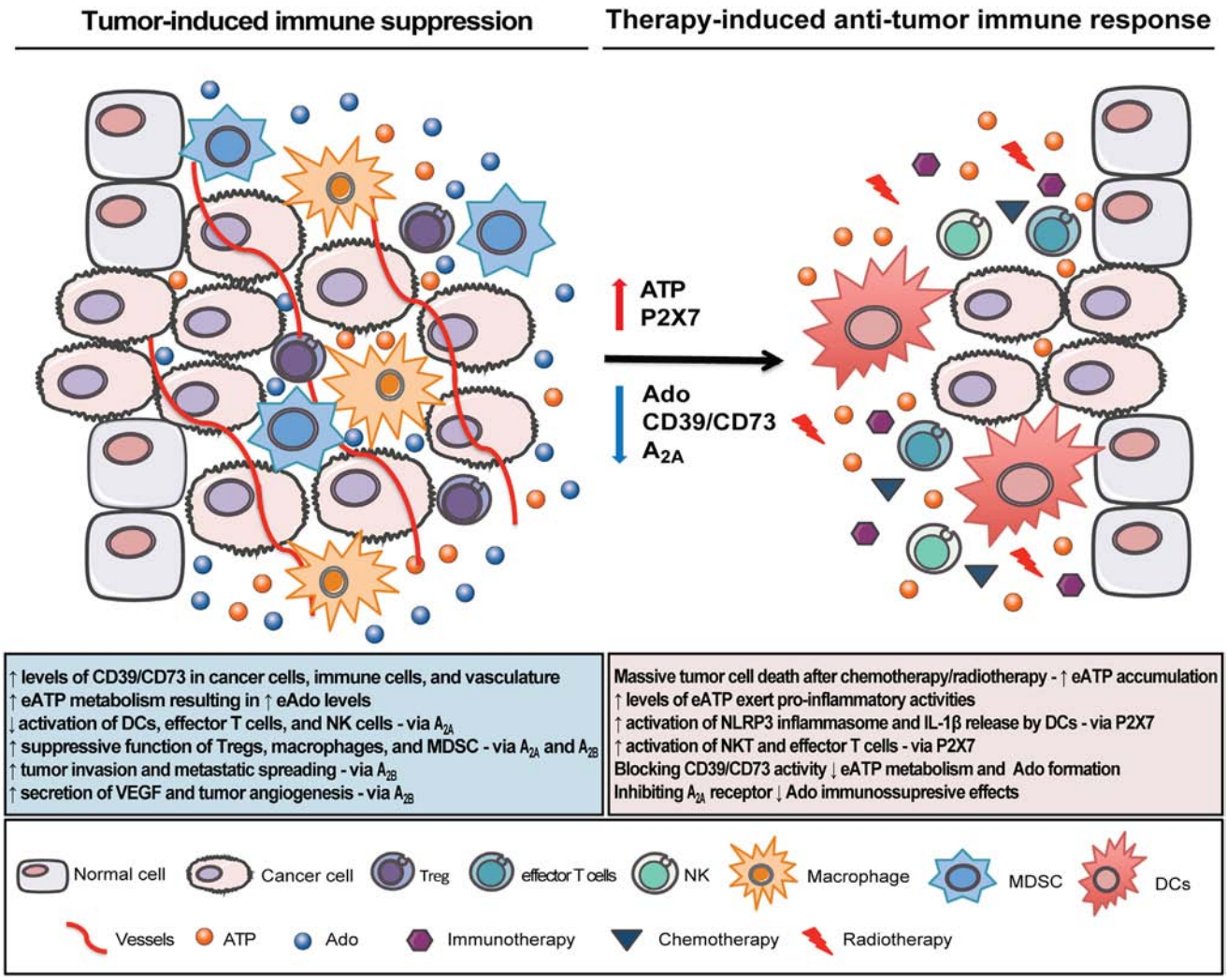

FIGURE 3 | Therapeutic strategies to overcome tumor immune escape and boost cancer immunosurveillance in the tumor microenvironment (TME). In the inflammatory TME, tumor and immune cells interact to produce a favorable immunosuppressive microenvironment. Extracellular adenosine triphosphate (eATP), a pro-inflammatory mediator, accumulates in the TME, but it is rapidly converted to the immunosuppressive factor adenosine (Ado) *via* the sequential enzymatic activity of CD39 and CD73. Ado acting through A_{2A} and A_{2B} receptors inhibits dendritic cells (DCs), NK, and effector T cells activation while it enhances the suppressive function of Tregs, macrophages, and myeloid-derived suppressor cell (MDSC). Strategies by targeting Ado formation, i.e., by blocking CD39/CD73 enzymes and Ado receptors (mainly A_{2A}) will build up eATP concentration and improve the antitumor immune response. Specifically on DCs, eATP acting through P2X7 receptor will trigger NLRP3 inflammasome activation and IL-1β release with consequent stimulation of $CD8^+$ and $CD4^+$ lymphocyte-mediated antitumor response, which is a critical step for the efficacy of chemotherapy and radiotherapy. Therefore, combining purinergic-targeting therapies with other anticancer modalities may be a new strategy to overcome immune escape, potentiate antitumor immune response, and consequently restrain tumor growth.

immune response against the tumor (153). Many studies have been shown the potential use of A_{2A} antagonists alone or in combination with other therapies to enhance antitumor immunity in preclinical models (125, 150, 257, 258). Combination therapies targeting both A_{2A} receptor and co-inhibitory molecules, such as CTLA4 and PD-1, have shown synergistic effects (256, 257, 259). Coadministration of A_{2A} antagonist with anti-CTLA4 mAb marked inhibited tumor growth and enhanced antitumor immune responses in a mouse melanoma model (256). Moreover, dual blockade of A_{2A} receptor and PD-1 significantly reduced $CD73^+$ tumor growth and metastasis spreading as well as prolonged mice survival (257, 259). The mechanism of the combination therapy was mainly dependent on NK cells, $CD8^+$ T, cells and IFN-γ. Importantly, the overexpression of CD73 by tumor cells was critical for the efficacy of the combined therapy, suggesting that CD73 might be a potential biomarker for the selection of patients undergoing this method of treatment. Supporting this statement, co-inhibition of CD73 and A_{2A} receptor by either gene deletion or pharmacological therapy limited tumor initiation, growth, and metastasis *in vivo* (260). In the double knockout (KO) mice, tumor control required $CD8^+$ T-cell and IFN-γ production within the core of tumors, while therapeutic activity of CD73 antibodies depend on Fc receptors binding. Interestingly, A_{2A} single KO mice showed a significant upregulation of CD73 expression in tumor cells and endothelial cells, suggesting that CD73 overexpression might be a mechanism of escape and resistance to monotherapy with A_{2A} antagonists. So far, two clinical trials (phase I) are currently underway to evaluate safety, tolerability, and antitumor activity of A_{2A} antagonists as a single agent and in combination with PD-1/PD-L1 inhibitors in patients (NCT02403193 and NCT02655822) (**Table 1**). Therefore, associating A_{2A} antagonist with other checkpoint blockade inhibitors appears to be a promising strategy to improve patient survival and yet many researchers have pointing the anti-adenosinergic signaling as the next-generation target in immuno-oncology.

CONCLUSION

Despite its complexity and contradictory effects, purinergic signaling has emerged as a novel targetable therapy to improve other anticancer modalities and cannot be underestimated considering its role in carcinogenesis. Strategies by blocking Ado formation and its immunosuppressive effects in the TME favoring eATP accumulation, and its pro-inflammatory effects appears to be the most promising approach to maximize the efficacy of other therapies such as immunotherapy, radiotherapy, and chemotherapy (**Figure 3**). However, considering the multifaceted effects of eATP and Ado in the TME, where host immune and stromal cells as well as tumor cells are modulated in different ways, choosing the most feasible purinergic target will be a challenging task. Ongoing and upcoming clinical trials will hopefully identify the best combinatorial approach to boost antitumor immune response and successfully restrain tumor growth.

AUTHOR CONTRIBUTIONS

PM, RC-S, and LS wrote the article. All the authors contributed to the study conception and design, and critically revised the manuscript.

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Interleukin-6 *Trans*-Signaling Pathway Promotes Immunosuppressive Myeloid-Derived Suppressor Cells *via* Suppression of Suppressor of Cytokine Signaling 3 in Breast Cancer

Mengmeng Jiang[1,2†], Jieying Chen[3†], Wenwen Zhang[1,2], Rui Zhang[1], Yingnan Ye[1], Pengpeng Liu[1], Wenwen Yu[2], Feng Wei[2], Xiubao Ren[2] and Jinpu Yu[1,2]*

[1] Cancer Molecular Diagnostics Core, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin's Clinical Research Center for Cancer, Tianjin, China, [2] Department of Immunology, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Immunology and Biotherapy, Tianjin's Clinical Research Center for Cancer, Tianjin, China, [3] Department of Urology, Tianjin Medical University General Hospital, Tianjin, China

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***Correspondence:**
Jinpu Yu
yujinpu@tjmuch.com

[†]These authors have contributed equally to this work.

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Interleukin-6 (IL-6) has been reported to stimulate myeloid-derived suppressor cells (MDSCs) in multiple cancers, but the molecular events involved in this process are not completely understood. We previously found that cancer-derived IL-6 induces T cell suppression of MDSCs *in vitro via* the activation of STAT3/IDO signaling pathway. In this study, we aimed to elucidate the underlying mechanisms. We found that in primary breast cancer tissues, cancer-derived IL-6 was positively correlated with infiltration of MDSCs *in situ*, which was accompanied by more aggressive tumor phenotypes and worse clinical outcomes. *In vitro* IL-6 stimulated the amplification of MDSCs and promoted their T cell suppression ability, which were fully inhibited by an IL-6-specific blocking antibody. Our results demonstrate that IL-6-dependent suppressor of cytokine signaling 3 (SOCS3) suppression in MDSCs induced phosphorylation of the JAK1, JAK2, TYK2, STAT1, and STAT3 proteins, which was correlated with T cell suppression of MDSCs *in vitro.* Therefore, dysfunction in the SOCS feedback loop promoted long-term activation of the JAK/STAT signaling pathway and predominantly contributed to IL-6-mediated effects on MDSCs. Furthermore, IL-6-induced inhibition of SOCS3 and activation of the JAK/STAT pathway was correlated with an elevated expression of IL-6 receptor α (CD126), in which the soluble CD126-mediated IL-6 *trans*-signaling pathway significantly regulated IL-6-mediated effects on MDSCs. Finally, IL-6-induced SOCS3 dysfunction and sustained activation of the JAK/STAT signaling pathway promoted the amplification and immunosuppressive function of breast cancer MDSCs *in vitro* and *in vivo*, and thus blocking the IL-6 signaling pathway is a promising therapeutic strategy for eliminating and inhibiting MDSCs to improve prognosis.

Keywords: breast cancer, interleukin-6, myeloid-derived suppressor cells, suppressor of cytokine signaling 3, the JAK/STAT signaling pathway, *trans*-signaling pathway

INTRODUCTION

Increasing evidence has highlighted the importance of crosstalk between cancer cells and the surrounding microenvironment in the initiation and progression of various cancers (1–3). The tumor microenvironment is composed of multiple immunosuppressive cells, among which myeloid-derived suppressor cells (MDSCs) play a vital role in promoting tumor invasion and metastasis (3).

Myeloid-derived suppressor cells are a heterogeneous population of immature myeloid cells with suppressive effects on both innate and adaptive immunity; therefore, they are regarded as a major obstacle in antitumor immunotherapy (4). Different MDSCs subsets display varied phenotypes in mice or in humans. For example, monocytic MDSCs (M-MDSCs) express $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ in mice and $CD11b^{+}HLA\text{-}DR^{-/lo}CD14^{+}CD15^{-}$ in human; while polymorphonuclear MDSCs (PMN-MDSCs) express $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ in mice and $CD11b^{+}HLA\text{-}DR^{-/lo}CD14^{-}CD15^{+}$ in human. Additionally, early-stage MDSCs (eMDSCs), which comprised more immature progenitors than M-MDSCs and PMN-MDSCs, are defined as specific MDSCs subset expressing $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$ in human tumors (5). In our previous study, we identified a subset of poorly differentiated eMDSCs in breast cancer that expressed an immature phenotype of $Lin^{-}HLA\text{-}DR^{-}CD45^{+}CD33^{+}CD13^{+}CD14^{-}CD15^{-}$ and displayed potent suppression of T cells *in vitro* and *in vivo* (6). We found that cancer-derived interleukin-6 (IL-6) induces the immunosuppressive ability of MDSCs by activating the STAT3/IDO signaling pathway, but the detailed molecular events are unclear (7).

Interleukin-6 is known as a key regulator of immunosuppression in advanced cancer and is responsible for the development of pro-inflammatory and metastatic tumor microenvironments (8). Numerous studies have reported significant correlations between IL-6 and circulating MDSCs in both human and mouse models (9–14). IL-6 increased circulating $CD11b^{+}CD14^{+}HLA^{-}DR^{-}$ cells in squamous carcinoma of the esophagus (9) and prostate cancer (13). Though previous studies reported that IL-6 restored MDSC accumulation in a mouse model of mammary carcinoma (14), a few studies have focused on the relationship between IL-6 and MDSCs in human breast cancer. Our previous study demonstrated that in breast cancers, IL-6 stimulates STAT3-dependent, nuclear factor-κB-mediated indoleamine 2,3-dioxygenase (IDO) upregulation in MDSCs (7); this triggers immunosuppressive effects of MDSCs *in vitro* and *vivo* (6). Although abnormal accumulation of MDSCs *via* the IL-6/STAT3 pathway was reported in multiple cancers (9, 13, 15), the major regulatory mechanisms remain unclear.

It is well-established that the interaction between IL-6 and IL-6R initiates the activation of the JAK/STAT signaling pathway, which transduces the IL-6 signal in both normal and malignant cells. In contrast to the rapid and reversible activation of STAT proteins in normal cells, phosphorylation of STAT proteins is sustained for a long time in malignant cells (16, 17). The dysfunctional negative feedback loop in the JAK/STAT signaling pathway induces constitutive activation of STAT proteins, oncogenic transformation, tumor invasion, and metastasis (18).

Suppressor of cytokine signaling (SOCS) proteins, particularly SOCS3, are major negative feedback regulators of the JAK/STAT signaling pathway (19). Under physiological conditions, IL-6 stimulates the expression of SOCS3 and inhibits phosphorylation of STAT proteins (20). This attenuates IL-6-induced activation of the JAK/STAT signaling pathway and inhibits expression of downstream functional genes (17, 21). It has been reported that constitutive defects in the expression of SOCS3 protein is frequent in malignant cells and is associated with dysregulation of cell growth, migration, and apoptosis (19).

However, only short-term and reversible suppression of SOCS was detected in certain types of immune cells in cancer, such as tumor-infiltrated T cells, dendritic cells (DCs), and macrophages (22, 23). It has been demonstrated that knockdown of SOCS3 in macrophages is beneficial for inhibiting tumor metastases in mice (24). However, it has also been reported that SOCS3 deficiency in myeloid cells promotes tumor development by inducing MDSCs in the tumor microenvironment (25). Therefore, it is urgent to elucidate the biological significance of SOCS3 deficiency in MDSC development and tumor progression, which may provide insight into potential therapeutic targets for breast cancer.

In this study, we evaluated the expression of SOCS proteins and their effects on IL-6-induced activation of the JAK/STAT signaling pathway in breast cancer MDSCs. We found that more MDSCs were recruited in IL-6 high-expressing breast cancer tissues, in which SOCS3 inhibition was detected. IL-6 promoted the amplification of MDSCs and enhanced their suppressive effects on T cells immunity *in vitro*. IL-6 stimulated SOCS3 suppression and thus induced long-term activation of the JAK/STAT signaling pathway in breast cancer MDSCs. IL-6-induced SOCS3 dysfunction and sustained activation of the JAK/STAT signaling pathway predominantly contributed to IL-6-mediated effects on MDSCs. Furthermore, we observed that IL-6-induced suppression of SOCS3 and activation of the JAK/STAT pathway were correlated with an elevated expression of IL-6 receptor α (IL-6-Rα, CD126), which was regulated by the soluble CD126-mediated IL-6 *trans*-signaling pathway. Summarily, we found that IL-6-induced SOCS3 dysfunction and sustained activation of the JAK/STAT signaling pathway promoted the amplification and immunosuppressive function of breast cancer MDSCs *in vitro* and *vivo*. Thus, blocking IL-6 signaling pathway is a promising therapeutic strategy for eliminating and inhibiting MDSCs to improve prognosis.

MATERIALS AND METHODS

Clinical Samples and Healthy Donors

In this study, we collected 253 primary breast cancer tissue samples from two cohorts for immunohistochemistry (IHC) analysis. Cohort 1 included 113 primary breast cancer patients who received surgical resection at the Department of Breast Oncology of Tianjin Medical University Cancer Institute and Hospital from October 2012 to October 2014. Cohort 2 included 140 breast cancer cases whose tumor tissues were assembled on tissue arrays after surgical removal between January 2001 and

August 2004 from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). All patients were women with a median age of 52 years (29–79 years) in cohort 1 and 51 years (29–83 years) in cohort 2. Among the patients, infiltrated mammary-ductal carcinoma accounted for 78.8% (89/113) in cohort 1 and 93.6% (131/140) in cohort 2. Cohort 1 included 9, 72, and 26 patients with histological grade I, II, and III cancer, respectively. Cohort 2 included 19, 111, and 7 patients with histological grade I, II, and III cancer, respectively. Cohort 1 included 18, 65, and 29 patients with clinical stage I, II, and III cancer, respectively. Cohort 2 included 11, 80, and 47 patients with clinical stage I, II, and III cancer, respectively (**Table 1**). Three patients in cohort 2 were excluded from the study because of non-cancer related death.

In addition, 20 fresh primary breast cancer tissue samples and the corresponding adjacent tissues were obtained for isolation of primary MDSCs *in situ*. Peripheral blood (PB) samples (40) from healthy donors were collected to enrich $CD3^+$ T cells to study the immunosuppressive ability of MDSCs *in vitro*. Additionally, $CD33^+$ and $CD14^+$ cells isolated from healthy donors' PB samples were employed as myeloid progenitor and differentiated monocyte controls, respectively. This study was approved by the Medical Ethics Committee of Tianjin Medical University. All experiments were performed in accordance with the principles of the Declaration of Helsinki. Written consent was obtained from all patients and healthy donors.

TABLE 1 | Baseline of all patients.

Baseline	Cohort 1	Cohort 2
Total	113	140
Age		
≤52 years	57	77
>52 years	55	63
Pathology		
iDC	89	131
Non-iDC	24	9
Histology grade		
0–I	9	19
II	72	111
III	26	7
Lymph nodes		
Negative	81	84
Positive	28	52
Tumor size		
≤3 cm	79	81
>3 cm	30	59
Stage		
0–I	18	11
II	65	80
III–IV	29	47
ER		
Negative	37	51
Positive	76	81
PR		
Negative	44	61
Positive	69	71
HER2		
Negative	62	95
Positive	29	37

Isolation of Primary MDSCs and $CD33^+$ Progenitors

Twenty primary breast cancer tissues and their adjacent tissues were collected during surgery and cut into small pieces before being ground and filtered using a filter mesh to prepare a single-cell suspension. $CD33^+$ cells were isolated using human CD33 MicroBeads (130-045-501; Miltenyi Biotec, Bergisch Gladbach, Germany) as previously reported (6). The negative-selected cells were defined as breast cancer cells and analyzed for the expression of IL-6. Thirty cases of PB samples were collected from healthy donors for PBMC isolation. $CD33^+$ and $CD14^+$ cells were enriched using human CD33 (130-045-501; Miltenyi Biotec) and human CD14 MicroBeads (130-050-201; Miltenyi Biotec), respectively, according to the manufacturer's instructions. Trypan blue staining was performed to ensure the full viability of each cell fraction.

IHC Assay

Fresh tissues were immediately fixed with formalin after surgical removal; tissues were made into paraffin embedded blocks and then sliced into 4-μm serial sections. The samples were heated for 1 h at 70°C, deparaffinized in xylene, and rehydrated using graded alcohol. Antigens were retrieved in citrate buffer (pH 6.0) for 2 min. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 20 min. We previously examined the expression a series of pan-myeloid and differentiated markers of myeloid linage and confirmed high expression of CD33 and CD13, low expression of HLA-DR and CD14, and negative expression of CD15 on the surface of breast cancer MDSCs (6). We also detected the expression of 3 pan-myeloid markers, including CD33 (26), CD13 (27), and CD11b (28) and found that non-specific staining of CD13 and CD11b on cancer cells, endothelial cells, and fibroblasts interfered with the specific staining on MDSCs (Figures S1B–C in Supplementary Material), indicating the feasibility of using CD33 to detect breast cancer MDSCs in an IHC assay. Therefore, all samples were incubated with mouse anti-human IL-6 (PeproTech, Rocky Hill, NJ, USA) and CD33 (Abcam, Cambridge, UK) monoclonal antibody (McAb) at a concentration of 1 μg/mL overnight at 4°C. A secondary antibody conjugated with streptavidin-horseradish peroxidase (Santa Cruz, Biotech, Dallas, TX, USA) was then added, and the mixture was incubated for 30 min before adding diaminobenzidine staining buffer (Maixin Biotechnology, Fuzhou, China). All images were captured using an Olympus BX51 microscope (Olympus, Tokyo, Japan). Five representative high-power fields (400× magnification) from each tissue section were selected for histology evaluation as previously described (6).

Flow Cytometry Analysis

Primary MDSCs were isolated from primary breast tumor tissues. To assess the proportions of $CD45^+CD13^+CD33^+CD14^-CD15^-$ MDSCs in cancerous and corresponding adjacent normal tissues, flow cytometry analysis was performed using a BD FACS Canto™ II flow cytometer (BD Biosciences, San Jose, CA, USA). The PerCp-conjugated anti-human CD45, phycoerythrin-conjugated

anti-human CD13 and CD33, and fluorescein isothiocyanate-conjugated anti-human CD14 and CD15 (BD Biosciences) antibodies were used to label the MDSCs. An isotype-matched IgG1 antibody (BD Biosciences) was used as a negative control. After incubation, cells were washed and resuspended in buffer, and the expression of cell surface markers was detected using the flow cytometer. The leukocyte population was gated using PerCp-labeled anti-human CD45, and breast cancer MDSCs were defined as $CD33^+CD13^+CD14^-CD15^-$ in the $CD45^+$ population. Furthermore, to detect the production of interferon (IFN)-γ and IL-10 in T cells co-cultured with or without MDSCs, we conducted an intracellular staining assay using flow cytometry. After co-culture, T cells were distinguished by allophycocyanin-labeled anti-CD3 McAb and the percentages of IFN-γ positive and IL-10 positive T cells were determined using PE/Cy7-labeled anti-IFN-γ and PE-labeled anti-IL-10 McAbs, respectively.

Cell Line and Cell Culture

The human breast cancer cell line MDA-MB-231 was obtained from the Chinese Academy of Medical Sciences (Resource number: 3111C0001CCC000014). The cell line was cultured in complete RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum in a 5% CO_2 incubator at 37°C. $CD33^+$ progenitors isolated from PBMCs of healthy donors were co-cultured with breast tumor cells to induce MDSCs with or without IL-6 antibody. $CD33^+$ and $CD14^+$ control cells were cultured in complete RPMI 1640 medium. After co-culture with MDSCs for 3 days, the proliferation, apoptosis, and cytokine secretion of T cells were studied to evaluate the immunosuppressive ability of MDSCs pretreated with or without IL-6 antibody.

Induction of MDSCs *In Vitro*

$CD33^+$ myeloid progenitors (2×10^6/mL) isolated from healthy PBMCs were added to multi-well plates and co-cultured with MDA-MB-231 breast cancer cells to induce MDSCs with or without IL-6 antibody (EMD Millipore, Billerica, MA, USA) at a concentration of 50 μg/mL. $CD33^+$ progenitors were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum as negative controls. After 48 h of culture, MDSCs were harvested for further analysis, and the supernatants were collected to detect soluble CD126 using the Human IL-6R ELISA Kit (GenWay Biotech, Inc., San Diego, CA, USA). The phenotype of harvested cells was examined by flow cytometry as previously described; the proportion of $CD45^+CD13^+CD33^+CD14^-CD15^-$ MDSCs was examined (6).

Cell Counting Kit 8 (CCK8) Assay

Cell Counting Kit 8 assay was used to detect the proliferation of T cells co-cultured with or without MDSCs. $CD3^+$ T cells were isolated from PBMCs of 10 healthy donors using the Human Pan T cell Isolation Kit II (130-091-156; Miltenyi Biotec). Both MDSCs and T cells with viability >95% were used for functional assays. Purified T cells (2×10^5) were plated in a 96-well plate and co-cultured with $CD33^+$ cells or MDSCs in the presence or absence of IL-6 antibody at ratio of 1:3 in triplicate. Cells were cultured in complete medium supplemented with 1,000 IU/mL recombinant human IL-2 (PeproTech) or anti-CD3/CD28 Abs (at bead/cell ratio of 1:1, Gibco) at 37°C in a 5% CO_2 incubator for 3 days. Next, 10 μL CCK8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was added to each well and incorporated into living cells during cell proliferation. Blank wells without cells were used as negative controls. T cells stimulated with IL-2 or anti-CD3/CD28 Abs were used as the T cell control. After 4 h of incubation, the optical density at 450 nm was measured using an enzyme immunoassay analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Cell proliferation was evaluated using stimulation index (SI), which was calculated using the following formula: SI = [(experimental counts)/(responder control counts + stimulator control counts)].

Annexin V Assay

Purified T cells (5×10^5) were plated in a 24-well plate and co-cultured with $CD33^+$ cells or MDSCs in the presence or absence of IL-6 antibody at ratio of 1:3 in triplicate. Cells were cultured in complete medium supplemented with 1,000 IU/mL recombinant human IL-2 at 37°C in a 5% CO_2 incubator for 3 days. The Annexin V assay was used to detect the apoptosis of T cells. We initially gated lymphocytes according to SSC and FSC features and then gated T cells using allophycocyanin-labeled anti-CD3 McAb (BioLegend, San Diego, CA, USA). The cells were stained with FITC-Annexin V and propidium iodide provided in an Apoptosis Detection Kit (BD Biosciences) as previously described (6). The positive expression of Annexin V and negative expression of PI represent apoptotic T cells.

Enzyme-Linked Immunosorbent Assay (ELISA)

Purified T cells (5×10^5) were plated in a 24-well plate and co-cultured with $CD33^+$ cells or MDSCs in the presence or absence of IL-6 antibody at ratio of 1:3 in triplicate. Cells were cultured in complete medium supplemented with 1,000 IU/mL recombinant human IL-2 or anti-CD3/CD28 Abs (at a bead/cell ratio of 1:1) at 37°C in a 5% CO_2 incubator for 3 days. The corresponding T cell culture supernatants were collected to detect cytokine levels in an ELISA assay. Levels of T cell-secreted cytokines, including IFN-γ and IL-10, were analyzed using the ELISA kits (Dakewe Biotech Co., Ltd., Shenzhen, China) as per the manufacturer's instructions.

Quantitative Real-time RT-PCR (qRT-PCR) Analysis

Interleukin-6, CD126, gp130, SOCS1, SOCS2, SOCS3, ADAM10, and ADAM17 mRNA expression in MDSCs isolated from primary breast cancer tissues and *in vitro*-induced MDSCs was analyzed by qRT-PCR. The mRNA levels of target genes were quantified using the SYBR Premix Ex Taq TM system (Takara Bio, Shiga, Japan) with an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). The primers for IL-6, CD126, gp130, SOCS1–3, ADAM10, ADAM17, and β-actin are shown

TABLE 2 | The RT-PCR primers of interested genes.

Genes	Primer sequences		Bases
Interleukin-6 (IL-6)	Up	CAATGAGGAGACTTGCCTGG	20
	Down	GGCATTTGTGGTTGGGTCAG	20
SOCS1	Up	GACGCCTGCGGATTCTACT	19
	Down	AGGCCATCTTCACGCTAAGG	20
SOCS2	Up	CGCTATCCTTCCCTGAACC	19
	Down	GTCCGAAATGGTGGCAGA	18
SOCS3	Up	AAGCACAAGAAGCCAACCAG	20
	Down	TTCCCTCCAACACATTCCAG	20
CD126	Up	TTGGACACTCACACGGACA	19
	Down	GAGGCTTTGGCTGGAAATC	19
gp130	Up	ACACCAAGTTCCGTCAGTCC	20
	Down	TACCATCACCGCCATCTACA	20
ADAM10	Up	GCTCATTGGTGGGCAGTATT	20
	Down	GTGGTTTAGGAGGAGGCAACT	21
ADAM17	Up	ACTGCACGTTGAAGGAAGGT	20
	Down	ACGCCTTTGCAAGTAGCATT	20
β-actin	Up	TGGCACCCAGCACAATGAA	19
	Down	CTAAGTCATAGTCCGCCTAGAAGCA	25

Up, upstream primer; down, downstream primer.

in **Table 2**. Relative mRNA levels in each sample were calculated based on their threshold cycle (Ct) values normalized to the Ct value of β-actin using the formula: $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{target\ gene} - Ct_{\beta\text{-}actin}$). All tests were conducted at least five times.

Western Blot Analysis

Western blot analysis was performed to detect the levels of CD126, gp130, ADAM10, ADAM17, and SOCS1–3 proteins, as well as total and phosphorylated JAK1, JAK2, TYK2, STAT1, and STAT3 in MDSCs and $CD33^+$ control cells. Cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes for western blot analysis using mouse anti-human CD126, gp130 (R&D Systems, Inc., Minneapolis, MN, USA), SOCS1 (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), SOCS2–3 (R&D Systems, Inc.), and β-actin. Rabbit anti-human antibodies were used to detect JAK1, JAK2, TYK2, STAT1, STAT3, p-STAT1, p-JAK1, p-JAK2, p-TYK2, and p-STAT3 (Cell Signaling Technology, Danvers, MA, USA). Membranes were incubated with primary antibodies overnight at 4°C, as described previously (7). Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG Ab (Zhongshanjinqiao, Beijing, China), and protein bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL, USA). The relative densities of protein bands were determined by comparing the band densities of proteins of interest to those of β-actin, using Quantity One software. We used the density ratio of phosphorylated protein to total protein to compare the expression of these phosphorylated proteins.

Statistical Analysis

Statistical analyses were performed using the SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (GraphPad, Inc., La Jolla, CA, USA). Measured data were presented as the mean ± SD; one-way analysis of variance and least significant difference tests were used to compare quantitative data. Categorical data were presented as the median, and the nonparametric χ^2 test was used to compare qualitative data. The cumulative survival probability was determined by the Kaplan–Meier method, and the log-rank test was used to compare overall survival (OS) of each subgroup of patients. *P*-values for each analysis are reported in the figure legends, and the level of statistical significance was set to $P < 0.05$.

RESULTS

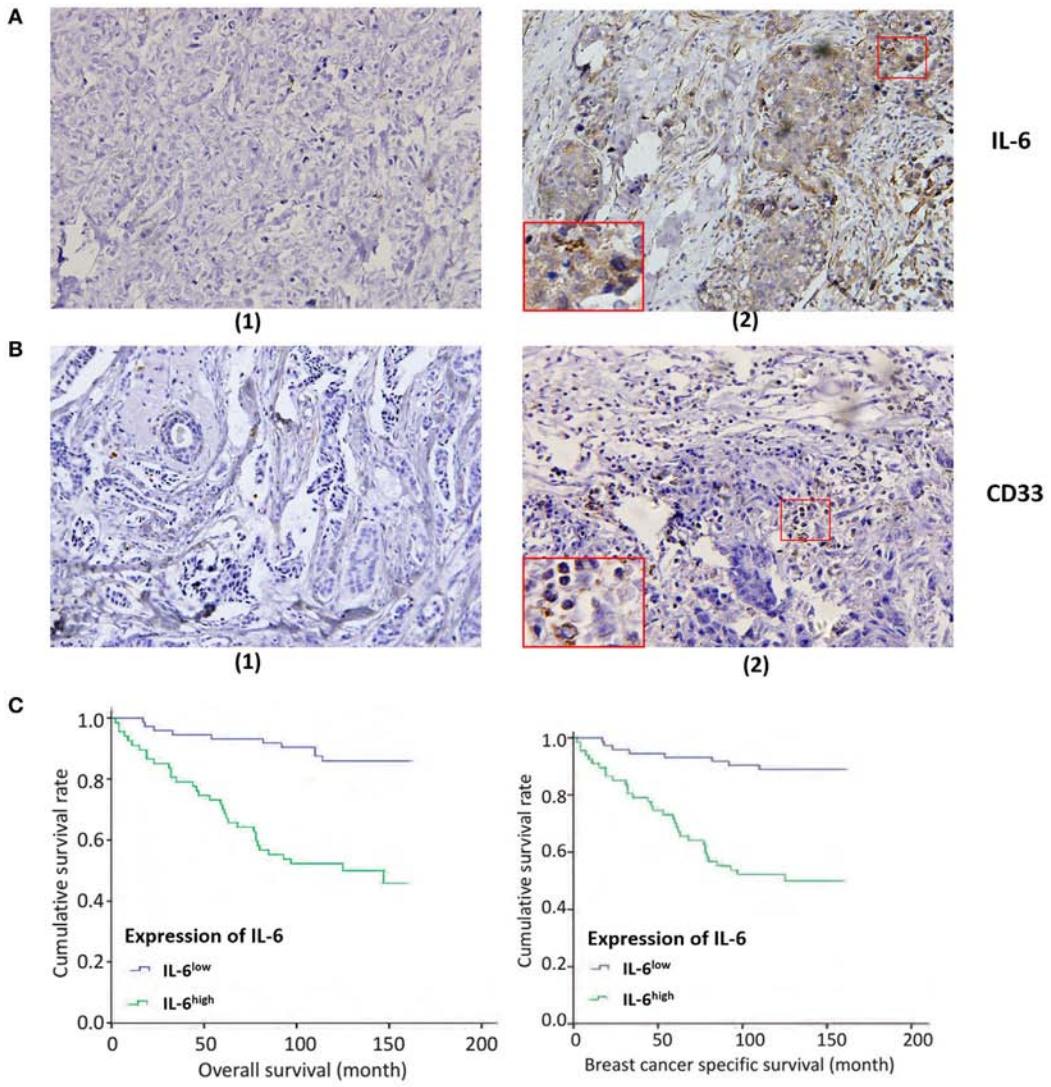
Tumor-Derived IL-6 and Local MDSCs Infiltration Are Significantly Correlated with Lymph Node Metastasis and Poor Prognosis in Breast Cancer Patients

Interleukin-6 was mainly expressed in the cytoplasm of breast cancer cells, as well as in some mesenchymal cells (**Figure 1A**). Based on the staining intensity and extent of IL-6 expression, breast cancer patients were divided into an IL-6 low expression group ($IL\text{-}6^{low}$) and high expression group ($IL\text{-}6^{high}$). The $IL\text{-}6^{high}$ cases accounted for 44.4% (48/108) of patients in cohort 1 and 50.5% (50/99) of patients in cohort 2. $CD33^+$ MDSCs were scattered in the stroma of breast cancer tissues with varying sizes and shapes (**Figure 1B**). According to the number of $CD33^+$ MDSCs that infiltrated locally, breast cancer patients were divided into lowly infiltrated MDSC group ($MDSCs^{low}$) and highly infiltrated MDSC group ($MDSCs^{high}$). The $MDSCs^{high}$ cases accounted for 52.0% of patients in cohort 1 and 50.9% of patients in cohort 2.

We next compared the correlations between IL-6 expression, MDSCs infiltration, other clinical pathological features (age, agenda, tumor size, tumor pathologic stage, tumor histological grade, and lymph node invasion), and expression of hormone receptors (estrogen receptor, progesterone receptor, and HER2) in the two cohorts. As shown in **Table 3**, tumor-derived IL-6 expression was significantly correlated with lymph node invasion and tumor histological grade; compared to $IL\text{-}6^{low}$ patients, $IL\text{-}6^{high}$ patients suffered from more aggressive histological features (cohort 1: $P < 0.001$; cohort 2: $P = 0.001$) and a higher risk of early lymph node invasion (cohort 1: $P < 0.001$; cohort 2: $P = 0.012$). Similar trends were observed in $MDSC^{high}$ patients as compared with $MDSC^{low}$ patients, where more infiltrated MDSCs were detected in cancer tissues at more advanced pathological stages, with higher histological grade, more lymph node metastasis, and larger tumor size ($P < 0.001$, $P < 0.001$, $P = 0.025$, $P = 0.018$; $P < 0.001$, $P = 0.007$; $P = 0.022$, $P = 0.032$, respectively, **Table 4**). These results demonstrated that breast cancers with higher IL-6 expression and greater MDSC infiltration possess a higher potential for invasion and metastasis.

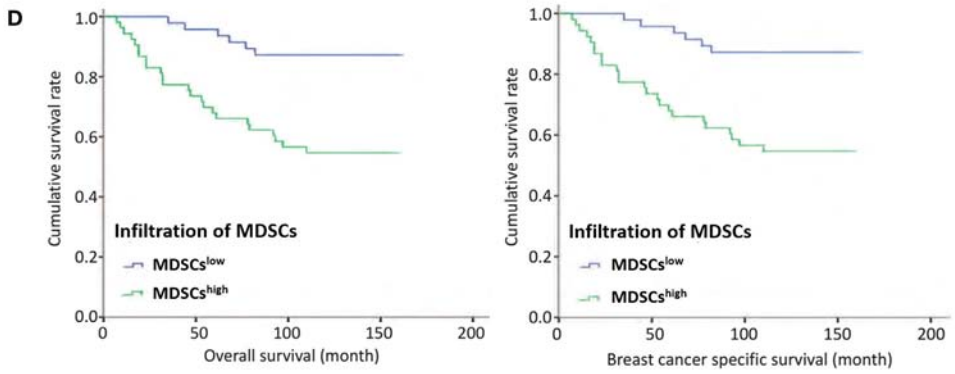
Next, we compared the OS of the 140 patients in cohort 2 with IL-6 expression and MDSC infiltration. We found that the OS, 3-, 5-, and 10-year survival of $IL\text{-}6^{high}$ patients, was significantly

shorter than those of IL-6^{low} patients ($P < 0.001$, **Figure 1C**). Similarly, IL-6^{high} patients displayed worse overall breast cancer-specific survival, 3-, 5-, and 10-year breast cancer-specific survival compared to IL-6^{low} patients ($P < 0.001$, **Figure 1C**). Similar results were observed in MDSC^{high} patients compared to MDSC^{low} patients ($P < 0.001$, **Figure 1D**). Thus, these findings indicate that tumor-derived IL-6 and MDSC infiltration are both unfavorable prognostic factors in breast cancer and are significantly correlated with aggressive tumor behavior and poor clinical outcomes in patients.



IL-6 expression	3-year (%)	5-year (%)	10-year (%)	*P*
OS				<0.001
IL-6^{low}	94.5	93.2	85.9	
IL-6^{high}	79.1	70.1	52.2	
BCSS				<0.001
IL-6^{low}	94.5	93.2	85.9	
IL-6^{high}	79.1	70.1	52.2	

FIGURE 1 | Continued



MDSCs infiltration	3-year (%)	5-year (%)	10-year (%)	*P*
OS				<0.001
$MDSCs^{low}$	97.9	95.7	87.2	
$MDSCs^{high}$	77.4	67.9	54.7	
BCSS				<0.001
$MDSCs^{low}$	97.9	95.7	87.2	
$MDSCs^{high}$	77.4	67.9	54.7	

FIGURE 1 | Tumor-derived interleukin-6 (IL-6) and local myeloid-derived suppressor cells (MDSCs) infiltration are significantly correlated with lymph node metastasis and poor prognosis in breast cancer patients. 253 breast cancer patients were selected for immunohistochemistry (IHC) staining of IL-6 and MDSCs, original magnification ×200. Five representative high-power fields (×400) for each tissue section were selected for histology evaluation. **(A)** (1) Low expression of IL-6; (2) high expression of IL-6. IL-6 was mainly expressed in the cytoplasm of breast cancer cells, as well as in some mesenchymal cells. **(B)** (1) Low infiltration of MDSCs; (2) The representative high infiltration of MDSCs. We defined the mesenchymal cells expressing CD33 antigen molecules as MDSCs and $CD33^+$ MDSCs were scattered in the stroma of breast cancer tissues with varying sizes and shapes. **(C)** The overall survival (OS) of the 140 patients in cohort 2 with IL-6 expression and MDSCs infiltration was analyzed using Kaplan–Meier and the Log rank test. The OS, BCSS, 3-, 5-, and 10-year survival rate of $IL\text{-}6^{high}$ patients were significantly shorter than those of $IL\text{-}6^{low}$ patients ($P < 0.001$). **(D)** Similar results were observed in $MDSCs^{high}$ patients as compared with $MDSCs^{low}$ patients ($P < 0.001$).

Tumor-Derived IL-6 Is Significantly Correlated with the Number of Infiltrated MDSCs *In Situ* Both at the mRNA and Protein Levels

We compared the correlation between the expression of IL-6 and number of infiltrated MDSCs *in situ* to evaluate the effects of IL-6 on MDSC accumulation in breast cancer tissues. We first studied the expression of IL-6 protein in 253 paraffin-embedded breast tissues from cohorts 1 and 2 by IHC. We found greater MDSC infiltration in cancer tissues with a high level of IL-6 (**Figure 2A**). The average number of MDSCs in the $IL\text{-}6^{low}$ group was significantly lower than that in the $IL\text{-}6^{high}$ group in both cohorts 1 and 2 [(1.95 ± 0.26) vs. (6.40 ± 0.48), $P < 0.001$; (1.31 ± 0.27) vs. (6.43 ± 0.79), $P < 0.001$, **Figure 2B**]. Pearson correlation analysis revealed a positive correlation between the expression of IL-6 and the number of MDSCs *in situ* in both cohorts (cohort 1, $R^2 = 0.3974$, $P < 0.0001$; cohort 2, $R^2 = 0.2812$, $P < 0.0001$, **Figure 2B**).

Twenty fresh breast cancer tissue samples were collected to study the correlation between RNA levels of tumor-derived IL-6 and percentages of $CD45^+CD33^+CD13^+CD14^-CD15^-$ MDSCs in breast cancer tissues by flow cytometry analysis. We observed a cluster of $CD33^+CD13^+CD14^-CD15^-$ cells in breast cancer tissue, which represented the predominant phenotype of MDSCs in breast cancer (**Figure 2C**). The percentage of $CD45^+CD33^+CD13^+CD14^-CD15^-$ MDSCs was 15.3–58.1% with a mean value of $29.82 \pm 11.463\%$. Based on the median relative RNA level of IL-6, breast cancer samples were divided into $IL\text{-}6^{high}$ and $IL\text{-}6^{low}$ groups. The average IL-6 mRNA level in the $IL\text{-}6^{high}$ group was 37.25-fold higher than that in the $IL\text{-}6^{low}$ group ($P = 0.0093$, **Figure 2D**). Higher frequency of MDSCs was detected in the $IL\text{-}6^{high}$ group compared to in the $IL\text{-}6^{low}$ group [$(13.75 \pm 3.44\%)$ vs. $(4.31 \pm 1.50\%)$, $P = 0.03$, **Figure 2E**]. Furthermore, Pearson correlation analysis revealed a strong positive correlation between the expression of IL-6 mRNA and number of MDSCs in fresh breast cancer tissues ($R^2 = 0.4399$, $P = 0.0014$, **Figure 2F**).

IL-6 Enhanced the Generation and T Cell Immunosuppressive Ability of MDSCs *In Vitro*

To mimic the breast cancer cell-conditioned microenvironment *in vitro*, $CD33^+$ myeloid progenitors were isolated from healthy donors' PMBCs and co-cultured with MDA-MB-231 breast

TABLE 3 | Correlations of interleukin-6 (IL-6) with clinicopathological features of breast cancer patients.

Baseline	Cohort 1			Cohort 2		
	IL-6^{low}	IL-6^{high}	*P*	IL-6^{low}	IL-6^{high}	*P*
Age			0.870			0.284
≤52 years	33	24		37	40	
>52 years	31	24		36	27	
Histology grade			0.208			0.523
0–I	7	2		11	8	
II–III	55	43		59	59	
Lymph node			<0.001			0.012
Negative	58	23		51	33	
Positive	6	22		20	32	
Tumor size			0.060			0.936
≤3 cm	50	29		42	39	
>3 cm	13	17		31	28	
Stage			<0.001			0.001
0–II	59	24		56	35	
III–IV	5	24		15	32	
ER			0.986			0.059
Negative	21	16		21	30	
Positive	43	33		47	34	
PR			0.256			0.232
Negative	22	22		28	33	
Positive	42	27		40	31	
HER2			0.242			0.681
Negative	38	24		50	45	
Positive	14	15		18	19	

TABLE 4 | Correlations of myeloid-derived suppressor cells (MDSCs) with the clinicopathological characteristics of breast cancer patients.

Baseline	Cohort 1			Cohort 2		
	MDSCs^{low}	MDSCs^{high}	*P*	MDSCs^{low}	MDSCs^{high}	*P*
Age			0.503			0.952
≤52 years	25	30		26	29	
>52 years	27	25		21	24	
Histology grade			0.025			0.018
0–I	7	1		10	3	
II–III	44	51		36	50	
Lymph node			<0.001			0.007
Negative	50	28		34	26	
Positive	2	24		11	27	
Tumor size			0.022			0.032
≤3 cm	42	33		33	26	
>3 cm	9	20		14	27	
Stage			<0.001			<0.001
0–II	51	28		40	26	
III–IV	1	27		5	27	
ER			0.586			0.074
Negative	16	20		14	25	
Positive	36	36		30	25	
PR			0.287			0.215
Negative	18	25		19	28	
Positive	34	31		25	22	
HER2			0.404			0.177
Negative	29	28		33	31	
Positive	12	17		11	19	

cancer cells. After 48 h of culture, the proportion of MDSCs possessing the $CD45^+CD33^+CD13^+CD14^-CD15^-$ phenotype was increased from 15.6 ± 2.6 to 30.83 ± 1.595% ($P = 0.015$; **Figure 3A**).

To determine whether IL-6 plays a major role in promoting MDSC differentiation *in vitro*, an IL-6 neutralizing antibody was added to the cancer-conditioned MDSC culture. The proportion of $CD45^+CD33^+CD13^+CD14^-CD15^-$ cells in IL-6-neutralizing antibody-treated MDSCs (Ab-treated MDSCs) was dramatically decreased compared to in untreated MDSCs (Ab-untreated MDSCs) [(11.98 ± 3.479%) vs. (30.83 ± 1.595%), $P = 0.0007$, **Figure 3A**]. These results indicate that breast cancer-induced IL-6 secretion significantly promotes the differentiation and accumulation of MDSCs *in vitro*.

To examine whether IL-6 regulates MDSCs-mediated immunosuppressive effects on T cells *in vitro*, we co-cultured both Ab-treated MDSCs and Ab-untreated MDSCs with T cells isolated from exogenous PMBCs and examined the proliferation, apoptosis, and cytokine production of T cells. MDSCs stimulated more apoptotic T cell compared to $CD33^+$ controls [(19.17 ± 2.12%) vs. (10.28 ± 1.26%), $P = 0.0240$, **Figure 3B**]. In addition, the IL-6 antibody dramatically abolished MDSCs-induced T cell apoptosis [(9.797 ± 0.6411%) vs. (19.20 ± 2.13%), $P = 0.0151$, **Figure 3B**]. Accordingly, compared to $CD33^+$ controls, MDSCs significantly inhibited IL-2-induced proliferation of T cells at a ratio of 1:3 (0.9452 ± 0.1721 vs. 0.3410 ± 0.02694, $P = 0.0256$, **Figure 3C**). The IL-6-blocking antibody fully reversed MDSCs-mediated inhibition on T cell proliferation (0.9655 ± 0.1131, $P = 0.0058$, **Figure 3C**). Similarly, anti-CD3/CD28 Abs-induced T cell proliferation was significantly inhibited by MDSCs ($P = 0.0416$), and the IL-6-blocking antibody reversed MDSCs-mediated inhibition on anti-CD3/CD28 Abs-induced T cell proliferation ($P = 0.0404$, **Figure 3C**).

Finally, we evaluated whether IL-6 modulates MDSCs-mediated suppression of cytokine secretion in T cells. IFN-γ secretion in IL-2-stimulated T cells was inhibited by MDSCs from 293.7 ± 17.47 to 168.6 ± 9.498 pg/mL ($P < 0.01$, **Figure 3D**). However, IL-6 blocking antibody eliminated MDSCs-mediated suppression on IFN-γ secretion, which increased to 310.0 ± 15.57 pg/mL ($P = 0.0015$, **Figure 3D**) after IL-6 antibody pretreatment. Consistently, anti-CD3/CD28 Abs-induced IFN-γ secretion of T cells was suppressed (1,094 ± 113.4 vs. 602.0 ± 120.5 pg/mL, $P = 0.0410$, **Figure 3D**) by MDSCs, but after IL-6 antibody pretreatment, the secretion of IFN-γ increased (992.8 ± 57.90 pg/mL, $P = 0.0238$, **Figure 3D**). In contrast, MDSCs stimulated more IL-10 secretion in IL-2-simulated T cells than $CD33^+$ controls, which increased from 434.8 ± 34.52 to 165.4 ± 23.39 pg/mL ($P < 0.001$, **Figure 3E**). However, IL-6-blocking antibody eliminated MDSCs-mediated increase of IL-10, which significantly decreased to 205.7 ± 20.54 pg/mL ($P = 0.0013$, **Figure 3E**). Consistently, IL-10 secretion in anti-CD3/CD28 Abs-stimulated T cells was promoted by MDSCs compared to $CD33^+$ controls (345.4 ± 35.68 vs. 509.8 ± 52.25 pg/mL, $P = 0.0386$, **Figure 3E**) and IL-6-blocking antibody eliminated MDSCs-mediated effect on IL-10 secretion (295.4 ± 59.25 pg/mL, $P = 0.0349$, **Figure 3E**).

Furthermore, we detected IFN-γ and IL-10 production in anti-CD3/CD28 Abs-stimulated T cells using an intracellular staining method. The results showed that the proportion of IFN-γ-positive T cells decreased after co-culture with MDSCs compared to CD33$^+$ controls (72.40 ± 5.771 vs. 42.40 ± 8.965%, $P = 0.0481$, **Figure 3F**), but MDSCs-mediated inhibition of

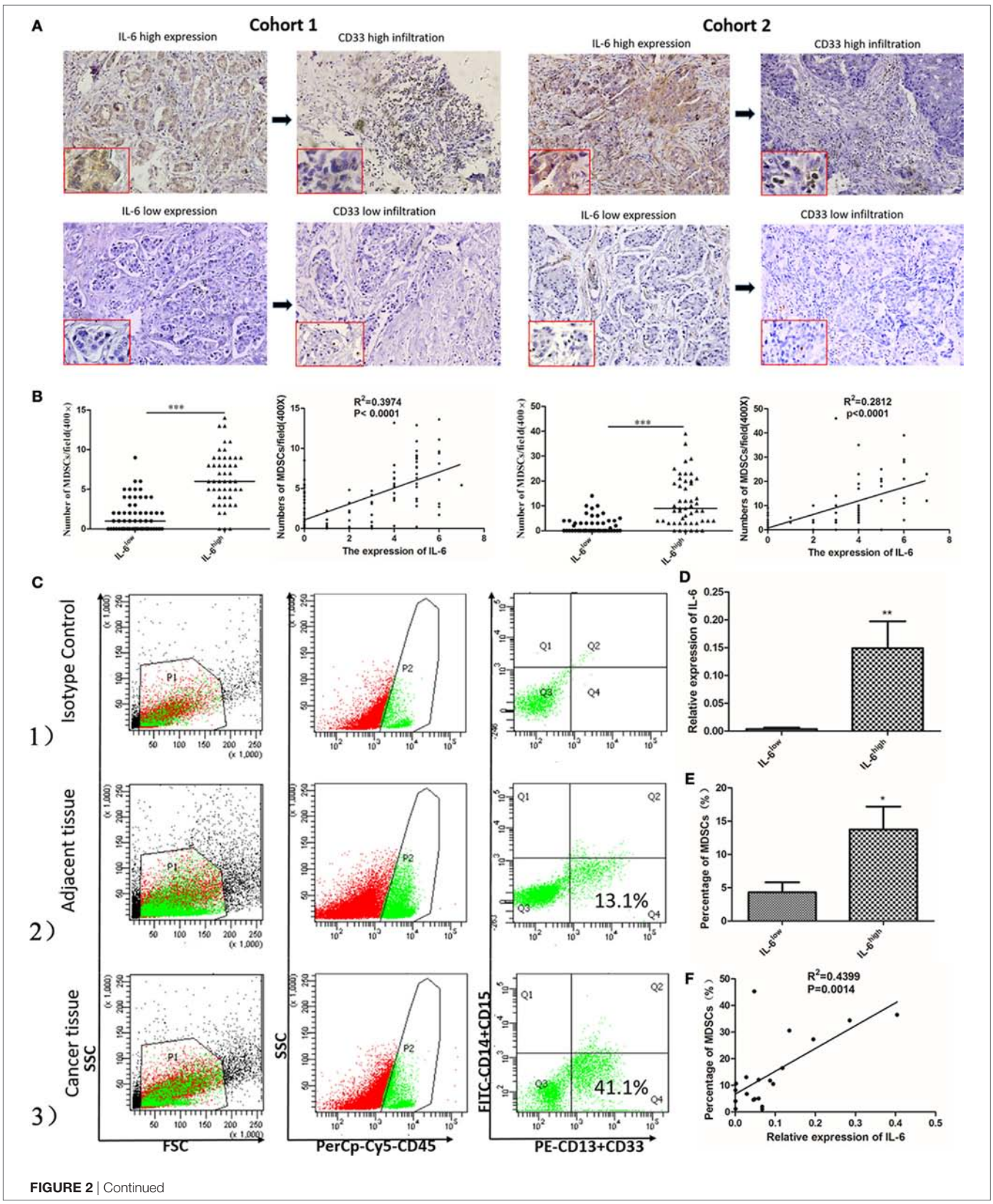


FIGURE 2 | Continued

FIGURE 2 | Tumor-derived interleukin-6 (IL-6) is significantly correlated with the number of infiltrated myeloid-derived suppressor cells (MDSCs) *in situ* both at the mRNA and protein levels. **(A)** The expression of the IL-6 protein and $CD33^+$ MDSCs infiltration in 253 paraffin-embedded breast tissues from cohort 1 and cohort 2 was studied by immunohistochemistry (IHC). We found greater MDSCs infiltration in cancer tissues with a high level of IL-6. **(B)** The correlation between the expression of IL-6 and MDSCs was compared both in cohort 1 and cohort 2 *in situ* ($n = 253$). The average number of MDSCs in the $IL\text{-}6^{low}$ group was significantly lower than that in the $IL\text{-}6^{high}$ group in both cohorts 1 and 2. Pearson correlation analysis revealed a positive correlation between the expression of IL-6 and the number of MDSCs *in situ* in both cohorts. **(C)** The infiltration percentage of the $CD45^+CD33^+CD13^+CD14^-CD15^-$ subpopulation in 20 fresh breast cancer tissue samples was detected using flow cytometry. (1) The subpopulation was gated using anti-CD45 mAb and isotype control was used; (2) The $CD45^+CD33^+CD13^+CD14^-CD15^-$ subpopulation in adjacent normal tissues. (3) The proportion of the interested subpopulation significantly increased in cancer tissues. **(D)** Based on the median relative RNA level of IL-6, breast cancer samples were divided into $IL\text{-}6^{high}$ and $IL\text{-}6^{low}$ groups. The average IL-6 mRNA level in the $IL\text{-}6^{high}$ group was 37.25-fold higher than that in the $IL\text{-}6^{low}$ group ($P = 0.0093$) ($n = 20$). **(E)** A higher frequency of MDSCs was detected in the $IL\text{-}6^{high}$ group compared to in the $IL\text{-}6^{low}$ group. **(F)** A correlation analysis on MDSCs number and IL-6 level was carried out ($R^2 = 0.4399$, $P = 0.0014$) ($n = 20$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

IFN-γ production in T cells was reversed by blocking IL-6 (73.20 ± 4.574%, $P = 0.0376$, **Figure 3F**). In contrast, the percentages of IL-10-positive T cells increased after co-culture with MDSCs (from 6.650 ± 1.751 to 16.91 ± 2.570%, $P = 0.0299$, **Figure 3G**) and blocking IL-6 inhibited IL-10 production in T cells (8.990 ± 1.123%, $P = 0.0476$, **Figure 3G**). These results indicate that MDSCs-induced immunosuppressive effects on T cells were IL-6-dependent and could be fully attenuated by blocking IL-6 signaling.

IL-6 Stimulated Sustained Activation of the JAK/STAT Pathway in MDSCs Displaying Persistent Phosphorylation of Downstream STAT Proteins

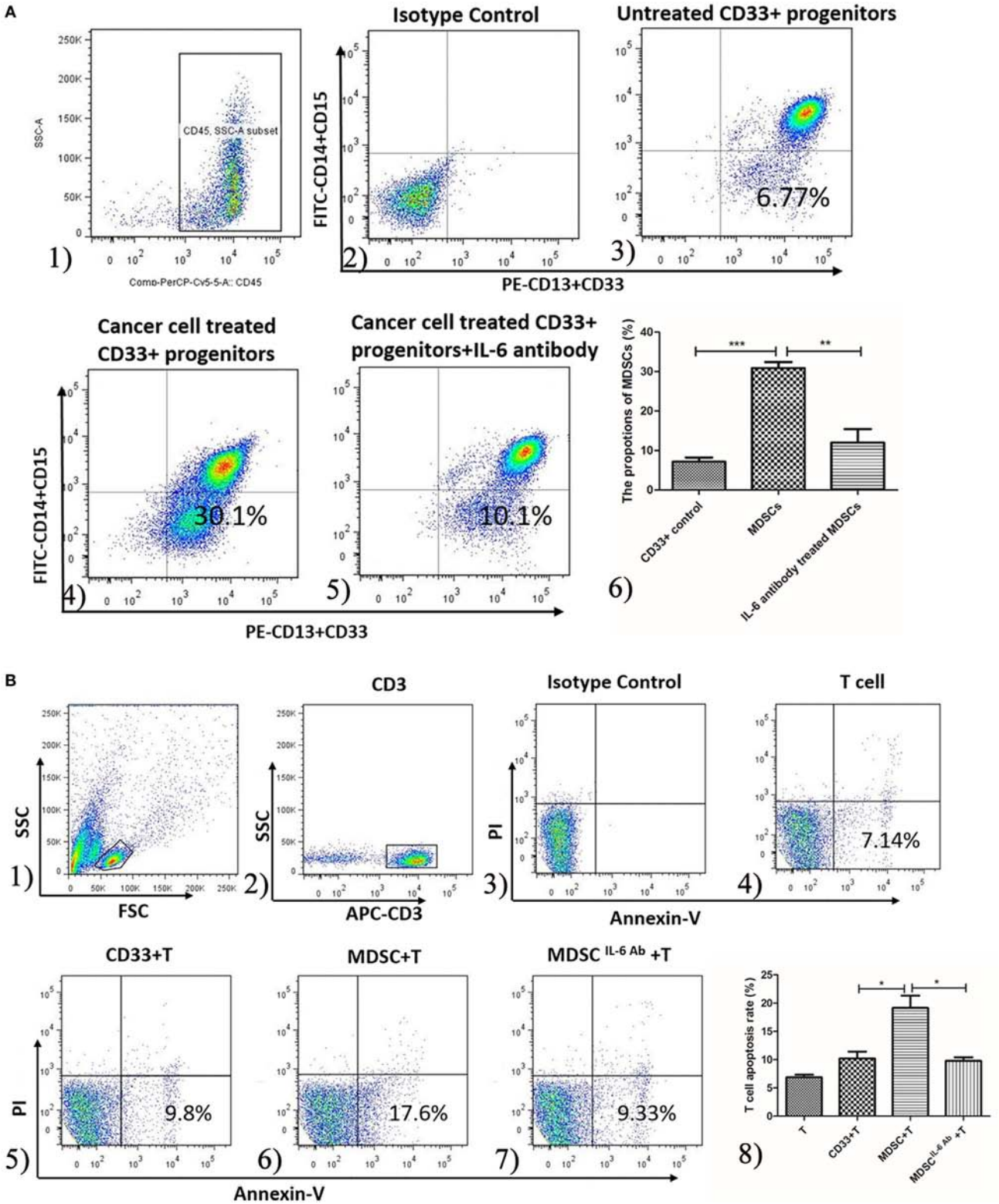
To elucidate the molecular mechanisms regulating IL-6-induced MDSC differentiation and immunosuppressive activities, we studied the activation status of the JAK/STAT pathway downstream of IL-6 signaling. We assessed the expression and phosphorylation of multiple functional proteins along the JAK/STAT pathway, such as JAK1, JAK2, TYK2, STAT1, and STAT3, using western blot assays. Comparable increases in phosphorylated JAK1, JAK2, TYK2, STAT1, and STAT3 proteins were detected in MDSCs as compared to that in $CD33^+$ controls (**Figure 4A**). Furthermore, sustained phosphorylation of STAT1 and STAT3 proteins was observed in MDSCs, which was maintained for a longer time than in normal IL-6-stimulated PBMCs (2 vs. 4 h, **Figure 4B**). In IL-6 (100 ng/mL)-stimulated PBMCs, the levels of phosphorylated STAT1 and STAT3 proteins were increased at 30 min, but decreased at 2 h, disappearing entirely at 4 h (**Figure 4B**). In contrast, persistent IL-6-induced STAT1 and STAT3 phosphorylation in MDSCs lasted for more than 4 h. After adding an IL-6 blocking antibody, phosphorylation levels of the above proteins were reduced significantly in MDSCs, including p-JAK1 (1.059 ± 0.06000 vs. 0.8431 ± 0.03423, $P = 0.0354$), p-JAK2 (1.093 ± 0.03076 vs. 0.8486 ± 0.07076, $P = 0.0340$), p-TYK2 (0.9248 ± 0.08132 vs. 0.6939 ± 0.01329, $P = 0.0487$), p-STAT1 (1.056 ± 0.07766 vs. 0.8229 ± 0.02599, $P = 0.0464$), and p-STAT3 (1.074 ± 0.03318 vs. 0.8247 ± 0.04921, $P = 0.0137$, **Figure 4C**). These data indicate that enhanced phosphorylation of STAT proteins in MDSCs is IL-6-dependent, although additional factors along the JAK/STAT pathway can manipulate persistent IL-6-induced activation of both STAT1 and STAT3 proteins.

IL-6-Induced Suppression of SOCS3 in MDSCs Was Determined at both the mRNA and Protein Levels

Because the loss of SOCS proteins has been reported to induce continuous activation of the JAK/STAT pathway in malignancy, we compared the expression of SOCS1, SOCS2, and SOCS3 between MDSCs and normal myeloid controls at both the mRNA and protein levels. Primary MDSCs isolated from 20 cases of primary breast cancer tissues were studied. $CD33^+$ and $CD14^+$ cells from healthy donors were regarded as normal myeloid-derived cell controls. We first detected the mRNA levels of SOCS1–3 and found that mRNA level of SOCS1 increased in MDSCs compared to in both $CD33^+$ ($P = 0.006$) and $CD14^+$ ($P = 0.003$) controls; the mRNA level of SOCS3 significantly decreased in MDSCs compared to in $CD33^+$ ($P < 0.001$) and $CD14^+$ ($P < 0.001$, **Figure 5A**) controls. An undetectable level of SOCS2 mRNA was observed in both MDSCs and controls (**Figure 5A**). We then compared the mRNA levels of SOCS1–3 between MDSCs from $IL\text{-}6^{high}$ tissues ($MDSC^{IL\text{-}6h}$) and MDSCs from $IL\text{-}6^{low}$ tissues ($MDSC^{IL\text{-}6l}$). The results demonstrated that the mRNA level of SOCS1 increased in $MDSC^{IL\text{-}6h}$ ($P = 0.0459$), while the mRNA level of SOCS3 decreased in $MDSC^{IL\text{-}6h}$ compared to that in $MDSC^{IL\text{-}6l}$ ($P = 0.0089$, **Figure 5B**). Linear regression analysis demonstrated that IL-6 expression was not correlated with SOCS1 ($R^2 = 0.09071$, $P = 0.2102$) but was negatively correlated with SOCS3 expression ($R^2 = 0.2205$, $P = 0.0367$, **Figure 5B**).

We then detected the mRNA levels of SOCS1–3 in induced MDSCs *in vitro*. Untreated $CD33^+$ myeloid progenitors were used as negative controls, while $CD14^+$ monocyte-derived immature DCs (iDC) were used as positive controls. The results were consistent with those observed in primary MDSCs, where the mRNA level of SOCS1 increased, while that of SOCS3 decreased in induced MDSCs (**Figure 5C**). The disparity between SOCS1 and SOCS3 expression was confirmed at the protein level. The expression of SOCS1 protein notably increased, while that of SOCS3 protein dramatically decreased in MDSCs compared to $CD33^+$ negative controls and iDC positive controls (**Figure 5D**).

To further verify the effect of IL-6 on SOCS3 expression, an IL-6-neutralizing antibody was added to block IL-6 signaling in MDSCs, and the synthesis and expression of SOCS was detected. Ab-treated MDSCs displayed slightly lower mRNA level of SOCS1 ($P = 0.0917$, **Figure 5E**), but significantly higher mRNA level of SOCS3 compared to that in Ab-untreated MDSCs



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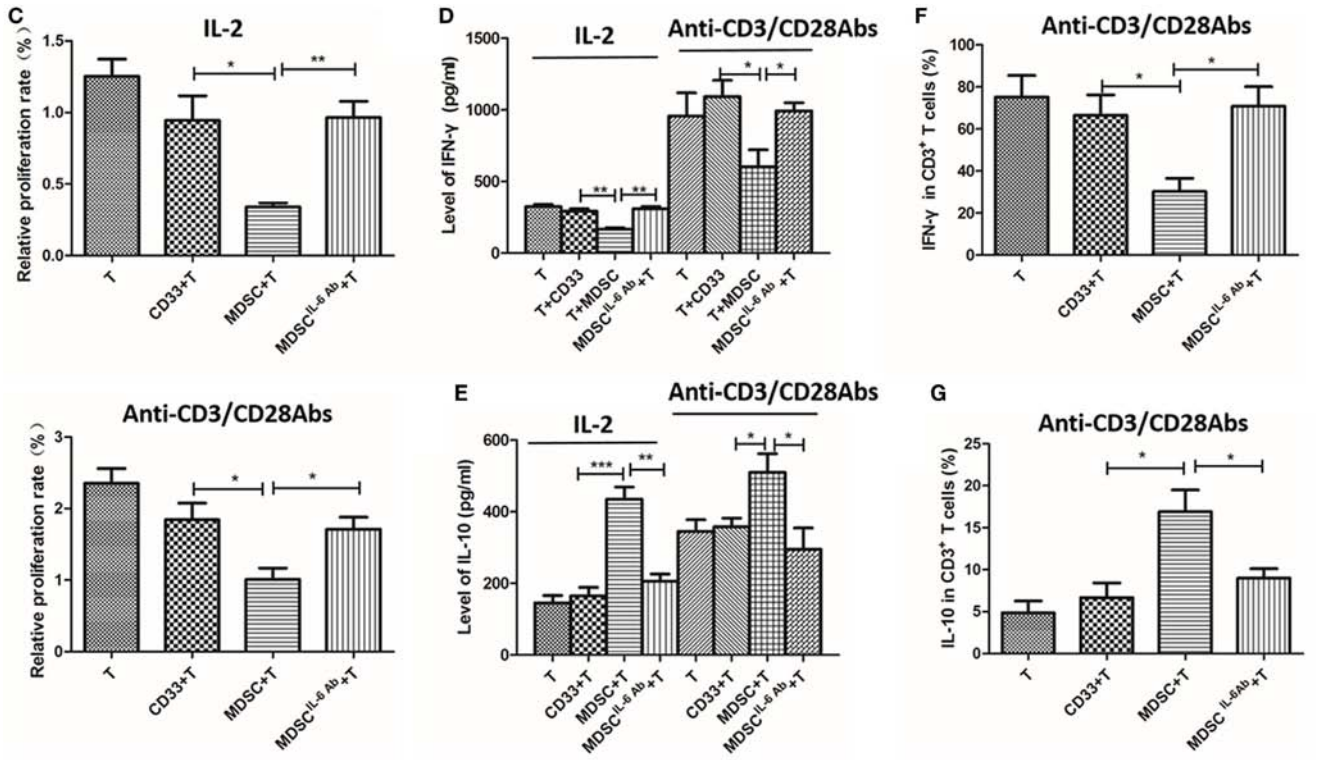


FIGURE 3 | Interleukin-6 (IL-6) enhanced the generation and T cells immunosuppressive ability of myeloid-derived suppressor cells (MDSCs) *in vitro.* **(A)** The proportion of healthy people's untreated CD33+ myeloid progenitors and the treated CD33+ cells was compared using flow cytometry method. Cells in Q4 represent MDSCs. (1) The subpopulation was gated using anti-CD45 mAb; (2) isotype control was used; (3) the proportion range of CD33+ was 15.6 ± 2.6%; (4) when treated with cancer cells the proportion of MDSCs was highly increased which was 30.83 ± 1.595%; (5) an IL-6 neutralizing antibody was added to the cancer-conditioned MDSCs culture and MDSCs decreased to 11.98 ± 3.479%; (6) the result of statistical analysis ($n = 6$). The effects of MDSCs and CD33+ cells on T cell proliferation, apoptosis and cytokine secretion were examined **(B–E)**. **(B)** T cells stimulated with 1,000 IU/ml IL-2 were co-cultured with CD33+ control cells or MDSCs at ratio of 1:3 for detecting apoptosis. T cells were gated using APC-labeled anti-CD3 mAb, and apoptotic cells were stained with FITC-labeled Annexin V. Cells in Q4 represent apoptotic T cells. (1) The lymphocytes were gated according to SSC and FSC features; (2) CD3 mAb labeled T cells; (3) isotype control; (4) T cells only; (5) CD33+ controls stimulated T cells; (6) MDSCs stimulated T cells; (7) T cells were co-cultured with MDSCs in the presence of IL-6 antibody; (8) Summary of (4–7) MDSCs stimulated greater T cell apoptosis compared to in CD33+ controls. In addition, the IL-6 antibody dramatically abolished MDSCs-induced T cell apoptosis ($n = 5$). **(C)** The proliferation of T cells was detected using CCK8 method. Compared to in CD33+ controls, MDSCs significantly inhibited IL-2 or anti-CD3/CD28 Abs-induced proliferation of T cells at a ratio of 1:3, which was attenuated by IL-6 blocking antibody ($n = 5$). **(D,E)** Supernatants were collected for detecting interferon (IFN)-γ and IL-10 level using ELISA assay ($n = 5$). **(D)** MDSCs inhibited IL-2 or anti-CD3/CD28 Abs-induced IFN-γ secretion, which was increased after IL-6 antibody pretreatment. **(E)** In contrast, MDSCs stimulated IL-10 secretion in IL-2 or anti-CD3/CD28 Abs simulated T cells compared to in CD33+ controls, which was dropped significantly after IL-6 blocking. **(F)** Flow cytometry was used to detect IFN-γ expression by intracellular staining. The proportion of IFN-γ-positive T cells decreased after co-culture with MDSCs compared to in CD33+ controls, but MDSCs-mediated inhibition of IFN-γ production in T cells was reversed by blocking of IL-6 ($n = 5$). **(G)** In contrast, the percentages of IL-10-positive T cells increased after co-culture with MDSCs and blocking of IL-6 inhibited IL-10 production in T cells ($n = 5$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

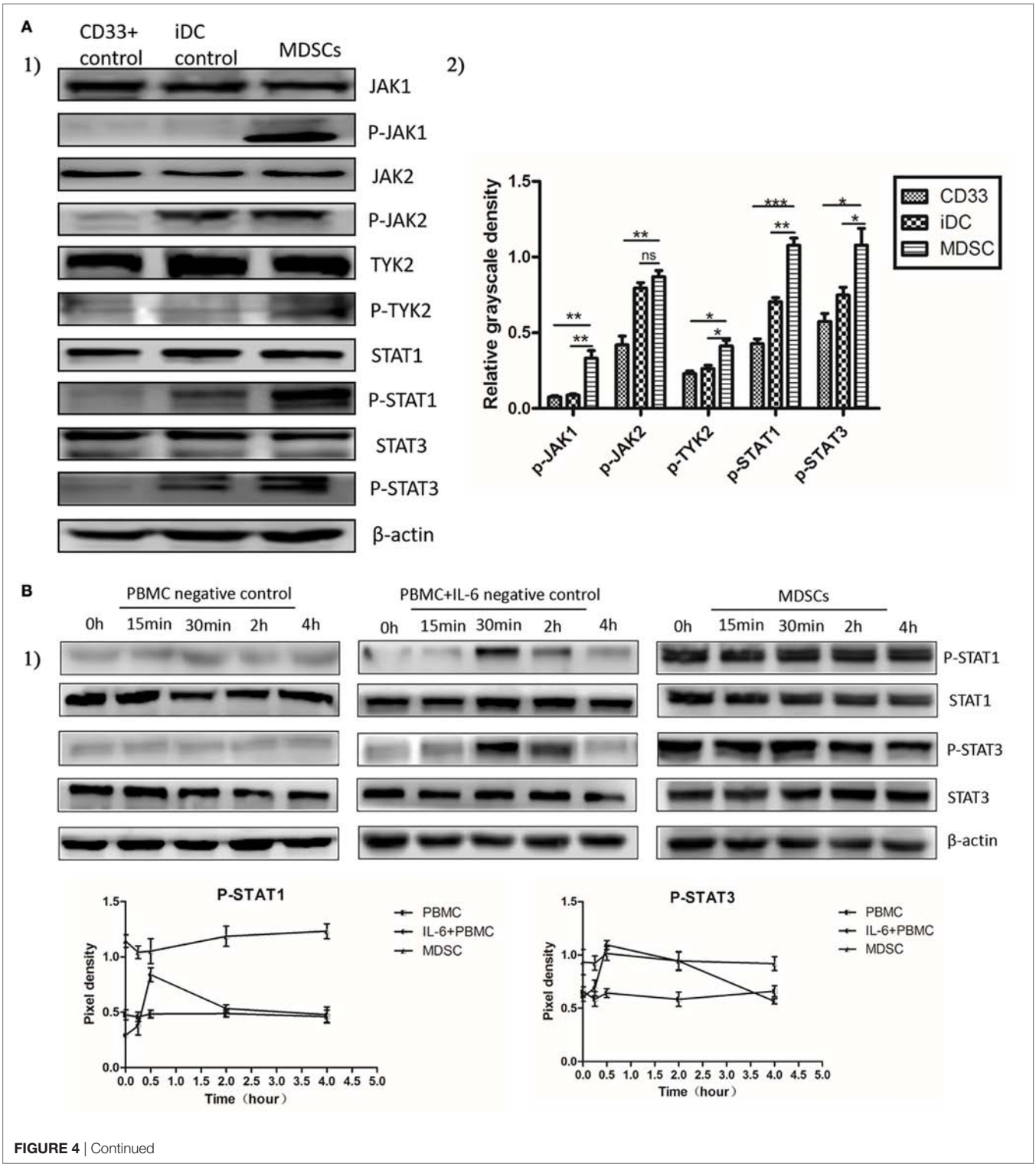
($P = 0.0117$, **Figure 5E**). Consistent results were confirmed at the protein level (**Figure 5F**). Therefore, our study suggests that IL-6 induces inhibition of SOCS3 expression in MDSCs at both the mRNA and protein levels *in vivo* and *vitro.*

IL-6-Dependent SOCS3 Suppression and Sustained Activation of the JAK/STAT Pathway Was Correlated with CD126 Upregulation

The IL-6 signal is transduced as a result of the interaction between IL-6 and IL-6R, which includes 2 subunits, CD126 and gp130 (8). We analyzed the expression of CD126 and gp130 in primary MDSCs and found that the mRNA levels of CD126 and gp130 in primary MDSCs were higher than those in CD33+ and CD14+ controls (**Figure 6A**). Furthermore, the mRNA levels of CD126 and gp130 in primary MDSCs$^{IL\text{-}6h}$ were higher than those in MDSCs$^{IL\text{-}6l}$ ($P = 0.010$, **Figure 6B**). Linear regression analysis demonstrated that CD126, rather than gp130, was positively correlated with IL-6 levels ($R^2 = 0.6717$, $P < 0.0001$, **Figure 6B**). Similarly, we examined expression of CD126 and gp130 in induced MDSCs, and found that these MDSCs exhibited higher mRNA levels of CD126 and gp130 than CD33+ controls ($P = 0.0145$, $P = 0.0011$, **Figure 6C**). Similar results were obtained

at the protein level, in which CD126 expression was significantly enhanced, while gp130 showed no significant changes in the expression (**Figure 6D**).

An anti-IL-6R (CD126) neutralizing antibody was used to block the interaction between IL-6 and IL-6R in MDSCs to study the effect of elevated CD126 on the JAK/STAT pathway. Phosphorylation levels of JAK1, JAK2, TYK2, and STAT3 proteins decreased after the addition of the anti-IL-6R neutralizing antibody (**Figure 6E**). This result indicates that CD126 plays a significant role in IL-6-dependent activation of the JAK/STAT pathway. Furthermore, the mRNA level of SOCS3 increased, while the mRNA level of SOCS1 decreased after blocking CD126



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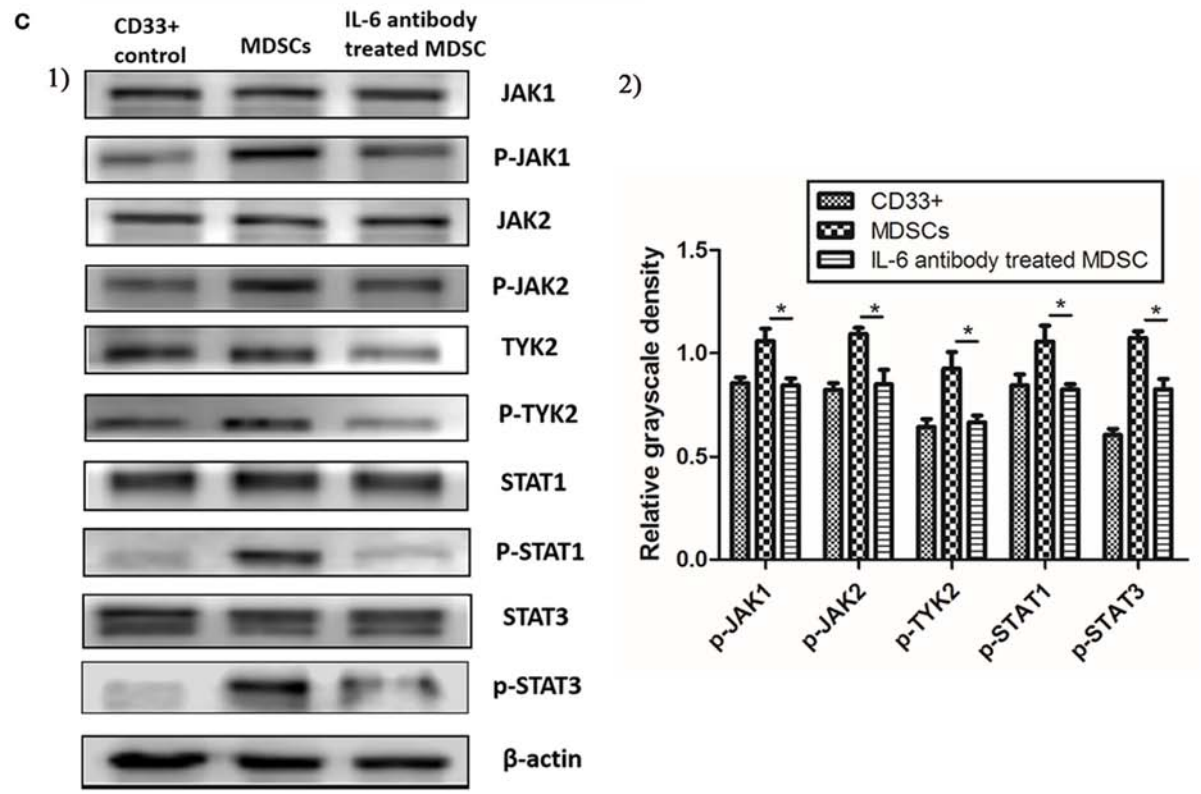


FIGURE 4 | Interleukin-6 (IL-6) stimulated the sustained activation of the JAK/STAT pathway in myeloid-derived suppressor cells (MDSCs) displaying persistent phosphorylation of downstream STAT proteins. The activation status of the JAK/STAT pathway was measured using Western blot. **(A)** Comparable increases in phosphorylated JAK1, JAK2, TYK2, STAT1, and STAT3 proteins were detected in MDSCs as compared to in CD33$^+$ controls ($n = 3$). **(B)** Furthermore, sustained phosphorylation of STAT1 and STAT3 proteins was observed in MDSCs, which was maintained for a longer time than in normal IL-6-stimulated PBMCs. In IL-6 (100 ng/mL)-stimulated PBMCs, the levels of phosphorylated STAT1 and STAT3 proteins were increased at 30 min, but decreased at 2 h, disappearing entirely at 4 h. In contrast, persistent IL-6-induced STAT1 and STAT3 phosphorylation in MDSCs lasted for more than 4 h ($n = 3$). **(C)** In contrast, persistent IL-6-induced STAT1 and STAT3 phosphorylation in MDSCs lasted for more than 4 h. After adding an IL-6-blocking antibody, phosphorylation levels of the above proteins were reduced significantly in MDSCs. They were compared using the density ratio of phosphorylated protein to total protein ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

($P = 0.0318$, $P = 0.0190$, **Figure 6F**). The expression of corresponding proteins in MDSCs displayed the same trend as that of mRNA after CD126 blocking (**Figure 6G**). These results indicate that suppressed expression of SOCS3 is significantly correlated with CD126 upregulation, which induces long-term activation of the JAK/STAT pathway.

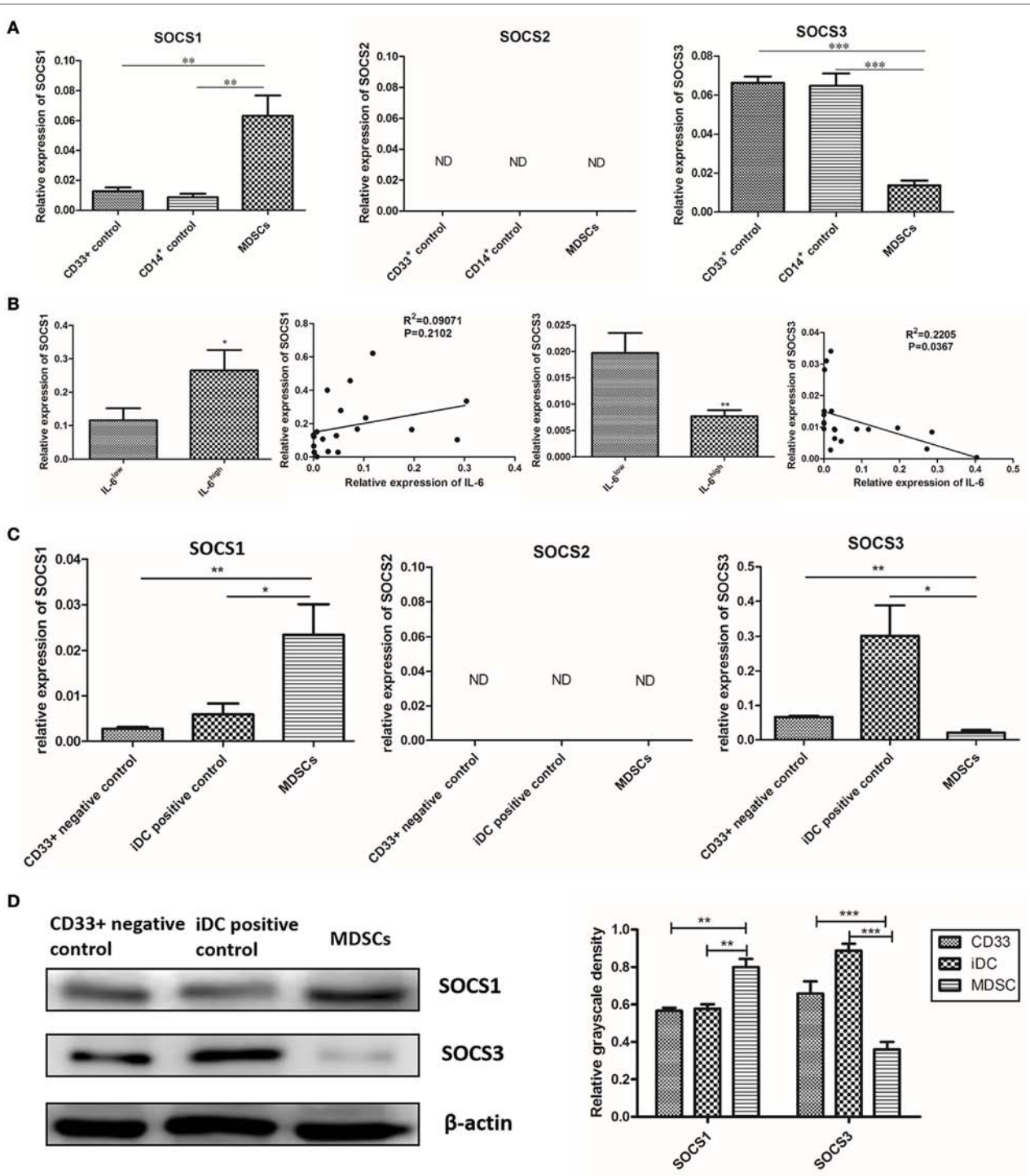
Soluble CD126-Mediated IL-6 *Trans*-Signaling Regulated IL-6 Dependent SOCS3 Suppression and Sustained Activation of the JAK/STAT Pathway in MDSCs

Signaling through membrane-bound and soluble IL-6R (CD126) is known as the *cis*- and *trans*-mediated signaling pathways, respectively (29). To investigate which type of CD126 mainly regulates IL-6-dependent activation of the JAK/STAT pathway, we measured the levels of membrane-bound and soluble CD126 in MDSCs. The results showed that MDSCs expressed lower levels of membrane-bound CD126 (7.667 ± 1.808 vs. 15.63 ± 1.200%, $P = 0.0214$, **Figure 7A**), but generated more soluble CD126 than those in CD33$^+$ controls (249.1 ± 24.35 vs. 165.6 ± 21.83 pg/mL, $P = 0.0236$, **Figure 7B**). These results demonstrate that soluble CD126 is significantly increased in MDSCs and may play major roles in suppressing SOCS3 expression and activating the JAK/STAT pathway in MDSCs.

To determine if soluble CD126 regulates SOCS3 expression and activation of the JAK/STAT pathway, we added ADAM proteases to the MDSC culture system *in vitro*. ADAM proteases, particularly ADAM10 and ADAM17, can induce shedding of membrane CD126 (30). We firstly detected the expression of ADAM10 and ADAM17 in MDSCs by RT-PCR and western blotting and found that the mRNA levels of ADAM10 and ADAM17 were clearly enhanced in MDSCs compared to those in CD33$^+$ controls ($P = 0.0064$; $P = 0.0297$, **Figure 7C**). But at the protein level, exclusively ADAM10 rather than ADAM17 significantly increased (**Figure 7D**). We then treated MDSCs with exogenous recombinant ADAM10 protein and measured the levels of soluble CD126 at different time points. The level of soluble CD126 in MDSCs significantly increased at 30 min (184.7 ± 5.066 vs.

142.0 ± 11.50 pg/mL, $P = 0.0273$) and decreased to pre-treatment levels at 2 h (125.1 ± 9.050 pg/mL, **Figure 7E**).

We next detected the activation of STAT and SOCS in MDSCs at different time points after adding exogenous ADAM10. We found that the levels of phosphorylated STAT1 and STAT3 proteins increased after ADAM10 treatment in MDSCs (**Figure 7F**). A slight increase in SOCS1 and decrease in SOCS3 protein were also detected after adding ADAM10 in MDSCs (**Figure 7F**). These results revealed that ADAM10 promotes the suppression of SOCS3 expression and phosphorylation of STAT proteins in MDSCs. This indicates that IL-6 *trans*-signaling is predominately mediated by soluble CD126 to regulate IL-6-dependent SOCS3



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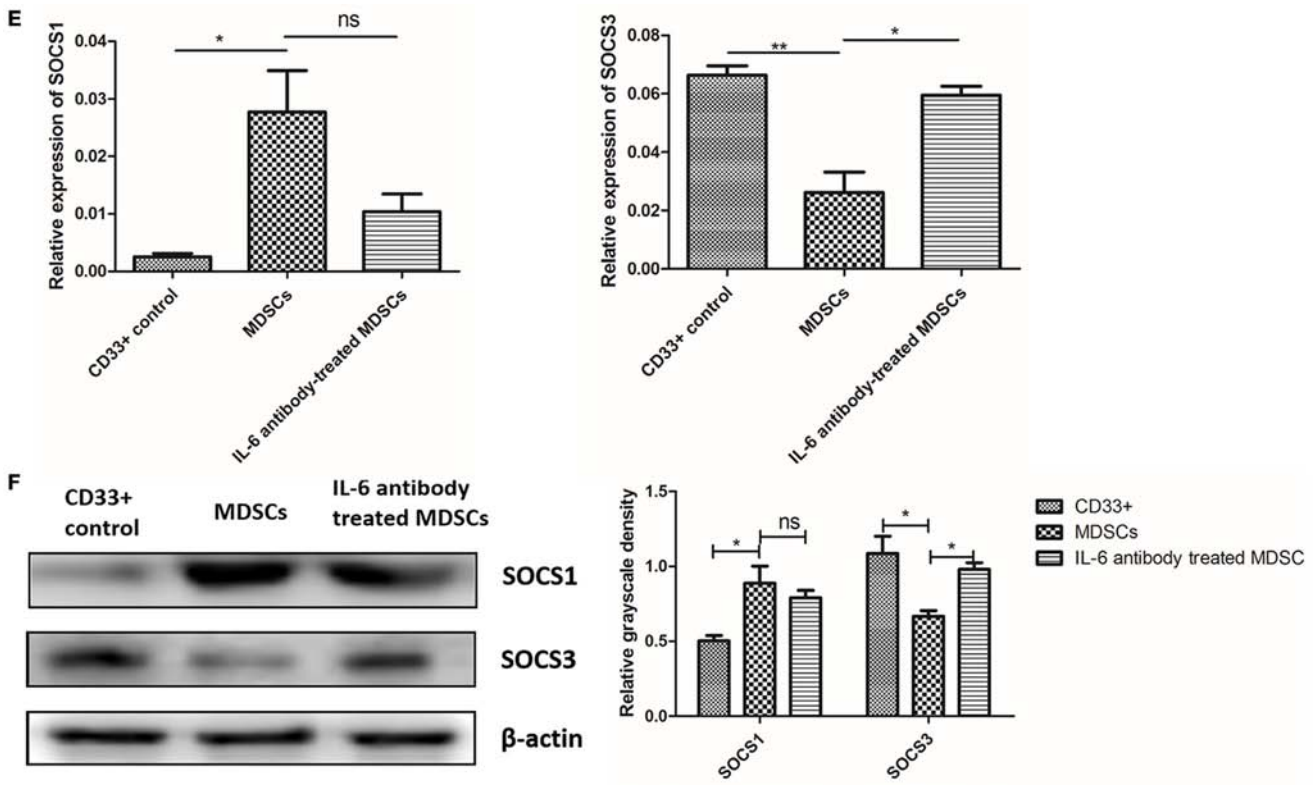


FIGURE 5 | Interleukin-6 (IL-6)-induced suppression of suppressor of cytokine signaling 3 (SOCS3) in myeloid-derived suppressor cells (MDSCs) was determined at both the mRNA and protein levels. **(A)** RT-PCR result showed mRNA level of SOCS1 was increased and SOCS3 was significantly decreased in MDSCs compared to in both $CD33^+$ and $CD14^+$ controls; an undetectable level of SOCS2 mRNA was observed in both MDSCs and controls ($n = 20$). **(B)** The mRNA levels of SOCS1–3 between MDSCs from IL-6^{high} tissues ($MDSC^{IL\text{-}6h}$) and MDSCs from IL-6^{low} tissues ($MDSC^{IL\text{-}6l}$) were compared. The mRNA level of SOCS1 was increased in $MDSC^{IL\text{-}6h}$ ($P = 0.0459$), while the mRNA level of SOCS3 was decreased in $MDSC^{IL\text{-}6h}$ compared to in $MDSC^{IL\text{-}6l}$ ($P = 0.0089$). Linear regression analysis demonstrated that IL-6 expression was not correlated with SOCS1 ($R^2 = 0.09071$, $P = 0.2102$), but demonstrated a significant negative correlation with SOCS3 expression ($R^2 = 0.2205$, $P = 0.0367$) ($n = 20$). **(C)** Then, we detected mRNA levels of SOCS1–3 in induced MDSCs *in vitro*. The results were consistent with those observed in primary MDSCs, where the mRNA level of SOCS1 was increased, while that of SOCS3 was decreased in MDSCs compared to in the controls ($n = 5$). **(D)** The whole cellular lysates were collected for western blot analysis. SOCS1 protein was notably increased, but SOCS3 protein was dramatically decreased in MDSCs as compared with those in $CD33^+$ negative controls and iDC positive controls ($n = 5$). **(E)** RT-PCR show that Ab-treated MDSCs displayed slightly decreased mRNA level in SOCS1 ($P = 0.0917$), but significantly higher mRNA levels in SOCS3 compared to in Ab-untreated MDSCs ($P = 0.0117$) ($n = 5$). **(F)** Consistent results were confirmed at the protein level ($n = 5$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

suppression and sustained activation of the JAK/STAT pathway in MDSCs, as well as coordinates the differentiation and immunosuppressive activity of MDSCs in breast cancer.

DISCUSSION

Multiple immunocytes recruited into the tumor microenvironment play pivotal roles in tumorigenesis (31). However, MDSCs represent a specific subset of heterogeneous immunosuppressive cells that enable cancer cells to escape immune surveillance and inhibit the host immune system attack on cancer cells (32). Bronte et al. recommended the characterization standards and nomenclature of MDSCs and indicated that MDSCs are often divided into two subtypes in humans: PMN-MDSCs and MO-MDSCs (5). In addition to these MDSCs subtypes, the eMDSC subtype is marked with $Lin^-HLA\text{-}DR^-CD33^+$ and comprised of more immature progenitors than M-MDSCs and PMN-MDSCs (5). However, the MDSC subset is tumor-dependent. Previous studies of breast cancer examined MDSCs in mouse models rather than in humans because of the uncertainty of cell phenotypes and complicated regulatory mechanisms in human MDSCs (33–35). Determining the precise phenotype of breast cancer MDSCs in humans improves the understanding of the crosstalk between cancer cells and the microenvironment in the initiation and progression of breast cancer.

In our previous study, we identified a subset of poorly differentiated eMDSCs in breast cancer displaying potent suppression of T cells *in vitro* and *vivo* (6). As a pan-myeloid marker, CD33 is expressed earlier and more extensively in the myeloid lineage, and we found that $CD33^+HLA\text{-}DR^-$ cells rather than $CD14^+HLA\text{-}DR^-$ cells and $CD11b^+HLA\text{-}DR^-$ cells were increased in patient blood samples compared to in healthy donor blood samples (6). We further detected the expression of a series of markers of myeloid linage, including HLA-DR, CD15, CD14, CD13, and CD11b. We

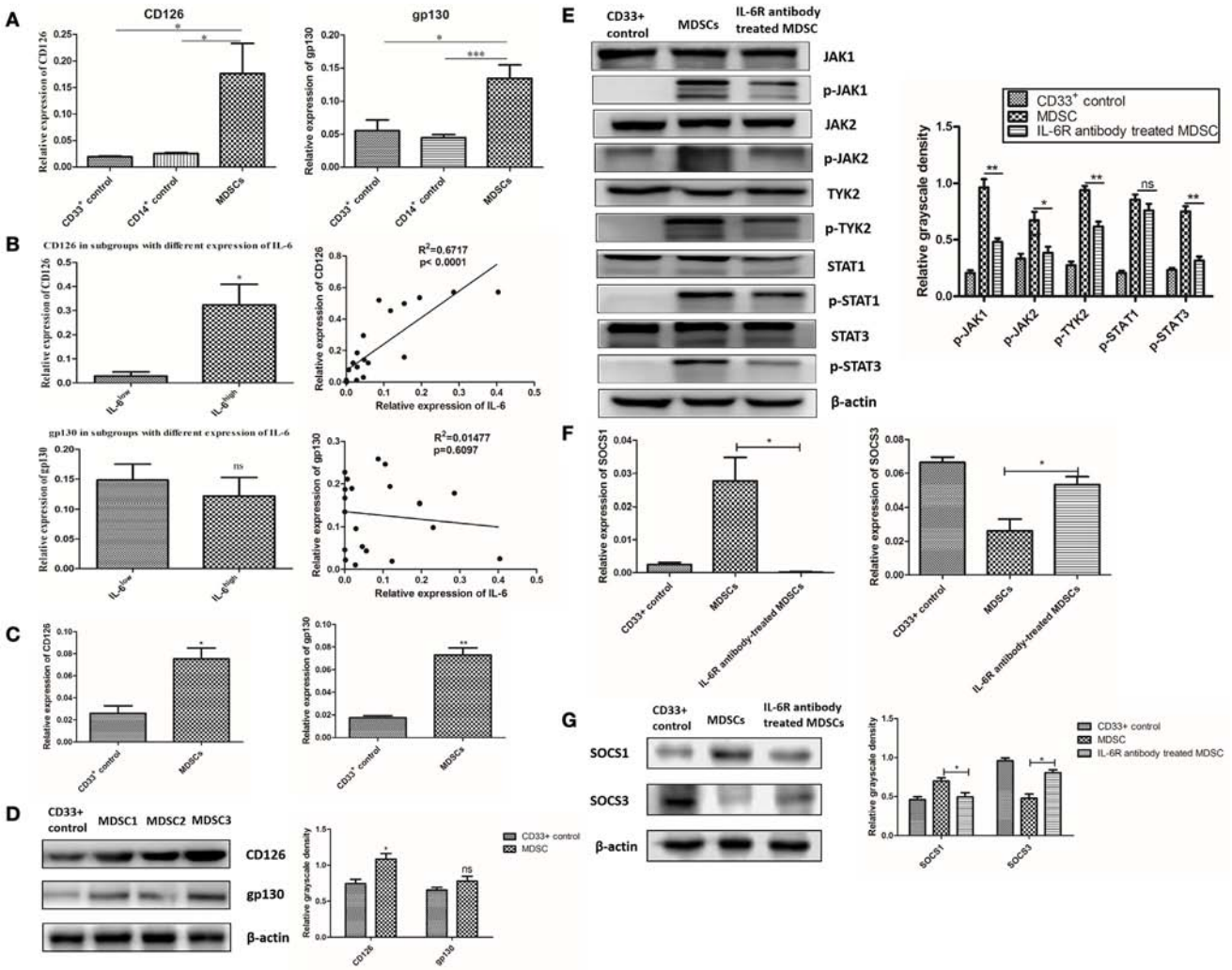


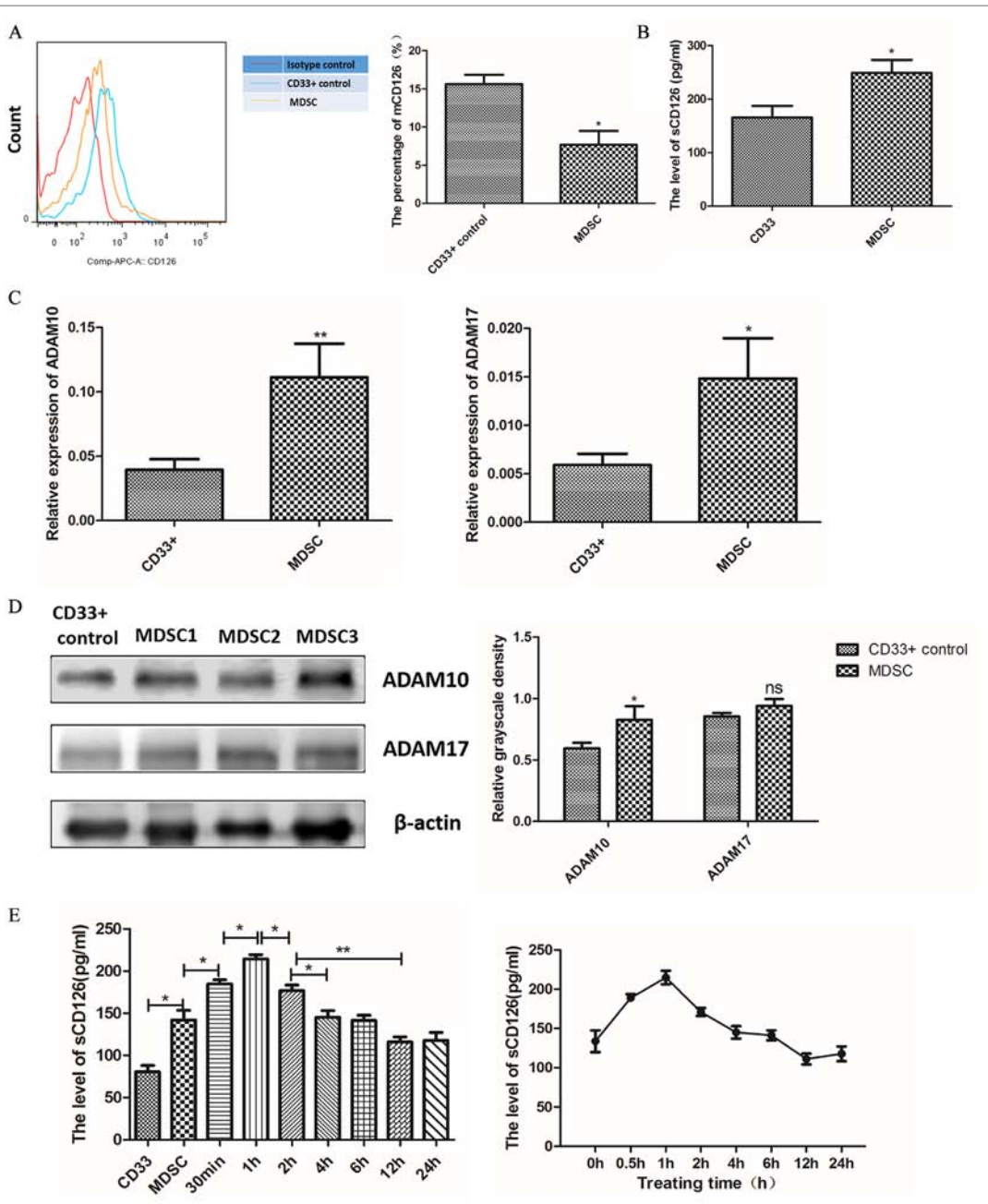
FIGURE 6 | Interleukin-6 (IL-6)-dependent suppressor of cytokine signaling 3 (SOCS3) suppression and sustained activation of the JAK/STAT pathway was correlated with CD126 upregulation. **(A)** The relative expression of interest genes were indicated by $2^{-\Delta Ct}$($\Delta Ct = Ct_{target\ gene} - Ct_{\beta\text{-actin}}$). We found the mRNA levels of CD126 and gp130 in primary myeloid-derived suppressor cells (MDSCs) were higher than those in $CD33^+$ and $CD14^+$ controls ($n = 5$). **(B)** The mRNA levels of CD126 and gp130 in primary $MDSCs^{IL\text{-}6h}$ were higher than those in $MDSCs^{IL\text{-}6l}$. Linear regression analysis demonstrated that CD126, rather than gp130, was positively correlated with IL-6 levels ($R^2 = 0.6717$, $P < 0.0001$) ($n = 20$). **(C)** The expressions of CD126 and gp130 in induced MDSCs were also examined. MDSCs exhibited higher mRNA levels of CD126 and gp130 compared to in $CD33^+$ controls ($n = 3$). **(D)** Similar results were obtained at the protein level, in which CD126 expression was significantly increased, while gp130 showed no significant changes in the expression ($n = 3$). **(E–G)** IL-6R antibody was used to block IL-6 signal and the downstream signaling pathway. **(E)** Phosphorylation levels of JAK1, JAK2, TYK2, and STAT3 proteins decreased after the addition of the anti-IL-6R neutralizing antibody ($n = 3$). **(F)** The mRNA level of SOCS3 was increased, while the mRNA level of SOCS1 was decreased after blocking of CD126 ($n = 3$). **(G)** The expression of corresponding proteins in MDSCs displayed the same trend as that of mRNA after CD126 blocking. β-actin blots were used as protein loading controls ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

confirmed low expression of HLA-DR and CD14, as well as negative expression of CD15 in breast cancer MDSCs. Additionally, both CD13 and CD11b expressed on breast cancer MDSCs, however, non-specific staining on the cancer cells, endothelial cells, and fibroblasts significantly interfered with the specific staining on MDSCs which were consistent with the previous reports (36, 37) (Figures S1B–C in Supplementary Material). Therefore, we defined the phenotype of $CD45^+CD13^+CD33^+CD14^-CD15^-$ to precisely distinguish breast cancer MDSCs.

In this study, we demonstrated the positive correlations between MDSCs *in situ* and numbers of metastatic lymph nodes, tumor volume, pathological stage, and histology grade. Furthermore, we confirmed the negative correlation between MDSCs and OS in breast cancer patients and found that patients with more MDSCs showed worse clinical outcomes. Similar findings were reported in other tumors, such as in digestive system malignant tumors (38), prostate cancer (39), and advanced melanoma (40). Our results indicate that MDSCs are unfavorable prognostic factors in breast cancer patients.

Numerous cytokines have been reported to recruit MDSCs in cancer tissues, such as IL-1β, IL-6, IL-4, macrophage colony-stimulating factor, and granulocyte macrophage

colony-stimulating factor (3, 14, 32). Among these tumor-derived cytokines, IL-6 has been proposed to be an efficient MDSCs inducer in solid tumors, such as esophageal cancer, prostate cancer, and melanoma (9, 28, 41–43). Circulating $CD11b^{+}CD14^{+}HLA^{-}DR^{-}$ cells were found to be significantly increased in esophageal cancer and were associated with circulating IL-6 levels (9). IL-6 induces MDSCs generation, and inhibition of IL-6 abrogates generation of MDSCs in tumor-bearing mice (13, 42). In this study, we evaluated the correlation between tumor-derived IL-6 and MDSC infiltration in 253 paraffin-embedded primary breast tissues and 20 fresh breast cancer tissues. We found that more MDSCs infiltrated IL-6 high-expressing cancer tissues, and that tumor-derived IL-6 displayed a strong positive correlation with the number of infiltrating MDSCs *in situ* at both the mRNA and protein levels. Furthermore, we demonstrated that tumor-derived IL-6 was essential for MDSCs amplification and function *in vitro*, including promoting T cells apoptosis, inhibiting T cell proliferation, decreasing IFN-γ secretion, and increasing IL-10 production. Therefore, determining the detailed molecular mechanisms that regulate IL-6-dependent recruitment and amplification of MDSCs in breast cancer may help screen for potential therapeutic targets to eradicate MDSCs



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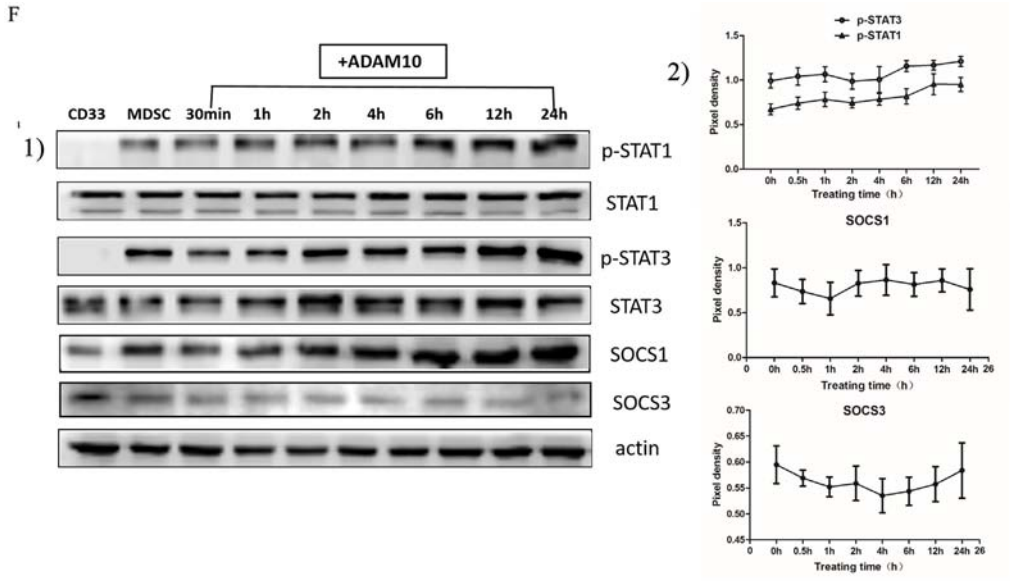

FIGURE 7 | Soluble CD126-mediated interleukin-6 (IL-6) *trans*-signaling regulated IL-6 dependent suppressor of cytokine signaling 3 (SOCS3) suppression, and sustained activation of the JAK/STAT pathway in myeloid-derived suppressor cells (MDSCs). **(A)** Flow cytometry showed that MDSCs expressed lower levels of membrane-bound CD126 than those in $CD33^+$ controls ($n = 5$). **(B)** The soluble CD126 secretion increased in MDSCs using Elisa assay method ($n = 5$). **(C)** RT-PCR method was used to detect ADAM10 and ADAM17 expression. The mRNA levels of ADAM10 and ADAM17 were clearly enhanced in MDSCs compared to in $CD33^+$ controls ($n = 3$). **(D)** Protein expression of ADAM10, but not ADAM17, was significantly increased ($n = 3$). **(E)** MDSCs were then treated with exogenous recombinant ADAM10 protein, and the change in soluble CD126 at different time points was measured. The level of soluble CD126 in MDSCs was increased at 30 min and decreased to pre-treatment levels at 2 h ($n = 3$). [**(F)**, 1] The activation of STAT and SOCS in MDSCs at different time points after adding exogenous ADAM10 were detected. We found that the levels of p-STAT1 and p-STAT3 proteins increased after ADAM10 treatment in MDSCs. A slight increase in SOCS1 and decrease in SOCS3 protein were also detected after adding ADAM10 in MDSCs. [**(F)**, 2] Quantification of immunoblot density was performed by normalizing the density of each band to STAT1, STAT3 or β-actin ($n = 3$). $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

and reverse MDSCs-mediated immune tolerance in breast cancer patients.

Interleukin-6 signals are transduced *via* the JAK/STAT signaling pathway in most cell types (44–46). Aberrant activation of the JAK/STAT signaling pathway in MDSCs has been reported in pancreatic cancer (15) and multiple myeloma (47). Physiologically, cytokine signal transduction can be switched off by SOCS proteins (48). Therefore, the activation of the JAK/STAT signaling pathway is rapid and reversible in normal cells. However, defects in SOCS expression frequently occur in malignant cells (16, 19), causing sustained phosphorylation of key proteins along the JAK/STAT signaling pathway (16, 17). In this study, we found that tumor-derived IL-6 triggers the differentiation and immunosuppressive activity of MDSCs. This was accompanied by sustained activation of the JAK/STAT signaling pathway, which led to phosphorylation of the STAT1, STAT3, JAK1, JAK2, and TYK2 proteins. Furthermore, the activation of the JAK/STAT signaling pathway in MDSCs was persistent, and lasted longer than that in normal myeloid controls. Accordingly, significant suppression of SOCS3 at both the RNA and protein levels was observed in MDSCs. Therefore, significant defects in the SOCS feedback loop may participate in the regulation of IL-6-dependent, sustained activation of the JAK/STAT signaling pathway in MDSCs.

The SOCS protein family consists of SOCS1–7 and CIS, which are divided into three subgroups: CIS and SOCS1–3, SOCS4/5, and SOCS6/7. CIS and SOCS1–3 are associated with the control of cytokine signaling, whereas the SOCS4–7 subgroup regulates the growth factor-induced receptor tyrosine kinase signaling (19). As reported previously, the expression of SOCS proteins is rapidly upregulated by IL-6, among which SOCS3 is the most important, and in turn, inhibits IL-6 cytokine signaling (48, 49). Numerous reports showed that SOCS3 defects are responsible for sustained IL-6/STAT3 signaling in human cancers (16, 50, 51). However, few studies have examined the expression of SOCS3 in immune cells. SOCS3 can also regulate the activation and differentiation of naïve $CD4^+$ T cells, preferentially by promoting Th2 and inhibiting Th1 differentiation (52). In addition, SOCS3 can regulate the activation of DCs and polarization of macrophages (53, 54). Regarding MDSCs, recent studies demonstrated that SOCS3 negatively regulates the development and function of MDSCs *via* inhibition of STAT3 activation in prostate cancer (25). SOCS3-deficient mice showed elevated $Gr\text{-}1^+CD11b^+$ MDSCs in tumors and exhibited heightened STAT3 activation (25). Consistent with the above results, we found that SOCS3 was significantly decreased in primary breast cancer MDSCs and induced MDSCs and was significantly correlated with sustained activation of the JAK/STAT signaling pathway and enhanced T cells immunosuppression in MDSCs. Furthermore, in a co-culture system *in vitro*, we demonstrated that suppressed expression of SOCS3 was initiated by IL-6. This explains the phenomenon observed in our previous study, which showed that cancer-derived IL-6-induced T cell suppression in primary MDSCs by activating STAT3-dependent,

nuclear factor-κB-mediated long-term IDO overexpression (7). Thus, SOCS3 defects may be the main cause of IL-6-induced persistent activation of the JAK/STAT signaling pathway and consequent enhanced differentiation and immunosuppressive activity of MDSCs.

Interestingly, in this study, we also found synchronous yet opposing changes in SOCS1 and SOCS3 expression at both the mRNA and protein levels. In contrast to SOCS3, SOCS1 expression was dramatically increased by IL-6-dependent sustained activation of the JAK/STAT signaling pathway in MDSCs. Both SOCS1 and SOCS3 have been demonstrated to inhibit phosphorylation of gp130, STATs, and JAK proteins along the JAK/STAT signaling pathway (48, 49). However, for IFN-α and IFN-γ secretion, SOCS1 is not as efficient as SOCS3 in inhibiting IL-6-dependent activation of the JAK/STAT signaling pathway (55). SOCS3 is associated with specific phosphotyrosine motifs within the activated IL-6 receptor gp130 (56–58), which directly inhibit the catalytic domains of JAK1, JAK2, and TYK2 (59). This may explain the relative specificity of SOCS3 in inhibiting IL-6 pathways. Therefore, the increase in SOCS1 may be a consequence of sustained IL-6 stimulation, which is consistent with the results of other studies (60, 61).

We further demonstrated that IL-6-induced inhibition of SOCS3 and activation of the JAK/STAT pathway was correlated with the elevated expression of CD126 *via* the IL-6 *trans*-signaling pathway. The IL-6 signaling complex assembly is composed of IL-6, CD126, and the shared signaling receptor gp130. CD126 exists in two forms, membrane-bound and soluble CD126. IL-6 signal transduction *via* membrane-bound CD126 is known as the *cis*-signaling pathway, while signal transduction *via* soluble CD126 is known as the *trans*-signaling pathway (8). The IL-6 *cis*-signaling pathway is mainly limited to hepatocytes, megakaryocytes, neutrophils, and certain T cell subsets (62). In contrast, the IL-6 *trans*-signaling pathway can potentially stimulate all types of cells that do not express membrane-bound IL-6R. During IL-6 *trans*-signaling, the soluble form of CD126 is generated either by alternative splicing or shedding of membrane-bound IL-6R, which is mediated by the metalloproteases ADAM10 and ADAM17 (29, 62, 63).

Previous studies of breast cancer indicated that MDSCs express ADAM-family proteases and IL-6Rα, which contribute to breast cancer cell invasiveness and distant metastasis through the IL-6 *trans*-signaling pathway in murine models (10). In our study, we compared the expression of IL-6R in MDSCs and found that both CD126 and gp130 were increased in MDSCs. However, while the soluble form of CD126 was increased, membrane-bound CD126 was decreased. Importantly, we reported that MDSCs express higher levels of ADAM10 as compared to that in $CD33^+$ controls. These results indicate that a higher level of soluble CD126 in MDSCs may be derived from enhanced shedding of membrane-bound IL-6R by ADAM10. To verify the effect of ADAM10, we added exogenous ADAM10 to increase the level of soluble CD126. This resulted in enhanced suppression of SOCS3 and phosphorylation of STAT1 and STAT3 in MDSCs. Although a previous study demonstrated that reduced expression of membrane-bound CD126 may result in impaired IL-6 classic signaling, followed by decreased phosphorylation of STAT3 and STAT1 (64), our results indicated that soluble CD126-mediated IL-6 *trans*-signaling pathway is sufficient for IL-6 signal transduction in MDSCs. Downstream effects include persistent activation of the JAK/STAT pathway and generation of more immunosuppressive MDSCs *via* suppression of SOCS3.

Taken together, this study provides insight into the cross-talk between breast cancer cells and regulatory immunocytes in local microenvironments. In breast cancer, tumor-derived IL-6 predominantly modulates the differentiation and immunosuppressive ability of MDSCs at both the tissue and cellular levels in which the soluble CD126-mediated IL-6 *trans*-signaling pathway and SOCS3 suppression are the most crucial molecular events orchestrating IL-6-dependent sustained activation of the JAK/STAT pathway in breast cancer MDSCs. Therefore, blocking the IL-6 signaling pathway is a promising therapeutic strategy for eliminating and inhibiting MDSCs, as well as reversing MDSCs-mediated immune escape in breast cancer.

ETHICS STATEMENT

This study was approved by the Medical Ethics Committee of Tianjin Medical University. All experiments were performed in accordance with the principles of the Declaration of Helsinki. Written consents were obtained from all patients and healthy donors.

AUTHOR CONTRIBUTIONS

MJ performed the research, data analyses, and wrote the manuscript. JC and RZ performed parts of the research and commented on manuscript. WZ, YY, PL, and WY performed parts of the research and data analyses. FW and XR contributed clinical information and samples for the study. JY designed the study and commented on manuscript.

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SUPPLEMENTARY MATERIAL

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Function of miR-146a-5p in Tumor Cells As a Regulatory Switch between Cell Death and Angiogenesis: Macrophage Therapy Revisited

*Elina Simanovich[1,2], Vera Brod[1], Maya M. Rahat[1] and Michal A. Rahat[1,2]**

[1] Immunotherapy Laboratory, Carmel Medical Center, Haifa, Israel, [2] The Ruth and Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

Tumors survive and progress by evading killing mechanisms of the immune system, and by generating a tumor microenvironment (TME) that reprograms macrophages *in situ* to produce factors that support tumor growth, angiogenesis, and metastasis. We have previously shown that by blocking the translation of the enzyme inducible nitric oxide synthase (iNOS), miR-146a-5p inhibits nitric oxide (NO) production in a mouse renal carcinoma cell line (RENCA), thereby endowing RENCA cells with resistance to macrophage-induced cell death. Here, we expand these findings to the mouse colon carcinoma CT26 cell line and demonstrate that neutralizing miR-146a-5p's activity by transfecting both RENCA and CT26 cells with its antagomir restored iNOS expression and NO production and enhanced susceptibility to macrophage-induced cell death (by 48 and 25%, respectively, $p < 0.001$). Moreover, miR-146a-5p suppression simultaneously inhibited the expression of the pro-angiogenic protein EMMPRIN (threefolds, $p < 0.001$), leading to reduced MMP-9 and vascular endothelial growth factor secretion (twofolds and threefolds, respectively, $p < 0.05$), and reduced angiogenesis, as estimated by *in vitro* tube formation and scratch assays. When we injected tumors with pro-inflammatory-stimulated RAW 264.7 macrophages together with i.v. injection of the miR-146a-5p antagomir, we found inhibited tumor growth (sixfolds, $p < 0.001$) and angiogenesis (twofolds, $p < 0.01$), and increased apoptosis (twofolds, $p < 0.01$). This combination therapy increased nitrites and reduced TGFβ concentrations in tumor lysates, alleviated immune suppression, and allowed enhanced infiltration of cytotoxic $CD8^+$ T cells. Thus, miR-146a-5p functions as a control switch between angiogenesis and cell death, and its neutralization can manipulate the crosstalk between tumor cells and macrophages and profoundly change the TME. This strategy can be therapeutically utilized in combination with the macrophage therapy approach to induce the immune system to successfully attack the tumor, and should be further explored as a new therapy for the treatment of cancer.

Keywords: miR-146a, antagomir, nitric oxide, EMMPRIN/CD147, tumor angiogenesis, tumor cell death, macrophage therapy, adoptive transfer

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***Correspondence:**
Michal A. Rahat
mrahat@netvision.net.il,
rahat_miki@clalit.org.il

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INTRODUCTION

By secreting a myriad of chemoattractants and growth factors, tumor cells actively recruit macrophages into the tumor mass and reprogram them *in situ* to produce elevated levels of growth factors, pro-angiogenic factors, and anti-inflammatory cytokines that collectively promote tumor growth and metastasis and mediate evasion of immune recognition (1–4).

One of the hallmarks of pro-inflammatory macrophages or M1-activated macrophages is the high expression of the enzyme inducible nitric oxide synthase (iNOS) that generates high amounts of the cytotoxic molecule nitric oxide (NO), as well as other cytotoxic molecules (e.g., TNFα) that serve as a killing mechanism (5). However, the infiltrating macrophages that encounter the tumor microenvironment (TME) lose this capability as they are rapidly skewed toward an activation mode approximating the M2-activation mode (6).

The role of NO production in the TME is very complex and depends on the relative concentrations generated by both macrophages and tumor cells. Tumor-associated macrophages and myeloid-derived suppressor cells, both of which are M2-like activated, secrete low levels of NO that are pro-angiogenic and immunosuppressive (7, 8). Tumor cells can also produce low amounts of NO (9), however, it has been demonstrated that in some types of tumors, tumor cells of higher grade and stage as well as metastatic cells tend to reduce or completely lose their iNOS expression in order to resist immune killing (10). We have recently demonstrated that in the mouse renal cell carcinoma cell line RENCA, a specific microRNA molecule—miR-146a-5p—mediates the translational inhibition of iNOS (11).

In many tumors, the expression of the potent pro-angiogenic factors vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) is upregulated by the protein extracellular matrix metalloproteinase inducer (EMMPRIN/CD147). EMMPRIN is a surface multifunctional protein, expressed on both tumor and stroma cells (12, 13), that can induce the expression of both VEGF and MMP-9 and enhance angiogenesis, probably through homophilic interactions (14, 15). EMMPRIN is also found secreted, and its overexpression in many types of tumors was correlated to enhanced levels of VEGF and MMP-9 and to increased invasiveness (16, 17). We have recently demonstrated, in the human renal and breast tumor cells lines A498 and MCF7, that neutralization of miR-146a-5p reduces the expression of EMMPRIN in these cells (17).

The cytotoxic capacity of macrophages and their ability to home to sites of inflammation, including cancerous lesions, rendered these cells a favorable target for therapy. However, once recruited into the tumor, the immunosuppressive TME polarizes and activates those cells to promote tumor growth. One of the therapeutic strategies used was to activate autologous immune cells *ex vivo* with IFNγ or combination of LPS and IFNγ, and then reinfuse then back into the patient. Such clinical trials were well-tolerated and showed feasibility, safety, and minimal adverse effects of the treatment (18–20). However, they also demonstrated a limited anti-tumoral activity, suggesting that the activation was not sufficient to overcome the immunosuppressive TME (21). As part of the TME, the ability of hypoxia, which is a dominant characteristic of solid tumors, to shift M1-activated macrophages to M2-like activated macrophages, and in particular to inhibit iNOS activity, certainly contributes to this failure (6, 10, 11). Thus, the macrophage therapy approach has been abandoned, until a way was found to overcome the influence of the immunosuppressive TME.

MicroRNA are small non-coding RNA strands that regulate gene expression, and their aberrant expression play a crucial role in cancerous diseases. Therefore, several therapeutic approaches designed to regulate their expression were developed, including antisense oligonucleotides (antagomirs). The RNA backbone of these antagomirs is often chemically modified [by replacing the oxygen in the phosphate group with sulfur, adding 2′-*O*-methyl group to non-bridging oxygen, connecting the 2′-oxygen to the 4′-carbon to lock the bridge-locked nucleic acids (LNA), or by adding a peptide], to increase their stability, specificity, and binding affinity [reviewed in Ref. (22, 23)]. Such modifications enabled the systemic intravenous administration of antagomirs in cancer, cardiovascular, and other preclinical disease models (24–26), which resulted in a specific reduction in the expression of the tested miRNAs and a marked effect on the expression of their target genes. This opened the door for microRNA-based therapy approaches, where specific miRNAs can be suppressed as needed.

Since we separately demonstrated the ability of miR-146a-5p to regulate the expression of two of the key mediators of angiogenesis and death, EMMPRIN and iNOS, we now ask whether miR-146a-5p can serve as regulatory switch between apoptosis and angiogenesis through its simultaneous and opposite effects on iNOS and EMMPRIN expression in the tumor cell. More importantly, we explore the possible use of miR-146a-5p neutralization as a possible new therapeutic approach for the inhibition of tumor growth in combination with the adoptive transfer of stimulated macrophages.

RESULTS

Pro-inflammatory Stimulation of RENCA and CT26 Cells Elevates the Expression of miR-146a-5p and the Transcription, but Not the Expression, of iNOS

The combination of IFNγ and LPS is the strongest known stimulation for mouse iNOS expression and NO production in many cell types, but not in all. Moreover, the effects of this combination on EMMPRIN expression have not been explored. We used the macrophage-like cell line RAW 264.7 as a positive control (**Figure 1**) and compared it to the three mouse tumor cell lines, the renal (RENCA), colon (CT26), and prostate (TRAMP-C2) carcinoma cell lines. We show here that the TRAMP-C2 cells responded to the combined stimulation by increasing their iNOS mRNA and protein expression (22-folds, $p < 0.01$), as well as their NO production (17-folds, $p < 0.001$). By contrast, the CT26 colon tumor cells did not express the protein or produced nitrites (**Figures 1A,C,D**), similar to the RENCA cells (11), despite elevated iNOS mRNA levels (**Figure 1E**).

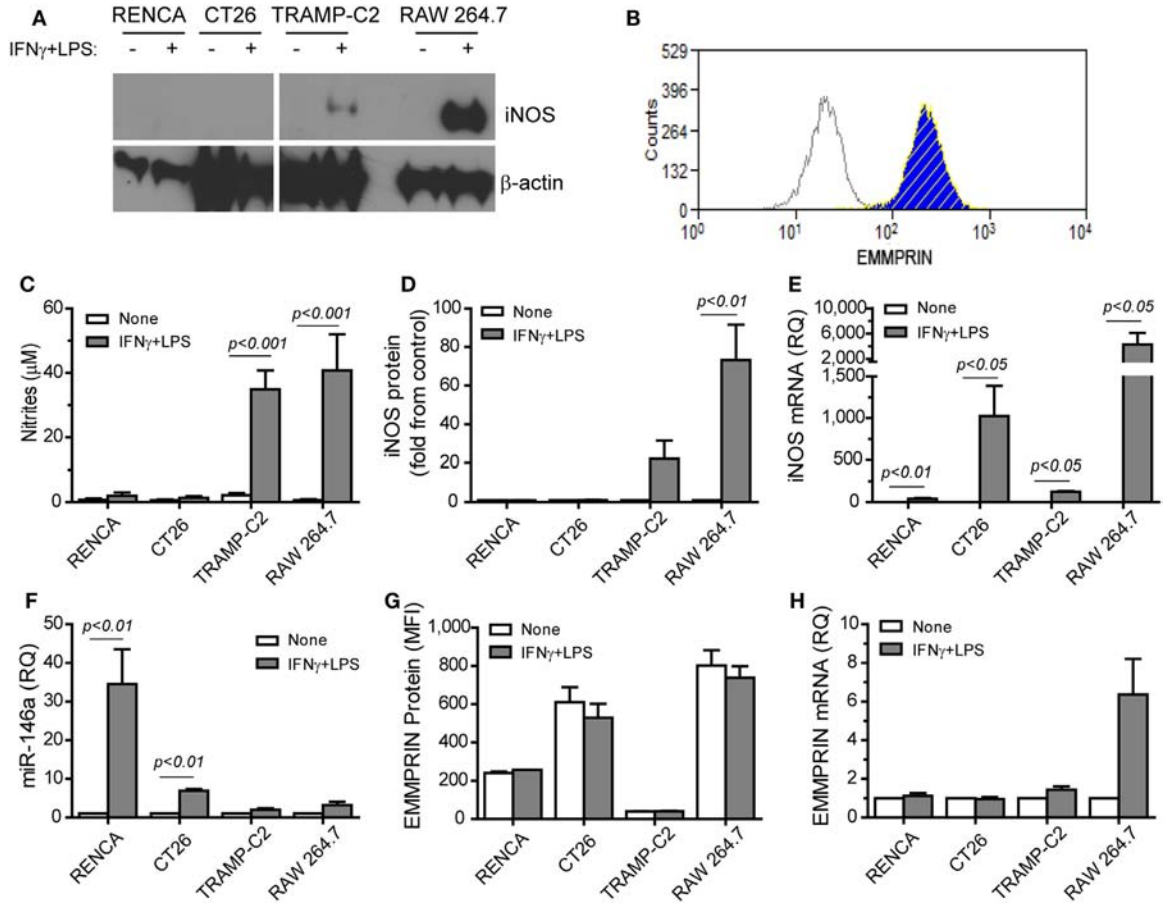

FIGURE 1 | Translational inhibition of inducible nitric oxide synthase (iNOS) is reversely correlated with miR-146a expression. RENCA, CT26, and TRAMP-C2 tumor cells lines (10^6 cells), and RAW 264.7 macrophage-like cells (10^6 cells) were incubated with or without the combined stimulation of IFNγ (100 U/ml) and LPS (1 μg/ml) for 24 h. **(A)** A representative western blot analysis for iNOS expression and **(B)** a representative histogram depicting EMMPRIN expression in RENCA cells (light gray, isotype control; blue, no stimulation; hatched yellow, with the combined stimulation). **(C)** Accumulation of nitrites, the stable product of nitric oxide (NO), reflecting inducible nitric oxide synthase (iNOS) activity; **(D)** densitometric analysis of western blots for iNOS protein expression; **(E)** iNOS mRNA accumulation; **(F)** accumulation of miR-146a-5p expression; **(G)** mean fluorescence of EMMPRIN protein expression; **(H)** accumulation of EMMPRIN mRNA (n = 5–6 in each group).

Since iNOS mRNA was increased in all three cell types, but protein expression was not, we reasoned that a post-transcriptional regulation of iNOS exists in CT26 and RENCA cells, but not in TRAMP-C2 cells. Indeed, the combined stimulation increased the expression of miR-146a-5p only in the RENCA and CT26 cells (by 34- and 7-folds, $p < 0.01$, **Figure 1F**). We also observed that the combined stimulation did not change the accumulation of EMMPRIN mRNA or protein in the three tumor cell lines (**Figures 1B,G,H**). Thus, the expression of iNOS is inversely correlated with miR-146a-5p expression in the three tumor cells, and EMMPRIN expression does not correlate to the stimulation or to miR-146a-5p expression, probably as it is already maximally expressed.

Neutralization of miR-146a-5p by Its Antagomir Restores iNOS Expression and Reduces EMMPRIN Expression

To demonstrate that miR-146a suppresses iNOS expression in CT26 tumor cells, we neutralized its activity by transfecting the cells with its antagomir, as we have done before in RENCA cells (11). We used the mirVana™ anti-miR-146a-5p inhibitor, a potent, chemically modified single-stranded RNA molecule with a sequence complementary to that of miR-146a-5p (anti-miR-146a-5p). The combined stimulation markedly elevated iNOS mRNA in both cell lines when transfected by either the antagomir or its negative control ($p < 0.05$, **Figure 2B**). However, the negative control did not induce iNOS protein expression or NO production in both cell lines, even in the presence of the combined stimulation, as evident by immunofluorescence (**Figure 2C**, red staining, and the relevant parts of **Figure 2D**) and nitrite accumulation (**Figure 2A**). Likewise, transfection with the antagomir in the absence of the combined stimulation did not induce iNOS expression (**Figure 2A**). Only transfection with the antagomir in the presence of the combined stimulation restored iNOS protein induction and NO production (threefolds and eightfolds for RENCA and CT26, respectively, $p < 0.05$, **Figures 2A,C,D**). Thus, iNOS expression and NO production in tumor cells require a strong pro-inflammatory stimulation, together with neutralization of miR-146a-5p activity, in both RENCA and CT26 cells.

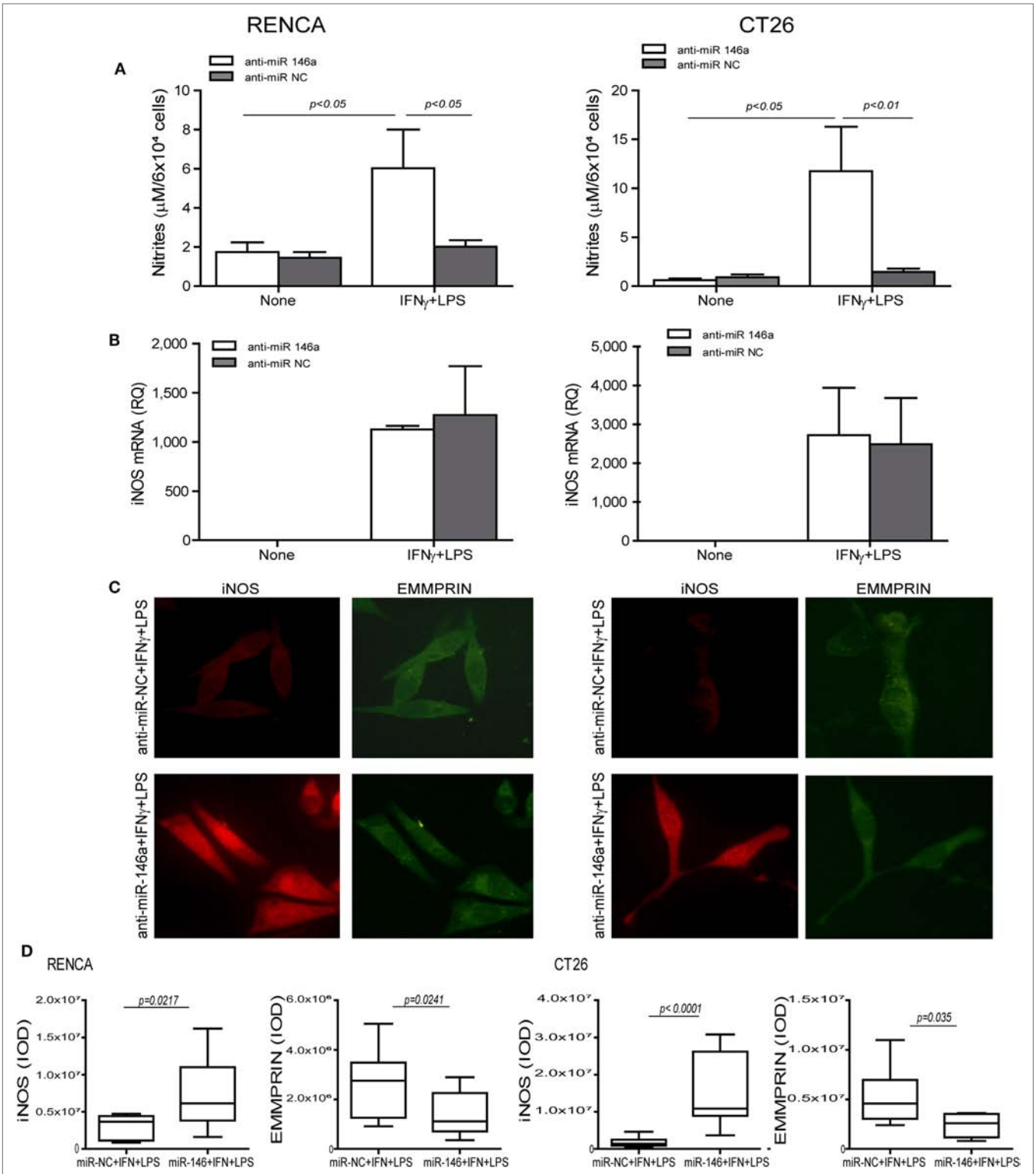

FIGURE 2 | Translational inhibition of inducible nitric oxide synthase (iNOS) in RENCA and CT26 cells is mediated by miR-146a-5p. RENCA or CT26 cells (5×10^4 cells) were transfected with anti-miR-146a-5p antagomir or with its negative control (anti-miR-NC) 24 h before they were stimulated with IFNγ (100 U/ml) and LPS (1 μg/ml) for 24 h. **(A)** Accumulation of nitrites and **(B)** accumulation of iNOS mRNA suggest a post-translational regulation. **(C)** Immunofluorescent staining **(D)** and their quantitation of cells transfected with the negative control (upper panel) or with the miR-146a-5p antagomir (lower panel) and stimulated with IFNγ and LPS, and were stained for iNOS (red staining) or EMMPRIN (green staining), magnification 200×. Only cells that were both transfected with anti-miR-146a-5p and incubated with the combined stimulation restored iNOS expression and accumulated nitrites, whereas the constitutive expression of EMMPRIN was reduced with the antagomir, regardless of the combined stimulation ($n = 4$ in all groups).

To explore the effects of the combined stimulation and miR-146a-5p on EMMPRIN expression in the same transfected cells, we stained for EMMPRIN as well. EMMPRIN was constitutively expressed in both RENCA and CT26 cells, and no change was visible upon incubation with the combined stimulation. However, transfection of the antagomir resulted in a decrease in the intensity of EMMPRIN staining compared to the cells transfected with the negative control (**Figure 2C**, green staining, and the relevant parts of **Figure 2D**). This effect was also quantified by evaluating the amounts of the secreted protein (3-fold decrease for both cell lines, $p < 0.05$, **Figure 3A**), and by assessing the membranal expression of the protein by flow cytometry (1.5- to 2-fold decrease, $p < 0.05$, **Figures 3B,C**). However, EMMPRIN mRNA was unaffected by the combined stimulation or the transfection of the antagomir (**Figure 3D**). Thus, EMMPRIN expression is also post-transcriptionally regulated in both tumor cell lines.

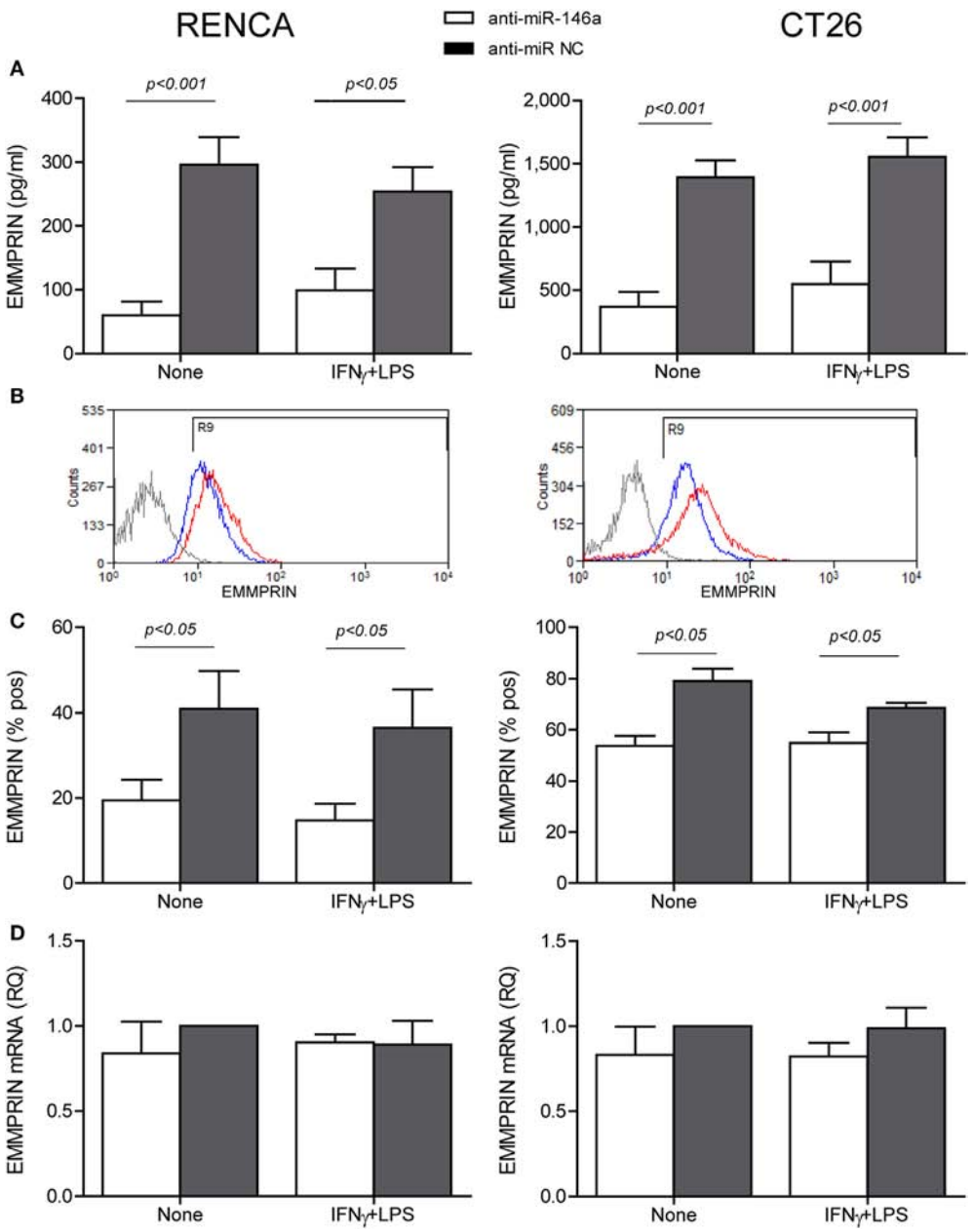

FIGURE 3 | Anti-miR-146a inhibits EMMPRIN expression in RENCA and CT26 cells. RENCA or CT26 cells (5×10^4 cells) were transfected and stimulated as described in **Figure 2**. **(A)** Accumulation of soluble EMMPRIN measured by ELISA; **(B)** representative histograms of surface EMMPRIN expression (gray line, isotype control; red line, cells transfected with the anti-miR-NC; blue line, cells transfected with anti-miR-146a-5p), and **(C)** percentage of positive cells expressing membranal EMMPRIN. **(D)** Accumulation of EMMPRIN mRNA detected by quantitative real-time PCR. The difference between the mRNA and protein expression levels suggests a post-translational regulation ($n = 4$–5 in each group).

In Vitro Neutralization of miR-146a-5p by Its Antagomir Leads to Enhanced Tumor Cell Death and Reduced Angiogenesis

Some tumor cells lose iNOS expression in order to escape immune-mediated death (10), and we have shown that despite the high levels of NO secreted by stimulated macrophages, they cannot kill RENCA cells that do not express iNOS, unless iNOS expression is restored by transfecting the cells with the miR-146a antagomir (11). When RENCA, CT26, or TRAMP-C2 cells were cocultured with the RAW 264.7 macrophages in the presence of the combined stimulation, only TRAMP-C2 cells exhibited increased death [48 ± 13% increase (**Figure 4A**), $p < 0.001$], despite the high NO levels accumulated in all cocultures

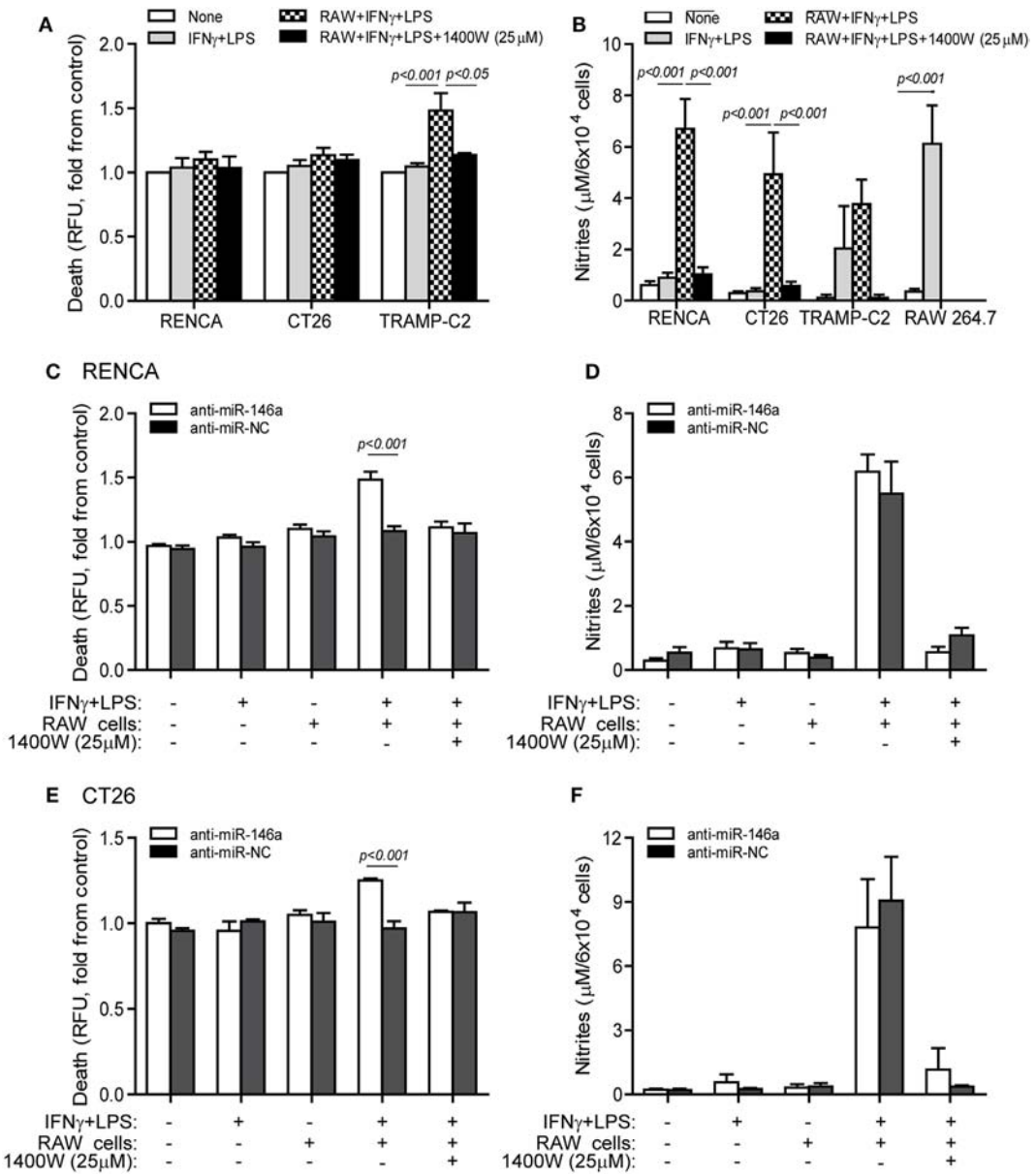

FIGURE 4 | Cytotoxic activity of macrophages depends on endogenous nitric oxide (NO) production in the tumor cells. **(A,B)** RENCA, CT26, or TRAMP-C2 cells (5×10^4 cells) were labeled with Cell Tracker Orange, and then cocultured for 24 h with unlabeled RAW 264.7 cells at a 2:1 ratio, with or without IFNγ (100 U/ml) and LPS (1 μg/ml), and with or without the addition of the 1400W inducible nitric oxide synthase (iNOS) inhibitor (25 μM). **(A)** Supernatants were collected and fluorescence was determined as a measure for tumor cell death, and calculated as fold change compared to non-stimulated cells. **(B)** Nitrite accumulation was measured in the supernatants. **(C–F)** RENCA and CT26 cells were labeled as before, and transfected with either anti-miR-146a-5p or its negative control (anti-miR-NC) 24 h before exposure to the combined stimulation and RAW 264.7 cells. Fluorescence was determined and reflected **(C)** RENCA cell death, and **(E)** CT26 cell death. Nitrite accumulation in the supernatants of **(D)** RENCA cells and **(F)** CT26 cells. TRAMP-C2 cells that produced endogenous NO upon stimulation exhibited increased death when cocultured with the macrophages, whereas RENCA and CT26 cells did not die despite the high NO accumulation, unless they were first transfected with anti-miR-146a-5p ($n = 6$ in all groups).

(**Figure 4B**). Cell death was abrogated by the addition of the selective iNOS inhibitor 1,400W ($p < 0.05$), suggesting that it was NO dependent.

To show that the induction of death by pro-inflammatory macrophages depends on the activity of miR-146a in the tumor cells, we next cocultured RAW 264.7 macrophages with RENCA (**Figure 4C**) or CT26 (**Figure 4E**) cells transfected with anti-miR-146a, and assessed tumor cell death relative to cells transfected with the negative control. RENCA cell death was increased by 48 ± 6% ($p < 0.001$) and CT26 cell death was increased 25 ± 1.3% ($p < 0.001$), only when anti-miR-146a-5p was introduced and when the combined stimulation was present. Again, this was abolished by the (1400W) iNOS inhibitor, demonstrating an NO-dependent effect. Despite the difference in cell death, we did not detect a difference in nitrite accumulation between the anti-miR-146a-5p and anti-miR-NC transfected cells, suggesting that the macrophages contributed the bulk of nitrites (**Figures 4D,F**).

Changes in the angiogenic activity of EMMPRIN were detected by the concentrations of its induced pro-angiogenic factors VEGF and MMP-9 in the supernatants of transfected cells. In comparison to cells transfected with the negative control, marked reduction in MMP-9 levels (about 2-folds, $p < 0.05$, **Figure 5A**) and VEGF levels (about 34-folds, $p < 0.05$, **Figure 5B**) were observed after transfection of anti-miR-146a-5p, regardless of the presence of the combined stimulation. Likewise, relative to cells transfected with the negative control, the supernatants from cells transfected with the antagomir caused a 40% reduction ($p < 0.05$) in the number of closed lumens (**Figure 6A**), and a 20–30% inhibition ($p < 0.05$) in endothelial cell proliferation and migration in the wound assay (**Figure 6B**). In both assays, the combined stimulation had no additional effects.

In Vivo Neutralization of miR-146a-5p by Its Antagomir Reduces Tumor Growth and Angiogenesis and Increases Apoptosis

To examine if the miR-146a-5p can be *in vivo* manipulated to reduce tumor size, we next subcutaneously implanted RENCA tumor cells in the syngeneic wild-type BALB/c mice. When tumors became palpable, we injected either the antagomir or its negative control to their circulation, with or without the simultaneous injection of RAW 264.7 cells that were previously *in vitro* stimulated with IFNγ and LPS for 24 h, to the rims of the tumors, where they would be least exposed to the hypoxic microenvironment. This was repeated three times every 7 days. Injection of the antagomir's negative control (anti-miR-NC) with or without stimulated RAW264.7 cells did not affect tumor growth rate, and at the end of the experiment, the average tumor size was 1.54 ± 0.3 cm^3 (**Figure 7A**). Injection of the antagomir

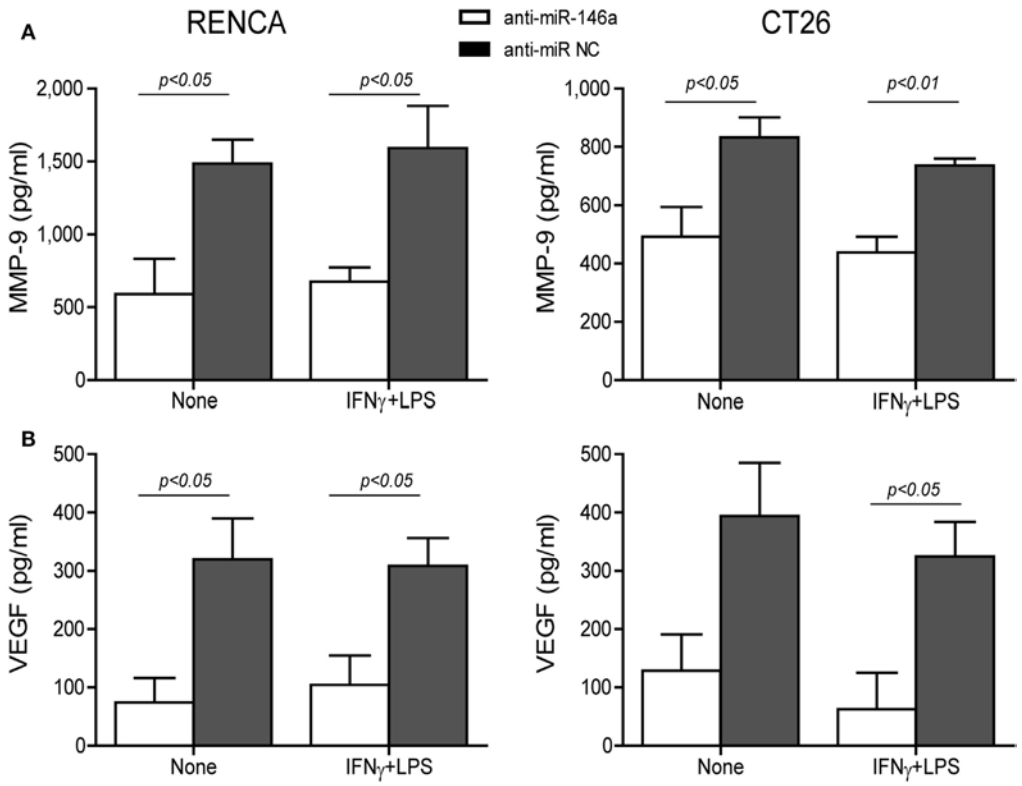

FIGURE 5 | Neutralization of miR-146a-5p by its antagomir reduces matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) concentrations in the supernatants. RENCA or CT26 cells (5×10^4 cells) were transfected with anti-miR-146a-5p or with anti-miR-NC 24 h before they were stimulated with IFNγ (100 U/ml) and LPS (1 μg/ml). **(A)** Accumulation of MMP-9 and **(B)** VEGF in the supernatants was measured by ELISA (n = 4–5 in each group). The antagomir reduced MMP-9 and VEGF levels regardless of the combined stimulation.

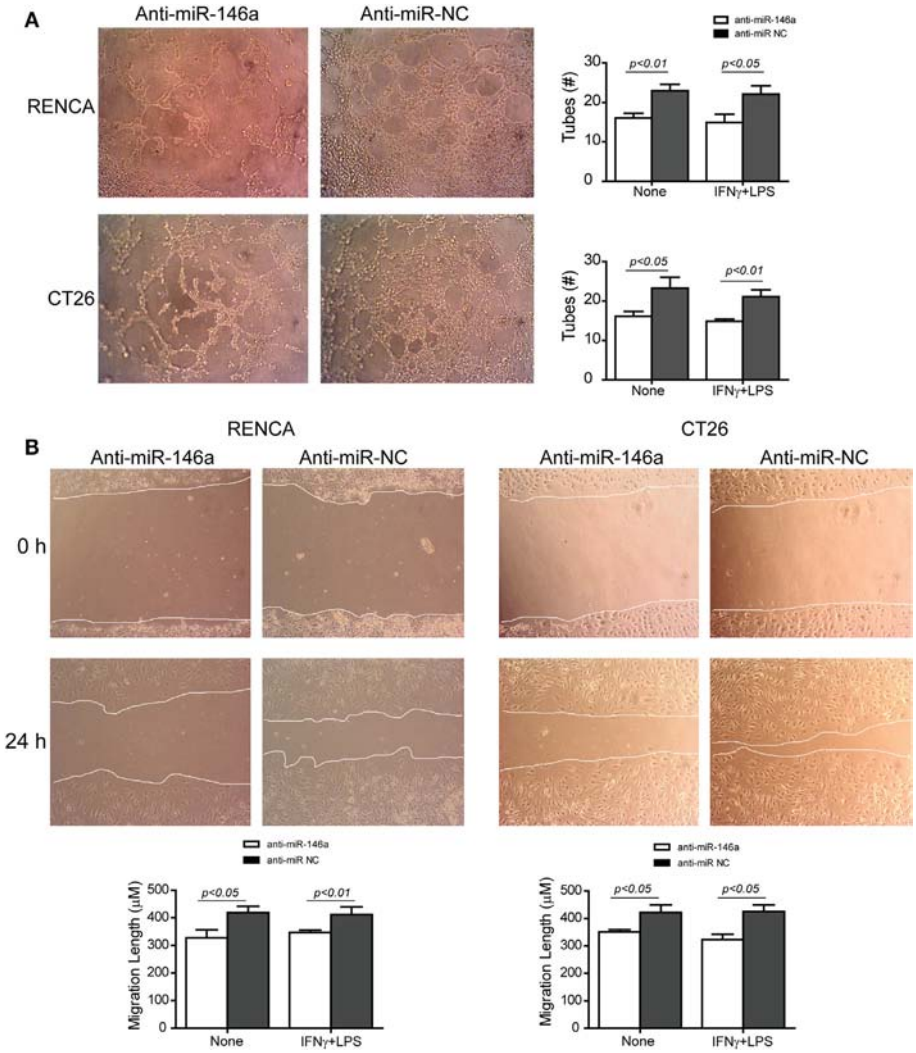

FIGURE 6 | Neutralization of miR-146a-5p with its antagomir reduces angiogenesis. **(A)** Supernatants from single cultures of tumor cells obtained from the previous experiments (described in **Figures 1**–**3**) were diluted 1:2 in full medium and incubated with the mouse bEND3 endothelial cells (8×10^4 cells) that were seeded on wells coated with Coultrex®. Images of the cells were taken after 6 h and the number of tubes with closed lumens was counted. **(B)** Confluent bEND3 endothelial cells were scratched and washed, and images were obtained at the beginning of the experiment (time 0 h) and 24 h later (magnification 20×). The length of endothelial cell migration was measured (n = 5–6 in each group).

alone resulted in a 1.4-folds reduction of tumor size ($p < 0.05$), whereas the combination of the antagomir and the stimulated macrophages resulted in a considerable slowing of the growth rate and about 6-folds reduction in tumor size ($p < 0.001$).

In mice injected with anti-miR-NC negative control, iNOS expression was not detected in the tumor cells, but macrophages that infiltrated the tumor after being injected to its rims expressed it in high levels, as evident by the intense staining (**Figure 7D**, low left panel). By contrast, iNOS expression was induced in RENCA tumor cells after anti-miR-146a-5p was injected i.v. (10-folds induction, $p < 0.05$, **Figure 7D**, right panels, **Figure 7E**).

EMMPRIN expression exhibited an inverse pattern to iNOS expression. Constitutive high expression levels of EMMPRIN were observed in the negative control group, and these were markedly reduced when anti-miR-146a-5p was injected, regardless of the injection of stimulated RAW 264.7 cells (about twofolds, $p < 0.01$, **Figures 7B,C**).

The effects of the treatment on angiogenesis were first estimated by the change in the mean vessel density (MVD) by staining for the endothelial marker CD31 (**Figure 8A**). Blood vessels in the negative control group injected with the anti-miR-NC were long, branched, and continuous (**Figure 8A**, top left panel), whereas in the group injected with both the anti-miR-146a-5p and stimulated macrophages, blood vessels were short, discontinuous, and with wider gaps between them (**Figure 8A**, bottom right panel). The vessel surface area, a measure of MVD, was gradually reduced (**Figure 8B**), culminating in a twofold decrease relative to the group receiving both anti-miR-146a-5p and stimulated macrophages ($p < 0.001$). A reduction in the

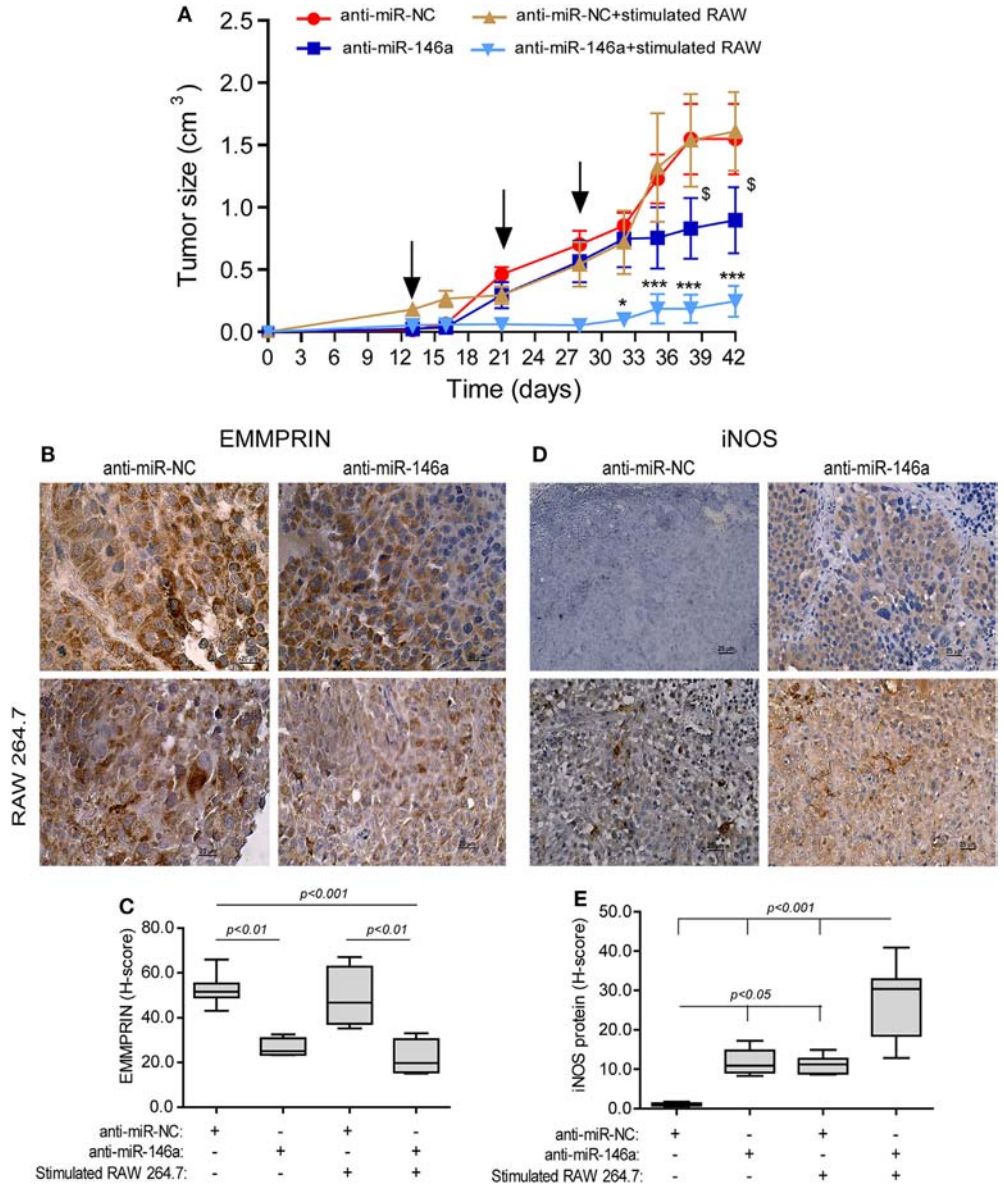

FIGURE 7 | Neutralization of miR-146a-5p, together with pro-inflammatory stimulated macrophages, inhibits tumor growth. **(A)** RENCA cells (2×10^6 cells) were injected to the flank of BALB/c mice. After tumors became palpable, mice were i.v. injected every 7 days (black arrows), with either anti-miR-146a-5p or its negative control anti-miR-NC (0.025 mg/g BW each), alone or together with injections of RAW 264.7 (10^6 cells) that were stimulated *in vitro* with IFNγ (100 U/ml) and LPS (1 μg/ml) for 24 h prior to injection. $^*p < 0.05$, $^{***}p < 0.001$ relative to the control group, $, $p < 0.05$ relative to the anti-miR-146a-5p group. Representative images of tissue sections immunohistochemically stained for **(B)** EMMPRIN protein expression and **(C)** its evaluation by the h-score, and **(D)** inducible nitric oxide synthase (iNOS) protein expression and **(E)** its evaluation by the *H*-score ($n = 6$ in the miR-NC+stimulated RAW 264.7 group, and $n = 5$ in each of the other groups, in two biological replicates).

levels of the pro-angiogenic factors VEGF (by 6.7-folds, $p < 0.01$, **Figure 8C**) and MMP-9 (by 5-folds, $p < 0.05$, **Figure 8D**) was observed in the tumor lysates between the groups receiving the anti-miR-NC and the group receiving anti-miR-146a-5p and stimulated macrophages.

The treatment with the antagomir and the stimulated macrophages reduced tumor cell proliferation (by twofolds, $p < 0.05$, **Figures 9A,B**) relative to the group receiving the anti-miR-NC alone, as assessed by the Ki-67 index. Complementarily, the rate of apoptosis was increased in this group, as evaluated by the TUNEL assay (2-folds, $p < 0.01$, **Figures 9C,D**) and the levels of activated caspase-3 (13-folds, $p < 0.01$, **Figure 9E**), relative to the negative control group.

Lastly, to detect immune-related changes in the TME, we measured the infiltration of $CD8^+$ T cells. In mice receiving anti-miR-NC alone $CD8^+$ T cells were few and mostly limited to the rims of the tumors (**Figure 10A**, top left panel). By contrast, in the group receiving both the antagomir and the stimulated

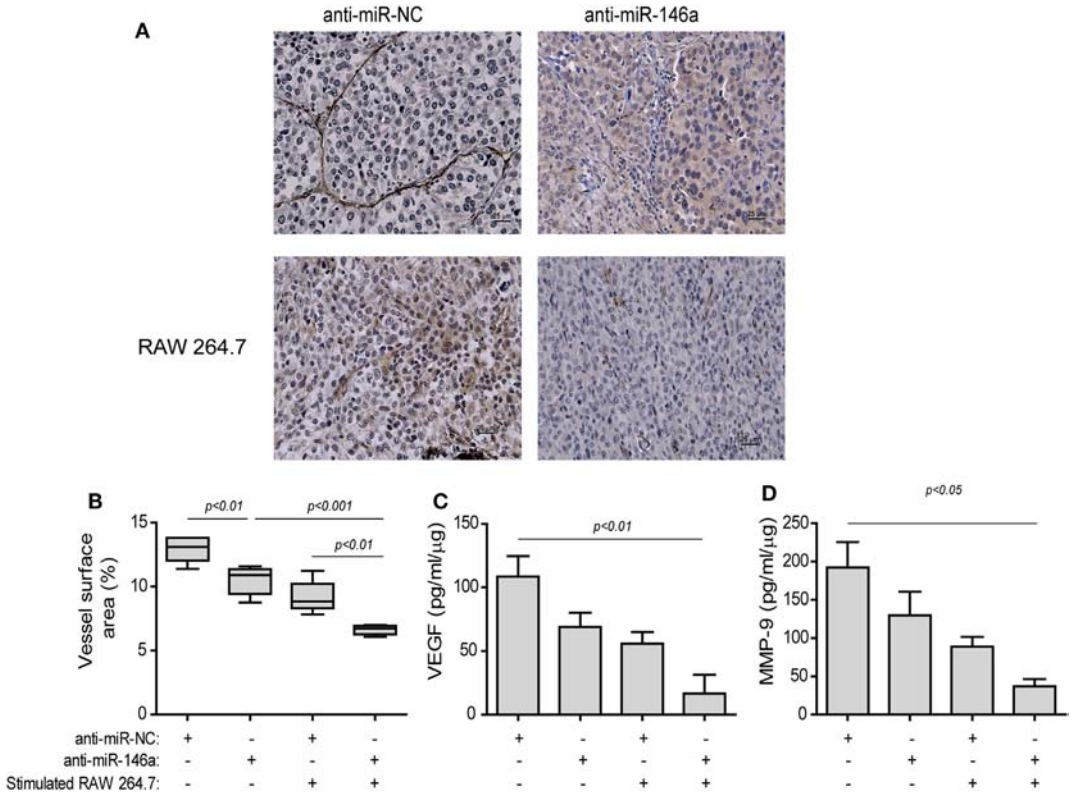

FIGURE 8 | Stimulated macrophages and miR-146a-5p neutralization reduce angiogenesis. Tumors were harvested after 42 days, paraffin-embedded, sectioned, and stained for the expression of the endothelial cell marker CD31. **(A)** Representative images (scale bar = 25 μm) and **(B)** the estimation of vessel surface area. Tumor sections were lysed and concentrations of **(C)** matrix metalloproteinase-9 (MMP-9) and **(D)** vascular endothelial growth factor (VEGF) were determined by ELISA (n = 3–5 in each group).

macrophages many $CD8^+$ T cells infiltrated the tumor tissue, resulting in an increase in the positively stained area (by fivefolds, $p < 0.001$, **Figure 10A**, bottom right panel, **Figure 10B**). Since we injected stimulated macrophages into the rims of the tumor, we saw no point in staining for their presence. However, nitrite concentrations, reflecting the macrophage mode of activation, were measured in the tumor lysates and showed an increase (3.7-folds, $p < 0.05$, **Figure 10C**) in the group receiving both the antagomir and stimulated macrophages, although the absolute levels were low. The same group also showed reduced levels of TGFβ, a dominant M2-related cytokine, relative to the other groups [by 5.7-folds (**Figure 10D**), $p < 0.05$]. Hence, we believe that these changes indicate immune modulation and the alleviation of immune suppression.

DISCUSSION

In this study, we show that miR-146a-5p simultaneously and oppositely regulates the tumor cell expression of two key mediators of the inflammatory response in cancer: iNOS, which can potentially mediate tumor cell death, and EMMPRIN, which can enhance survival and angiogenesis though induction of VEGF and MMP-9. Thus, miR-146a-5p works as a regulatory switch between death and survival of tumor cells.

Here, we expand our previous findings in the mouse renal cell carcinoma RENCA (11) to the mouse colon cell carcinoma CT26, and show that these two tumor cells can escape macrophage-induced cell death if their iNOS protein expression is completely lost. Reduced iNOS expression in tumor cells has been associated with their ability to resist immune killing (10). We show that this ability is achieved by the post-translational inhibition exerted by high levels of miR-146a-5p. We show that the presence of M1-activated macrophages that produce high levels of NO is necessary for tumor cell death, but if the tumor cell does not endogenously produce NO, even in minute amounts, it remains resistant to the cytotoxic effects of NO produced by the macrophages. Although NO is a gaseous molecule that can easily transverse membranes, there is a distinction between its exogenous high production by the macrophages and the limited endogenous production by the tumor cells, which is critical in the determination of tumor cell survival or death. However, the precise mechanism that distinguishes between NO produced endogenously and exogenously is still unclear and merits further investigation.

Of note, NO has been shown to sensitize refractory tumors to radio- and chemotherapy (27–29), but increase their resistance to photodynamic therapy (30). However, the actual biological effect depends greatly on the concentrations of NO, the measure

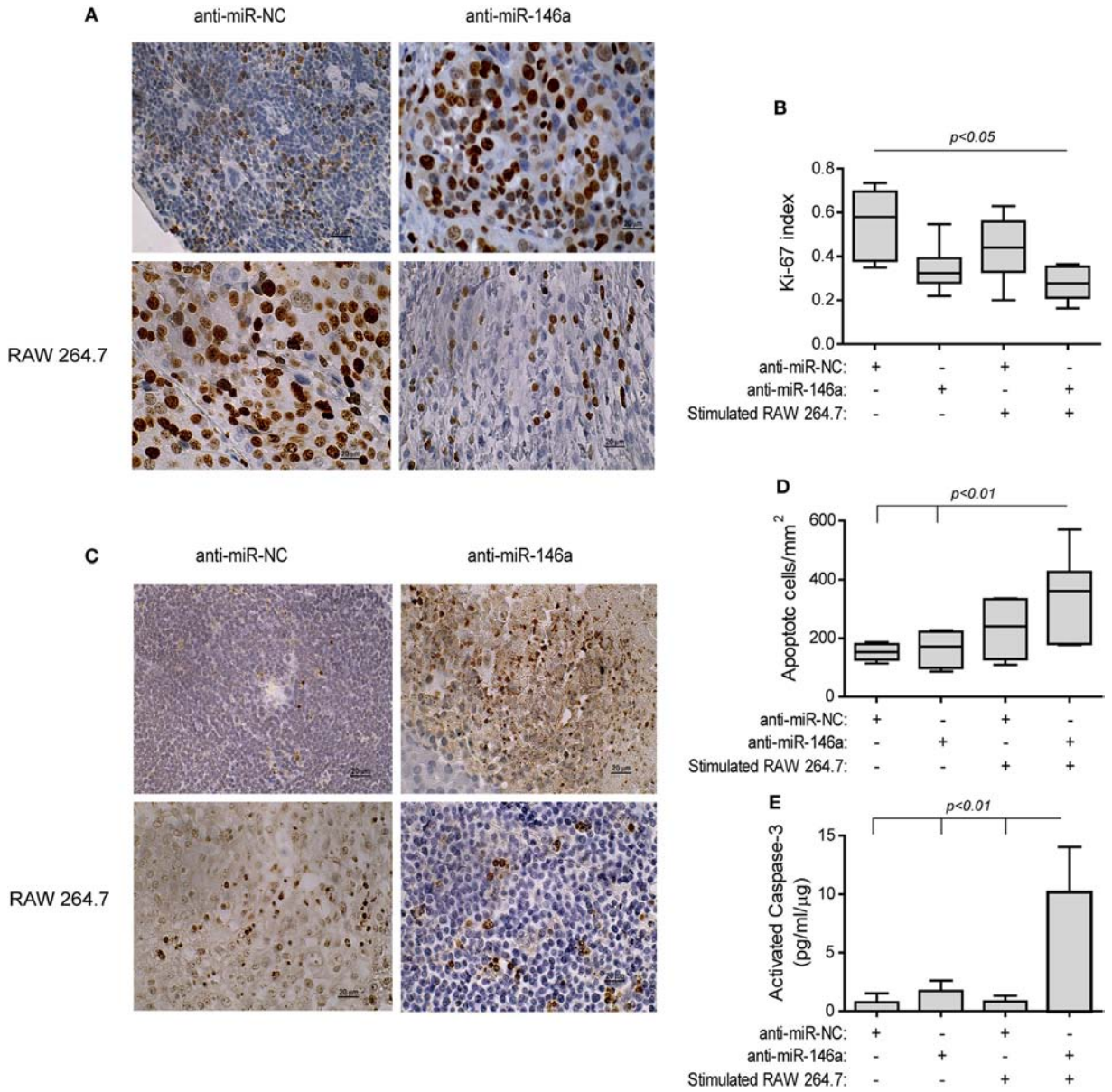

FIGURE 9 | Stimulated macrophages and miR-146a-5p neutralization increases tumor cell apoptosis. Tumors were harvested, paraffin-embedded, sectioned, and stained for Ki-67 or DNA strand breaks (TUNEL assay). **(A)** Representative images of Ki-67 staining (scale bar = 20 μm) and **(B)** their quantitation. **(C)** Representative images of TUNEL assay and **(D)** their quantitation. **(E)** Tumor sections were lysed and concentrations of cleaved, activated caspase-3 were determined (n = 3–5 in each group).

of hypoxia in the local site, and the cell type producing it. Our results, demonstrating sensitization of the RENCA and CT26 cells to macrophage-induced cell death only after restoration of iNOS and NO production, suggest that endogenous tumor NO production may activate pro-apoptotic pathways. Thus, in tumors that lost their iNOS and NO production, antagomir therapy may restore this production and serve to sensitize tumors to other treatment modalities, such as radio- or chemotherapies.

Simultaneously, high levels of miR-146a-5p also raise EMMPRIN expression in the same tumor cell, thus inducing angiogenesis by enhancing VEGF and MMP-9 secretion and by directly affecting endothelial cells, as observed in the *in vitro* tube formation and wound assays and suggested before (31). However, since we did not observe any presence of miR-146a-5p in the supernatants (data not shown), we negate the possibility that tumor cells export miR-146a-5p as a means to reprogram the neighboring macrophage. Thus, the effects of miR-146a-5p are limited to the tumor cells.

As miR-146a-5p emerged as a regulatory switch of tumor cell behavior, we next examined the potential therapeutic effects of neutralizing it by using a miR-146a-5p antagomir as a means to modulate tumor behavior and its microenvironment. First,

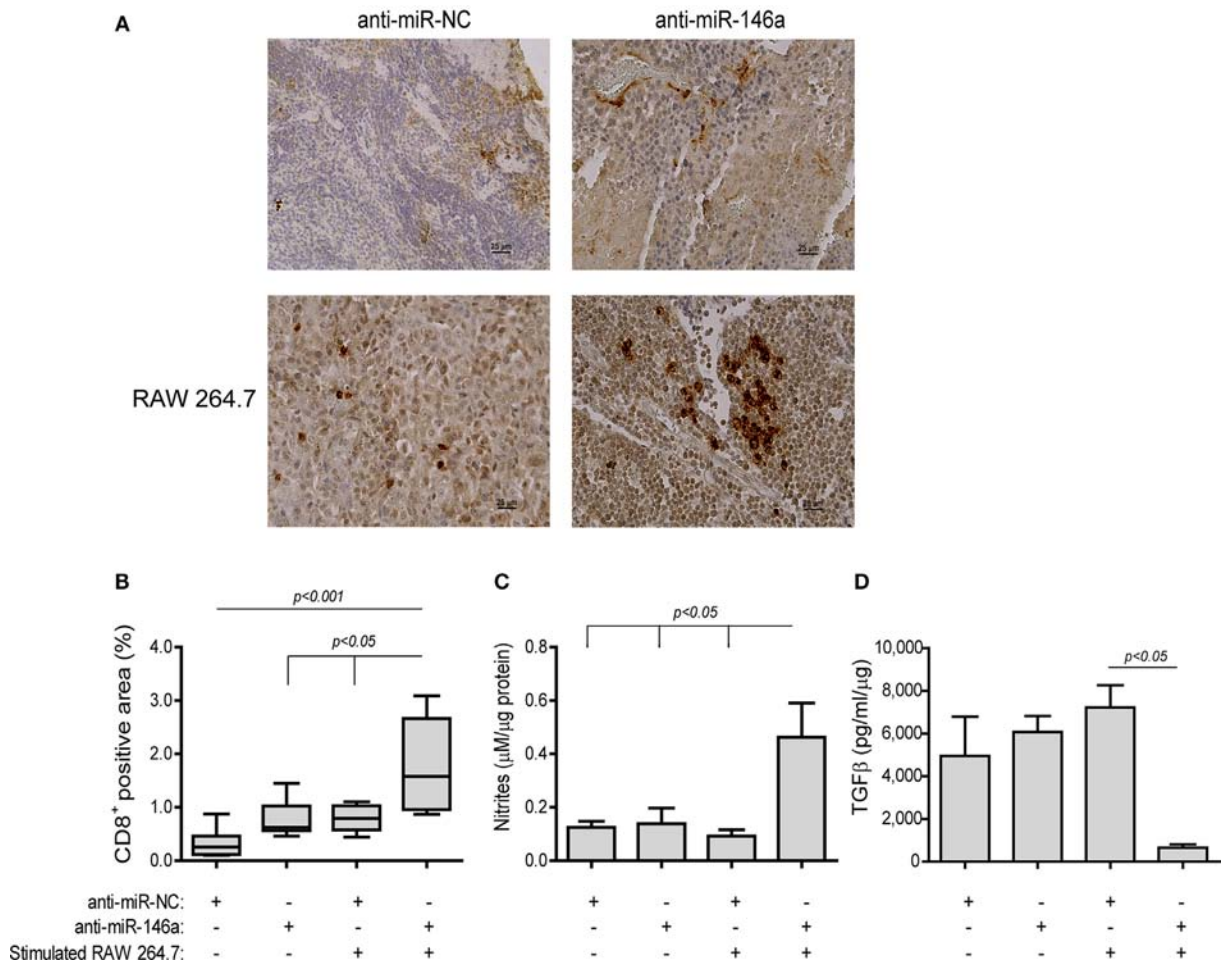

FIGURE 10 | Stimulated macrophages and miR-146a-5p neutralization immune-modulate the tumor microenvironment. Tumors were harvested, paraffin-embedded, sectioned, and stained for the expression of CD8. **(A)** Representative images (scale bar = 25 μm) and **(B)** their quantitation. Tumor sections were lysed and concentrations of **(C)** nitrites and **(D)** TGFβ were determined (n = 3–5 in each group).

we chose to inject anti-miR-146a-5p directly to the tail vein, as the chemical modification introduced to the mirVana antagomir increases its stability, and the tumor leaky vasculature enables its diffusion to the tumor cells [reviewed in Ref. (32–35)]. Next, we injected the stimulated macrophages into the rims of the tumor, as we have done before (11), to allow them to gradually exert their cytotoxic function, from the rims toward the tumor core, before they encounter the immunosuppressive effects of the hypoxic microenvironment.

Previously, the use of anti-miRNAs for therapy was hampered by several problems, especially the degradation of the anti-miRNA molecules in the circulation and their poor delivery to target sites. However, introduction of chemical modifications, such as the LNA technique that bridges the 2′-oxygen and the 4′-carbon, and the addition of a 2′-*O*-methyl group, markedly stabilized these molecules (22). Furthermore, anti-miRNAs were conjugated to different nanoparticles to improve delivery, including neutral lipid emulsions (e.g., DOPC), polyethylenimine, polyethylene glycol, and bacterium-derived particles coated with antibodies for specific target sites, to name just a few (22). Preclinical experiments using several specific modified anti-miRNAs delivered with different nanoparticles have already shown reduction in tumor growth, reduced metastasis, cell viability, and angiogenesis, without accumulating damage to normal tissues, indicating low toxicity (22, 23). However, antagomir therapy can be successfully used even without such delivery methods, and we show here that directly injecting the modified antagomir intravenously still inhibited tumor growth, bypassing this question.

Side effects or adverse responses were not reported when the expression of miR-146a was targeted in mice for therapy of different conditions, whether administered locally or systemically (36–38). However, in one case miR-146a antagomir successfully ameliorated the clinical symptoms in a myasthenia gravis model, but caused functional defects in B cells, including reduced antibody production, reduced number of plasma and memory cells, and reduced class switching (39). However, we do not believe that such effects, which are at the core of the B-cell

driven autoimmune disease as myasthenia gravis, are relevant in our model, which rely mostly on the interaction between macrophages and tumor cells.

Since miR-146a is an inflammatory miRNA that regulates the NF-κB pathway among other influences, targeting it may be highly context dependent. In the *in vitro* experiments, we transfected only the tumor cells with the antagomir, causing a reduction of EMMPRIN expression and an increase in iNOS expression. However, when delivered systemically *in vivo,* both the tumor cells and the macrophages were exposed to the antagomir, and could potentially respond differently. In macrophages, the effects of the antagomir could potentially disrupt the negative regulation on the components of the NF-κB pathway TRAF6 and IRAK-1, which are verified targets of miR-146a-5p (40). Thus, the NF-κB pathway, which is needed for the induction of iNOS, should be enhanced, and the overall effects of iNOS expression should only increase. However, we could not conclusively discern whether the macrophages were in fact affected by the antagomir: first, because the levels of EMMPRIN expression were reduced in the presence of the antagomir to a level comparable to that of the negative control. Second, because the adoptively transferred macrophages were stimulated *ex vivo* with LPS, so their increased iNOS expression could be the result of either the combined stimulation or the effect of the antagomir.

We show that the combined treatment with anti-miR-146a-5p and the stimulated macrophages resulted in reduction of the anti-inflammatory cytokine TGFβ and concurrent increase in the infiltration of $CD8^+$ cytotoxic T cells into the tumors. In addition, nitrites were accumulated in tumors receiving the combined stimulation, suggesting a shift in macrophage activation. We have recently shown that TGFβ is the dominant cytokine in the RENCA TME (41, 42). Therefore, its reduced levels together with the increased macrophage production of NO, altered the TME, alleviated immune suppression and allowed $CD8^+$ T cells to infiltrate deep into the tumor and eradicate tumor cells. Signaling pathways leading to TGFβ activation are not yet fully understood, and although Smad4, which is part of the downstream TGFβ signaling pathway, has been identified as a direct target of miR-146a (43, 44), no regulatory loop has been established. Therefore, we could only speculate that either miR-146a indirectly affects TGFβ activation, or that the *ex vivo* stimulation of the macrophages that shifts them toward M1-activation, together with the administration of the antagomir, contributes, and gradually amplifies the reduction in TGFβ levels. Furthermore, we have not yet performed this experiment with implanted CT26 tumor cells, and due to their immunological status resulting from high expression of gp70, the product of the envelope protein of the murine leukemia virus retrovirus (45, 46), we cannot predict the outcome of such an experiment.

The concept of macrophage therapy was studied mostly in the 80s and 90s [reviewed in Ref. (47)]. The ability of macrophages to produce strong cytotoxic mediators, their ability to home directly into the core of tumors, and the easy protocols for their isolation from peripheral blood made them preferable instruments of therapy. However, all attempts to stimulate monocytes *ex vivo* with IFNγ or a combination of IFNγ and LPS, and then reinfuse them into the patient, failed. They did not produce beneficial effects in human patients, whereas in mice they exhibited a limited success to delay, but not regress, tumor growth (10). These disappointing results led researchers to abandon the concept. However, improved understanding of how the tumor-cell-driven immunosuppressive microenvironment shifts pro-inflammatory macrophages into a pro-angiogenic, M2-like mode of activation (6) may now enable us to alleviate immune suppression and allow macrophages and other immune cells to kill tumor cells, re-enabling this modified approach.

Our study demonstrates that inhibition of miR-146a-5p in combination with the adoptive transfer of stimulated macrophages can "turn off" angiogenesis and "turn on" tumor killing mechanisms such as iNOS, enabling the recruitment of additional activated immune cells that can now kill tumor cells. In other words, we can now re-visit macrophage therapy and improve it by manipulating miR-146a-5p levels. Naturally, such an approach should be further studied in different tumor models and eventually in clinical trials.

MATERIALS AND METHODS

Cells

The tumorigenic mouse renal (RENCA, ATCC CRL-2947) and colon (CT26, ATCC CRL-2638) carcinoma cell lines were cultured in RPMI-1640 medium, 10% fetal calf serum (FCS), 1% L-Glutamine and antibiotics, with addition of 100 mM HEPES buffer (pH 7.4) for the RENCA cells, or 1% sodium pyruvate for the CT26 cells. The mouse TRAMP-C2 prostate cancer cell line (ATCC CRL-2731), the macrophage-like RAW 264.7 cell line (ATCC TIB-71), and the endothelial bEND3 cells (ATCC CRL-2299) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, 1% L-glutamine and antibiotics, with addition of 5 μg/ml insulin and 10^{-8} mol/l methyltrienolone (R1881), the dihydrotestosteron analog (NLP005, Perkin-Elmer) for the TRAMP-C2 cells. All cell lines were used at passages 3–15 and regularly tested for morphological changes and presence of mycoplasma, RAW 264.7 cells were identified as macrophages by their ability to phagocytose zymosan particles, and tumor cells were tested as cells of epithelial origin by their expression of cytokeratin 18.

When indicated, cells were stimulated with IFNγ (100 U/ml, 485-MI-100, R&D systems, Minneapolis, MN, USA) and LPS (1 μg/ml, L-6529, *Escherichia coli* 055:B5, Sigma, St. Louis, MO, USA). To avoid immune stimulation or possible masking of signals by exogenous stimuli, cells were serum-starved before their exposure to the experimental conditions or their injection to mice.

Reverse Transfection and Inhibition of miR-146a-5p

Reverse transfection and inhibition of miR-146a-5p were performed exactly as before (11) for both RENCA and CT26 cells, only that the mirVana anti-miR-146a-5p inhibitor™ (4464084, Ambion, Austin, TX, USA) or its negative control (4464076, anti-miR-NC, Ambion), at 30 nmol/l each, were used instead of the first-generation inhibitors.

Quantitative Real-time PCR (qPCR) Analyses

Quantitative real-time PCR analyses were performed as described before (11). Total RNA was extracted from 10^6 RENCA or CT26 cells using the RNA extraction kit (17200, Norgen biotek, ON, Canada), and 500 ng of total RNA were transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (4368814, Applied Biosystems, Foster City, CA, USA). Expression of iNOS and EMMPRIN mRNAs and their reference gene PBGD, or miR-146a-5p and its reference gene U6 were determined by qPCR using TaqMan assay on demand kit with the StepOne system (Applied Biosystems) in triplicates according to the manufacturer's instructions.

Determination of Nitrites, Western Blots Analyses, and Cytotoxic Assays

Determination of nitrites, western blots analyses, and cytotoxic assays were performed as before (11). The optical density of the bands in western blots was quantified using ImageJ. For the cytotoxic assays, the iNOS inhibitor 1,400 W (25 μM, W4262, Sigma) was used.

Flow Cytometry

EMMPRIN expression was evaluated as before (17), using 1 μg of the FITC-conjugated anti-mouse CD147 or with its isotype control (123705, BioLegend, San Diego, CA, USA).

Immunofluorescence

RENCA or CT26 cells (6×10^4 cells) were transfected on cover slips with anti-miR-146a-5p as described above and fixed with cold methanol for 5 min at room temperature. Cells were permeabilized with 0.25% Triton-X 100 for 10 min, and incubated with blocking buffer (2% donkey normal serum, 0.1% Triton-X 100 in PBS) for 30 min at room temperature. Cells were stained with primary antibodies (rat anti-mouse EMMPRIN, MAB772, R&D systems, or rabbit anti-mouse iNOS, ab15323, Abcam, Cambridge, UK) diluted 1:250 in blocking buffer overnight at 4°C. Then secondary antibodies (Alexa 488-conjugated donkey anti-rat IgG, ab150153, Abcam, or Alexa 546-conjugated donkey anti-rabbit IgG, A10040, Thermo Fisher, Rockford, IL, USA) were diluted 1:500 in blocking buffer in the dark for 1 h at room temperature. Coverslips were mounted on a slide with fluoromount G. Three washes with PBS were applied after each step. Images were acquired by upright fluorescent trinocular microscope (Olympus BX-60, Tokyo, Japan) using the MS60 camera and the MShot Image Analysis System V1 (MSHOT, Guangzhou Micro-shot Technology Co., Guangzhou, China).

ELISA

The mouse MMP-9, VEGF, TGFβ, and activated caspase-3 concentrations were determined as before (42). EMMPRIN concentrations were measured using with an ELISA kits (ab215405, Abcam) at a dilution of 1:200, according to the manufacturer's instructions.

In Vitro Wound Scratch Assay

In vitro wound scratch assay was performed as described before (17), with the mouse bEND3 endothelial cell monolayers (10^5 cells) seeded in 24-well dishes and incubated with experimental supernatants derived from RENCA or CT26 cells transfected with anti-miR-146a-5p or its negative control (diluted 1:2 with medium). Images of the field of injury were acquired at the beginning of the experiment and after 24 h. The average distances between the two sides of the wound were measured along the scratch (at least eight locations per field) in both time periods using the ImagePro plus 4.5 software (Media Cybernetics, Inc., Rockville, MD, USA), and the difference, which reflects the length to which the cells migrated, is presented.

In Vitro Tube Formation Assay

Coultrex® reduced growth factor basement membrane extract (40 μl/well, 3433, Trevigen, Gaithersburg, MD, USA) was used to coat 96-well plates at 4°C, and incubated at 37°C for 2 h to polymerize. bEND3 cells (8×10^4) were seeded in triplicates in DMEM with 2% FCS and experimental supernatants diluted 1:2 with medium. After 6 h, the number of closed lumens per microscopic field, representing tube-like structures, was counted in two separate fields.

Experimental Mouse Model

Experimental mouse model BALB/c mice (female, 8 weeks old, Envigo, Jerusalem, Israel) were kept with a 12 h light/dark cycle and access to food and water *ad libitum*. Tumors were generated as before (11), and when they became palpable at day 14, mice were randomly assigned to four groups that received the following treatments every 7 days: three i.v. injections of 0.025 mg/g body weight of (a) anti-miR-146a-5p or (b) anti-miR-negative control (anti-miR-NC) to the tail vein. Groups (c) and (d) were treated as groups (a) and (b), respectively, with the addition of 10^6 RAW 264.7 cells stimulated with IFNγ (100 U/ml) and LPS (1 μg/ml) for 24 h, injected to the rims of the tumors. Tumors were measured every 3–4 days and their volume calculated (length × width × 0.5 cm^3). At the end of the experiment, or when tumors were greater than 1.5 cm^3, mice were euthanized and their tumor tissues were harvested. Part of the tumor was freshly frozen for evaluation of nitrite and cytokine concentrations in tumor lysates, while other parts were formalin-fixed and paraffin-embedded for later analysis by immunohistochemical staining.

Immunohistochemistry and Immune Reactive Score

Immunohistochemistry and assigning an immune reactive score was performed as described in Ref. (42). Antigen retrieval for iNOS was performed by microwave heating in citrate buffer pH 6.0, and the antibody used was rabbit anti-iNOS (Abcam). All sections were viewed under the bright field trinocular microscope (Olympus BX-60, Tokyo, Japan) and images were acquired with the MS60 camera and the MShot Image Analysis System V1 (MSHOT, Guangzhou Micro-shot

Technology Co., Guangzhou, China). Vessel densities assessed by CD31 staining and by using a Weibel grid to calculate vessel surface area (48), and the fraction of Ki-67-positive tumor cells was calculated by the digital image analysis web application ImageJS (49). EMMPRIN and iNOS expression were assessed using the modified H-score, which assigns an immune reactive score on a continuous scale of 0–300, based on the percentage of positive cells expressing the protein at different intensities. Staining was divided into three categories: 1 for "light staining," 2 for "intermediate staining," and 3 for "strong staining." The percentage of positive cells was determined according to the positive surface area of cells measured with ImagePro plus 4.5 software, and the score was calculated using the formula: 1 × (%1 positive cells) + 2 × (%2 positive cells) + 3 × (%3 positive cells).

Statistical Analyses

All values are presented as means ± SE. Significance between two groups was determined using the two-tailed unpaired *t*-test. Differences between three or more experimental groups were analyzed using one-way analysis of variance (ANOVA) and the *post hoc* Bonferroni's multiple comparison tests, and the two-way ANOVA following Bonferroni's post-tests for comparing time and groups. *P* values exceeding 0.05 were not considered significant.

ETHICS STATEMENT

Mice were cared for in accordance with the procedures outlined in the NIH Guideline for the Care and Use of laboratory Animals, and all experiments were performed under the approved protocol (IL-121-12-11) issued by the Animal Care and Use Committee of the Technion-Israel Institute of Technology.

AUTHOR CONTRIBUTIONS

ES performed the experiments; VB was in charge of the immunohistochemical staining; MMR performed the *in vivo* experiments; and MAR designed the study, analyzed and interpreted the results, and wrote the manuscript.

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REVIEW
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Recent Advances in Targeting CD8 T-Cell Immunity for More Effective Cancer Immunotherapy

*Aurélie Durgeau[1,2†], Yasemin Virk[1†], Stéphanie Corgnac[1] and Fathia Mami-Chouaib[1]**

[1] *INSERM UMR 1186, Integrative Tumor Immunology and Genetic Oncology, Gustave Roussy, EPHE, Fac. de Médecine – Univ. Paris-Sud, Université Paris-Saclay, Villejuif, France,* [2] *ElyssaMed, Paris Biotech Santé, Paris, France*

Recent advances in cancer treatment have emerged from new immunotherapies targeting T-cell inhibitory receptors, including cytotoxic T-lymphocyte associated antigen (CTLA)-4 and programmed cell death (PD)-1. In this context, anti-CTLA-4 and anti-PD-1 monoclonal antibodies have demonstrated survival benefits in numerous cancers, including melanoma and non-small-cell lung carcinoma. PD-1-expressing $CD8^+$ T lymphocytes appear to play a major role in the response to these immune checkpoint inhibitors (ICI). Cytotoxic T lymphocytes (CTL) eliminate malignant cells through recognition by the T-cell receptor (TCR) of specific antigenic peptides presented on the surface of cancer cells by major histocompatibility complex class I/beta-2-microglobulin complexes, and through killing of target cells, mainly by releasing the content of secretory lysosomes containing perforin and granzyme B. T-cell adhesion molecules and, in particular, lymphocyte-function-associated antigen-1 and CD103 integrins, and their cognate ligands, respectively, intercellular adhesion molecule 1 and E-cadherin, on target cells, are involved in strengthening the interaction between CTL and tumor cells. Tumor-specific CTL have been isolated from tumor-infiltrating lymphocytes and peripheral blood lymphocytes (PBL) of patients with varied cancers. TCRβ-chain gene usage indicated that CTL identified *in vitro* selectively expanded *in vivo* at the tumor site compared to autologous PBL. Moreover, functional studies indicated that these CTL mediate human leukocyte antigen class I-restricted cytotoxic activity toward autologous tumor cells. Several of them recognize truly tumor-specific antigens encoded by mutated genes, also known as neoantigens, which likely play a key role in antitumor CD8 T-cell immunity. Accordingly, it has been shown that the presence of T lymphocytes directed toward tumor neoantigens is associated with patient response to immunotherapies, including ICI, adoptive cell transfer, and dendritic cell-based vaccines. These tumor-specific mutation-derived antigens open up new perspectives for development of effective second-generation therapeutic cancer vaccines.

Keywords: immunotherapy of cancer, cytotoxic T lymphocytes, tumor antigens, neoantigens, T-cell receptor repertoire

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James Lorens, University of Bergen, Norway

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Alessandro Poggi, Ospedale Policlinico San Martino, Italy
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****Correspondence:***
Fathia Mami-Chouaib
fathia.mami-chouaib@gustaveroussy.fr,
fathia.mami-chouaib@inserm.fr

†These authors have contributed equally to this work.

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Abbreviations: ACT, adoptive cell transfer; CDR, complementarity-determining region; CTL, cytotoxic T lymphocyte; CTLA, cytotoxic T-lymphocyte associated antigen; PD, programmed cell death; DC, dendritic cell; HLA, human leukocyte antigen; ICAM-1, intercellular adhesion molecule 1; ICI, immune checkpoint inhibitors; IFN, interferon; LFA-1, lymphocyte-function-associated antigen-1; mAb, monoclonal antibody, NSCLC, non-small-cell lung carcinoma; MHC-I/β2m, major histocompatibility complex class I/beta-2-microglobulin; TAA, tumor-associated antigen; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte; TSA, tumor-specific antigen.

INTRODUCTION

$CD8^+$ T lymphocytes play a central role in immunity to cancer through their capacity to kill malignant cells upon recognition by T-cell receptor (TCR) of specific antigenic peptides presented on the surface of target cells by human leukocyte antigen class I (HLA-I)/beta-2-microglobulin (β2m) complexes. TCR and associated signaling molecules thus become clustered at the center of the T cell/tumor cell contact area, resulting in formation of a so-called immune synapse (IS) (1) and initiation of a transduction cascade, leading to execution of cytotoxic T lymphocyte (CTL) effector functions. Major CTL activities are mediated either directly, through synaptic exocytosis of cytotoxic granules containing perforin and granzymes into the target, resulting in cancer cell destruction, or indirectly, through secretion of cytokines, including interferon (IFN)γ and tumor necrosis factor (TNF). Adhesion/costimulatory molecules, mainly lymphocyte-function-associated antigen-1 (LFA-1, CD11a/CD18 or α_L/β_2) and CD103 (α_E/β_7) integrins, on CTL play a critical role in TCR-mediated killing by interacting with their cognate ligands, intercellular adhesion molecule 1 (or CD54) and E-cadherin, respectively, and directing exocytosis of lytic granules to the cancer cell surface at the IS (2, 3). NKG2D, a c-type lectin molecule expressed on activated lymphocytes (4, 5), also plays an important role in the induction of T-cell-mediated cytoxicity and in CTL-dependent rejection of cancer (6, 7). NKG2D ligands include major histocompatibility complex class I-related chain (MIC)A and MICB (8), and UL16-binding proteins 1, 2, and 3 (9). These ligands are upregulated upon cell stress, such as tumor transformation, and are expressed by most of the cancer cells (10) in particular those of epithelial origin (11).

Activation of naive CD8 T cells by antigen-presenting cells (APC) involves binding of TCR, that is associated with the CD3 complex, to specific peptide-major histocompatibility complex class I (pMHC-I) complexes and the interaction of the costimulatory molecules CD28 and CD2 with their respective ligands CD80/CD86 and LFA-3 (12). Costimulatory receptors such as TNF receptor family member 4 (TNFRSF4 best known as OX40 or CD134) and member 9 (TNFRSF9 best known as 4-1BB or CD137) also play an important role in T-cell priming and antitumor immune responses (13–17).

ANTITUMOR T-CELL RESPONSES

Evidence for antitumor $CD8^+$ T-cell immunity was provided by isolation of tumor-specific CTL from peripheral blood or tumor tissue of patients with diverse cancers, such as melanoma and lung carcinoma (18–22). The existence of a tumor-specific CTL response was further strengthened by identification of tumor-associated antigens (TAA) and detection of TAA-specific $CD8^+$ T cells in spontaneously regressing tumors (18). Moreover, a correlation between tumor progression control and the infiltration rate of $CD8^+$ T lymphocytes in the tumor was established (23). Efficacy of the antitumor immune response is negatively influenced by a hostile tumor microenvironment. Establishment of an immunosuppressive state within the tumor is mediated by diverse immunosuppressive factors released by cancer cells themselves, such as vascular endothelial growth factor, transforming growth factor-β (TGF-β) and indoleamine 2,3-dioxygenase, and/or by recruiting regulatory immune cells with immunosuppressive functions, such as regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSC) (24). Indeed, a role for Treg cells in modulating tumor-specific effector T lymphocytes by producing immunosuppressive cytokines, such as IL-10 and TGF-β, consuming IL-2 or expressing the inhibitory molecule cytotoxic T-lymphocyte associated antigen (CTLA)-4, has been reported (25, 26). MDSC are a heterogeneous group of myeloid progenitor cells and immature myeloid cells, including immature macrophages, granulocytes, and dendritic cells (DC), that impair T-lymphocyte functions by upregulating the expression of immune suppressive factors, such as arginase and inducible nitric oxide synthase, increasing the production of nitric oxide (NO) and reactive oxygen species, and inducing Treg cells (27). Moreover, it has been shown that predominant secretion of TNF by $CD4^+$ T cells in MHC class II-expressing melanoma promotes a local immunosuppressive environment, impairing effector $CD8^+$ T-cell functions (28).

While it is generally admitted that $CD8^+$ T cells are directly involved in antitumor cytotoxic responses, the role of $CD4^+$ T cells is more controversial. Involvement of $CD4^+$ T cells in regulating antitumor immunity was associated with their help in priming of $CD8^+$ T cells, through activation of APC and an increase in antigen presentation by major histocompatibility complex class I (MHC-I) molecules *via* secretion of cytokines such as IFNγ (29, 30). More recently, it has been shown that $CD4^+$ T-cell help optimized CTL in expression of cytotoxic effector molecules, downregulation of inhibitory receptors, and increased migration capacities (31). A role for the $CD4^+$ T-cell subset in optimizing the antitumor immune response was supported by *in vivo* studies demonstrating that depletion of $CD4^+$ T lymphocytes promotes tumor progression, whereas their adoptive transfer was correlated with improved tumor regression (32). Moreover, it has been reported that $CD4^+$ T cells recognize most tumor-specific immunogenic mutanomes, and that vaccination with such $CD4^+$ immunogenic mutations confers antitumor activity and broadens CTL responses in mice (33). Frequent recognition of neoantigens by $CD4^+$ T cells was also observed in human melanoma (34). Notably, $CD4^+$ CTL able to kill specific tumor cells have been described in several cancer types, including non-small-cell lung carcinoma (NSCLC), cutaneous T-cell lymphoma, and melanoma (35–39); for review, see Ref. (32). Elsewhere, TAA-specific $CD4^+$ T-cell clones were shown to mediate HLA-II-restricted cytotoxic activity, making them attractive effectors in cancer immunotherapy (39, 40). While $CD4^+$ CTL are able to lyse target cells *via* the granule exocytosis pathway (35, 36, 41, 42), they mainly use FasL- and APO2L/TRAIL-mediated pathways to kill their target cells (35, 43).

TUMOR ANTIGENS RECOGNIZED BY T CELLS

Our fundamental knowledge of the tumor-specific T-cell response came with the discovery of tumor antigens that differentiated malignant cells from their non-transformed counterparts and

provided important input in the field of tumor immunology and cancer immunotherapy. The first human tumor antigen recognized by CTL was identified in melanoma and was designated melanoma-associated antigen (MAGE)-1 (44). Subsequently, several other antigens of the MAGE family were characterized, most of which were identified through generation of tumor cell lines and isolation of reactive autologous CTL clones. Based on their expression profile, tumor antigens were initially classified into two categories: TAA and tumor-specific antigens (TSA). TAA are relatively restricted to tumor cells, and, to a limited degree, to normal tissues, whereas TSA are expressed only in tumor cells, arising from mutations that result in novel abnormal protein production.

At present, numerous TAA have been identified in a large variety of human cancer types. They are heterogeneous in nature and were classified into at least four groups according to their expression repertoire and the source of the antigen: antigens encoded by cancer-germline genes, differentiation antigens, overexpressed antigens, and viral antigens (**Table 1**). Antigens encoded by cancer-germline genes are expressed in tumor cells and in cells from adult reproductive tissues, including placenta and testicular cells, and are thus designated cancer testis antigens. Differentiation antigens are expressed only in tumor cells and in the normal tissue of origin, while overexpressed antigens are derived from proteins that are overexpressed in tumors, but are expressed at much lower levels in normal tissues. Viral antigens derive from viral infection and are associated with several human cancers, including cervical carcinoma, hepatocarcinoma, nasopharyngeal carcinoma, and adult T-cell leukemia (45, 46).

The first mutant TSA, also termed neoantigens, were identified by the genetic method (46) *via* isolation of reactive $CD8^+$ and $CD4^+$ T-cell clones (**Table 2**). Recent accessibility to next-generation sequencing (NGS) technology and improvement in *in silico* epitope prediction have contributed to identification of patient-specific tumor antigens generated by somatic mutations in individual tumors (**Table 3**). Notably, most mutations identified in tumor-expressed genes do not generate neoantigens recognized by cognate T lymphocytes. Moreover, a large fraction of these mutations are not shared between patients and may thus be considered patient specific (47). These neoantigens have opened up new perspectives in cancer immunotherapy. They were shown to be involved in the success of immune checkpoint inhibitor (ICI) (48–50), adoptive cell transfer (ACT) immunotherapy (51, 52), and even virally induced epithelial cancer (53) and DC-based immunotherapy (54, 55); thus, they might be of use as predictive biomarkers of the response to immunotherapy.

TABLE 1 | Classification of tumor-associated antigens.

Type of antigens	Antigen characteristics	Example of human tumor antigens
Cancer-germline	Expressed only by tumor cells and adult reproductive tissues	MAGE, BAGE, GAGE, NY-ESO-1
Differentiation	Expressed by tumors and a limited range of normal tissues	Tyrosinase, Melan-A, gp100, CEA, MART-1
Overexpressed	Expressed by both normal and tumor cells, but much highly expressed in tumor cells	HER2, WT1, MUC1, ppCT
Viral	Expressed only by tumor cells as a result of viral infection	HPV, HBV, EBV, HTLV

PROCESSING OF CD8 T-CELL EPITOPES

Most antigenic peptides recognized by $CD8^+$ T cells originate from degradation of intracellular proteins by proteasomes and translocation to the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP)1/TAP2 heterodimeric complex. Once in the ER, peptides larger than 11 residues are further cleaved by ER amino-peptidase (ERAP)1 and ERAP2 before being loaded onto MHC-I molecules and presented on the surface of target cells for CD8 T-cell recognition [for review, see Ref. (87, 88)].

Defects in the antigen-processing machinery and, in particular, in TAP subunits, have been described as a major mechanism used by several tumors to escape from CD8 T-cell immunity (89). In this context, alternative peptide degradation pathways permitting CD8 T cells to overcome this tumor evasion mechanism have been identified. Indeed, proteasome/TAP-independent CTL epitopes, generated either by the cytosolic metallopeptidase insulin-degrading enzyme or cytosolic endopeptidases nardilysin and thimet oligopeptidase, have been described (90, 91). Moreover, TAP-independent processing of antigenic peptides can be achieved by the so-called secretory pathway in which the proteolytic enzyme furine releases C-terminal peptides (92). Interestingly, peptide epitopes that emerge at the surface of cancer cells with impaired TAP function derived from self-antigens and act as immunogenic neoantigens, as they are not presented by normal cells (93). Our group identified a signal peptide-derived CD8 T-cell epitope processed independently of proteasomes/TAP, by a novel pathway involving signal peptidase and the signal peptide peptidase (94, 95). These signal sequence-derived peptides represent attractive T-cell targets that permit CTL to destroy TAP-impaired tumors and therefore correspond to promising candidates for cancer immunotherapy.

THE TCR REPERTOIRE AND ANTITUMOR T-CELL IMMUNITY

The TCR–CD3 complex, expressed on the T-cell surface, allows recognition of antigenic peptides bound to MHC molecules on target cells and APC, and transduction of the signal into the cytosol to initiate signaling events leading to T-cell activation (96). The TCRα- and β-chains are products of V(D)J recombination, a somatic rearrangement of the germline TCR loci occurring in T cells (97). This process leads to generation of a diverse TCR repertoire [$>10^{15}$ distinct αβ-receptors or clonotypes (98)] that enables T-cell recognition of numerous foreign or mutant antigens. The TCRα- and β-chains possess three hypervariable regions, referred to as complementarity-determining regions (CDR) 1, 2, and 3. CDR3 is highly polymorphic and is directly responsible for recognition of antigenic peptides. Immunoscope/spectratype

TABLE 2 | Mutant tumor antigens recognized by CD8 or CD4 T cells.

Gene/protein	Tumor type	Human leukocyte antigen (HLA)	Peptide	Position	Reference
		Class I	**CD8 T-cell epitope**		
LPGAT1	Bladder tumor	B44	AEPINIQTW	262–270	(56)
CASP-8	Head and neck SCC	B35	FPSDSWCYF	476–484	(57)
Beta-catenin	Melanoma	A24	SYLDSGIHF	29–37	(58)
CDK4	Melanoma	A2	ACDPHSGHFV	23–32	(59)
CDKN2A	Melanoma	A11	AVCPWTWLRG	125–133 (p14ARF-ORF3)	(60)
HLA-A11d	Melanoma				
CLPP	Melanoma	A2	ILDKVLVHL	240–248	(61)
GPNMB	Melanoma	A3	TLDWLLQTPK	179–188	(62)
RBAF600	Melanoma	B7	RPHVPESAF	329–337	
SIRT2	Melanoma	A3	KIFSEVTLK	192–200	
SNRPD1	Melanoma	B38	SHETVIIEL	11–19	
SNRP116	Melanoma	A3	KILDAVVAQK	668–677	
MART2	Melanoma	A1	FLEGNEVGKTY	446–455	(63)
MUM-1f	Melanoma	B44	EEKLIVVLF	30–38	(64)
MUM-2	Melanoma	B44	SELFRSGLDSY	123–133	(65)
		Cw6	FRSGLDSYV	126–134	
MUM-3	Melanoma	A68	EAFIQPITR	322–330	(66)
Myosin class I	Melanoma	A3	KINKNPKYK	911–919	(67)
N-ras	Melanoma	A1	ILDTAGREEY	55–64	(68)
OS-9	Melanoma	B44	KELEGILLL	438–446	(69)
Elongation factor 2	Lung SCC	A68	ETVSEQSNV	581–589	(70)
NFYC	Lung SCC	B52	QQITKTEV	275–282	(71)
Alpha-actinin-4	NSCLC	A2	FIASNGVKLV	118–127	(72)
Malic enzyme	NSCLC	A2	FLDEFMEGV	224–232	(20)
HLA-A2	RCC				(73)
Hsp70-2	RCC	A2	SLFEGIDIYT	286–295	(74)
		Class II	**CD4 T-cell epitope**		
COA-1	CRC	DR4	TLYQDDTLTLQAAGE	447–46	(75)
		DR13			
ARTC1	Melanoma	DR1	YSVYFNLPADTIYTNH		(76)
CDC27	Melanoma	DR4	FSWAMDLDPKGAE	760–771	(77)
FN1	Melanoma	DR2	MIFEKHGFRRTTPP	2050–2063	(78)
LDLR-FUT fusion protein	Melanoma	DR1	WRRAPAPGA	315–323	(79)
			PVTWRRAPA	312–320	
neo-PAP	Melanoma	DR7	RVIKNSIRLTLE	724–734	(80)
PTPRK	Melanoma	DR10	PYYFAAELPPRNLPEP	667–682	(81)
Triosephosphate isomerase	Melanoma	DR1	GELIGILNAAKVPAD	23–37	(82)

SCC, squamous cell carcinoma; RCC, renal cell carcinoma; CRC, colorectal carcinoma; NSCLC, non-small-cell lung carcinoma.
From: https://www.cancerresearch.org/scientists/events-and-resources/peptide-database (slightly modified).

technology was first used to probe the T-cell repertoire by analyzing the diversity of TCRVβ (99, 100) and, more recently, TCRVα (101, 102) chains without isolating peptide-reactive T cells and cloning TCR genes. It is based on the use of V and J gene-segment-specific primers for reverse transcription-polymerase chain reaction amplification of CDR3 of a bulk T-cell population from diverse biological materials such as blood and tumor tissues (103). Analyzing CDR3 polymorphisms and sequence length diversity served to follow up T-cell clonality in tumor-infiltrating lymphocytes (TIL) to investigate T-cell functions and the pattern of TCR utilization. It highlighted restriction of the CDR3 length of TCRβ- and TCRα-chains in T cells infiltrating solid tumors and hematological malignancies, including melanoma, renal cell carcinoma (RCC), neuroblastoma, NSCLC, and Sezary syndrome (19, 101, 104–109). TCRβ-chain gene usage also showed that antigen-specific T-cell clones with high functional avidity/tumor reactivity expanded only at the tumor site, but not in peripheral blood (108). Identification of TAA has led to improvement in procedures for detecting and monitoring specific antitumor T-cell responses. In this regard, combining a quantitative immunoscope approach with MHC–peptide multimer-based T-cell sorting led to more sensitive *ex vivo* follow-up, by quantitation of human $CD8^+$ T-cell responses and monitoring of T-cell subsets throughout immunotherapy clinical trials (110).

Tremendous progress in characterizing the size and dynamics of the T-cell repertoire has emerged from recent advances in

TABLE 3 | Validated mutant antigens identified by WES and recognized by CD8 T cells.

Gene/protein	Tumor	Human leukocyte antigen	Peptide	Position	Reference
SETDB1	Cervival cancer	B40	VESEDIAEL	17–25	(53)
METTL17	Cervival cancer	A32	RTKVVQTLW	277–285	
ALDH1A1	Cervival cancer	B35	IPIDGIFFT	66–74	
CDKN2A	Melanoma	A2	KMIGNHLWV	153–161	(55)
TKT	Melanoma	A2	AMFWSVPTV	435–443	
TMEM48	Melanoma	A2	CLNEYHLFL	161–169	
AKAP13	Melanoma	A2	KLMNIQQKL	278–286	
OR8B3	Melanoma	A2	QLSCISTYV	186–194	
SEC24A	Melanoma	A2	FLYNLLTRV	465–473	
EXOC8	Melanoma	A2	IILVAVPHV	649–658	
MRPS5	Melanoma	A2	HLYASLSRA	58–66	
PABPC1	Melanoma	A2	MLGEQLFPL	516–524	
KIF2C	Melanoma	A2	RLFPGLTIKI	10–19	(52)
POLA2	Melanoma	Cw7	TRSSGSHFVF	413–422	
CCT6A	Melanoma	B27	LRTKVYAEL	156–164	(54)
TRRAP	Melanoma	A2	LLYQELLPL	774–782	
DNMT1	Melanoma	A24	IYKAPCENW	835–843	
PABPC3	Melanoma	A24	YYPPSQIAQL	416–425	
MAGE-A10	Melanoma	A24	LYNGMEHLI	255–263	
FMN2	Melanoma	A3	HSVSSAFKK	843–851	
WASL	Melanoma	B7	YPPPPPALL	343–351	
MAGEA6	Melanoma	A1	KVDPIGHVY	168–176	(83)
		B15	LMKVDPIGHVY	166–176	
		Cw5	KVDPIGHVYF	168–177	
PDS5A	Melanoma	Cw3	FVVPYMIYLL	1000–1009	
MED13	Melanoma	A1	VSVQIISCQY	1685–1694	
		A30	VQIISCQY	1687–1694	
		B15			
FLNA	Melanoma	B7	CVRVSGQGL	2049–2057	
KIB1B	Melanoma	B7	APARLERRHSA	1009–1018	
KFI1BP	Melanoma	A24	AYHSIEWAI	243–251	
		B38	YHSIEWAI	244–251	
		Cw12	NAYHSIEWAI	242–251	
NARFL	Melanoma	A3	KSQREFVRR	62–70	(84)
PPFIA4	Melanoma	B39	MRMNQGVCC	706–714	
CDC37L1	Melanoma	A2	FLSDHLYLV	181–189	
MLL3	Melanoma	B7	KPSDTPRPVM	1026–1035	
FLNA	Melanoma	A2	HIAKSLFEV	364–372	
		B44	AGQHIAKSLF	361–370	
DOPEY2	Melanoma	B7	KPFCVLISL	362–370	
TTBK2	Melanoma	B7	RPHHDQRSL	1174–1182	
KIF26B	Melanoma	A11	SSYTGFANK	254–263	
SPOP	Melanoma	A2	FLLDEAIGL	141–149	
CDK4	Melanoma	A2	ALDPHSGHFV	23–32	
RETSAT	Melanoma	A68	HSCVMASLR	545–553	
		B37	HDLGRLHSC	539–547	
CLINT1	Melanoma	B57	VSKILPSTW	469–477	
COX7A2	Melanoma	A11	GVADVLLYR	80–88	
FAM3C	Melanoma	B44	TESPFEQHI	192–200	(48)
CSMD1	Melanoma		GLEREGFTF		
PPP1R3B	Melanoma	A1	YTDFHCQYV	172–180	(85)
CDK12	Melanoma	A11	CILGKLFTK	924–932	
CSNK1A1	Melanoma	A2	GLFGDIYLA	26–34	
GAS7	Melanoma	A2	SLADEAEVYL	141–150	
MATN	Melanoma	A11	KTLTSVFQK	226–234	
HAUS3	Melanoma	A2	ILNAMIAKIJ	154–162	
MTFR2	Non-small-cell lung carcinoma (NSCLC)		FAFQEYDSF	321–326	(50)
CHTF18	NSCLC		LLDIVAPK	765–772	
MYADM	NSCLC		SPMIVGSPW	22–30	
HERC1	NSCLC	A11	ASNASSAAK	3274–3282	(49)
HSDL1	Ovarian cancer	Cw14	CYMEAVAL	20–27	(86)

DNA and RNA sequencing (RNAseq) technologies (111, 112). High-throughput TCR sequencing (TCR-seq) involves NGS for generating DNA sequences covering TCR CDR3 and permits quantification of T-cell diversity at very high resolution (113). Another method for profiling the TCR repertoire relies on a TCR-specific short read assembly strategy based on 5′ amplification of cDNA ends (RACE), so as to obtain TCRβ CDR3 transcript sequences and massively parallel Illumina sequencing of TCRβ CDR3 amplification products (114). This strategy avoids potential bias associated with the use of multiple primer sets required to amplify CDR3 regions from all *TCRBV* sequences and takes advantage of the conserved sequences of *TCRBC1* and *TCRBC2* genes (115, 116). High-throughput DNA-based strategy for identifying antigen-specific TCR sequences was also developed by the capture and sequencing of genomic DNA fragments encoding TCR genes (117). More recently, an optimized approach to characterizing tissue-resident T-cell (T_{RM}) populations emerged from extraction of TCR CDR3 sequence information directly from RNAseq data sets of thousands of solid tumors and control tissues (118). This method circumvents the need for PCR amplification and provides TCR information in the context of global gene expression profiles.

Sequence-based immunoprofiling is a useful tool for monitoring the dynamics of the T-cell repertoire under physiological and pathological conditions, and in response to therapeutic interventions. In this respect, characterization of the TCR repertoire in TIL permits isolation of tumor-specific T-cell clones for use in cancer immunotherapy. TCR-seq can also be used to evaluate T-cell diversity and identify tumor-reactive T-cell clonotypes, along with potentially immunogenic neoantigen-reactive T cells (119). For instance, deep cDNA sequencing of TCR-α and β-chains enabled quantitative monitoring of the T-cell repertoire in lung cancer patients treated with cancer peptide vaccines (120). Another interesting parameter for follow-up by deep TCR-seq is the heterogeneity of T-cell density and clonality across tumor regions. Indeed, it has been shown that high intratumor heterogeneity of TCR is positively correlated with that of predicted neoantigens and has been associated with increased risk of disease progression (121). In contrast, maintenance of high-frequency TCR clonotypes alongside CTLA-4 blockade therapy was associated with improved overall survival in prostate cancer and melanoma (122). Moreover, high TCR clonality was associated with an increased response by melanoma patients to the programmed cell death (PD)-1 blockade, suggesting that TCR repertoire analysis could be used as a predictive marker in cancer immunotherapy (123). Indeed, elevated TCR clonality and significant T-cell clone expansion were observed in melanoma patients responding to anti-PD1 treatment (124). Overall, T-cell clonality and TCR repertoire diversity appear to be biomarkers of antitumor adaptive immunity and might also be predictive markers of responses to cancer immunotherapy.

T-CELL-BASED CANCER IMMUNOTHERAPIES

An understanding of regulation of the molecular interaction between T cells and tumor cells, together with refined T-cell engineering technologies and the discovery of TSA, gave rise to novel cancer immunotherapies with unprecedented clinical efficacy. These therapies are aimed at (re)activating and expanding tumor-specific CTL, with the goal of destroying primary cancer cells and metastases. The most effective current cancer immunotherapies include ICI, such as anti-PD-1 and anti-CTLA-4, ACT of *ex vivo*-expanded tumor-reactive T cells, either native (CTL clones or TIL) or engineered to express particular TCR or chimeric antigen receptors (CAR), and TSA-based cancer vaccines (peptide- or RNA-based) (84, 125–132). Moreover, increasing evidence of a link between CD8 and CD4 T-cell recognition of mutant neoepitopes and clinical responses to cancer immunotherapy strategies has been reported (34, 48–53, 55); for review, see Ref. (47).

ACT Immunotherapy

The possibility of expanding subsets of mature T cells *in vitro* led to development of ACT immunotherapy. The aim is to transfer a T-cell population enriched in potentially highly tumor-reactive effector cells (130, 131, 133, 134). In this context, re-infusion of *ex vivo*-expanded TIL displaying increased specificity toward cancer cells was developed as a means of strengthening patient spontaneous T-cell responses and overcoming tolerance to the tumor. Steven Rosenberg's team has been one of the pioneers in the development of ACT, mainly using selected tumor-reactive T cells and TIL. Thus, clonal repopulation of T cells directed against overexpressed self-derived differentiation antigens, in combination with chemotherapy and high doses of IL-2, led to tumor regression in patients with metastatic melanoma (135, 136). Similarly, treatment of patients with uveal melanoma by adoptive transfer of autologous TIL, administered together with IL-2, resulted in objective tumor regression (137). Clinical responses were associated with the presence of tumor-resident $CD8^+$ T lymphocytes that target tumor-specific mutant neoantigens and express the PD-1 checkpoint receptor (51, 52, 83, 138, 139). Moreover, neoantigen-reactive TCR have been identified from the most frequent clonotypes among TIL, opening up new avenues for developing a personalized TCR-gene therapy approach that targets individual sets of antigens presented by tumor cells without the need for determining their identity (140). Accordingly, neoantigen-reactive TCR have been identified, with the aim of treating patients with autologous T cells genetically modified to express such TCR (141). Nevertheless, analyses of neoantigen-specific T-cell responses in melanoma patients treated by ACT demonstrated that the T-cell-recognized neoantigens can be selectively lost over time emphasizing the importance of targeting broad TCR recognized neoantigens to avoid tumor resistance (142).

While ACT of tumor-specific T cells holds promise for melanoma treatment, significant challenges remain in clinical translation to other solid tumors. This can be explained by the observation that some tumors, referred to as "immune-desert tumors" or "cold tumors," are rarely infiltrated by T cells, and TIL often display an exhausted state acquired in the tumor microenvironment. Indeed, TIL are characterized by high expression levels of one or several inhibitory receptors such as PD-1, CTLA-4, Tim-3, LAG-3, and TIGIT, and often display altered production

of cytokines leading to weak antitumor reactivity (143, 144); for review, see Ref. (145). Moreover, the limited life span of TIL and difficulties linked to their production, including isolation from fresh patient tumor specimens and selection based on tumor-specificity, constrain their clinical routine use.

To overcome limitations of TIL-based ACT, and due to the availability of TAA-specific TCR or antibodies, genetically engineered T cells have been developed with either tumor-specific TCR or CAR (146–149). Therefore, desired specificity was achieved by genetically modifying T cells to express a TAA-specific TCR (150–153). Candidates are selected either from the native TCR repertoire or after mutagenesis of their antigen recognition domain, the CDR3 domain, to increase the affinity of T cells (154). Thus, T cells engineered to express TAA-specific TCR (recognizing Melan-A/MART1-, gp100-, NY-ESO-, or p53-derived peptides) resulted in objective regression of metastatic melanoma lesions in some patients (153, 155, 156). As an alternative, engineered T-cell strategy utilizes CAR comprising the antigen-binding domain of an antibody, fused with one or more immunostimulatory domains, to activate T cells once the recognition domain has bound to a target cell. Because such T cells are able to recognize tumor antigen-expressing cells in a MHC-independent manner, a single CAR can be used on all patients whose tumor expresses the target antigen (i.e., CD19, CD20). The therapeutic potential of CAR-expressing T cells, especially in patients with hematological malignancies such as B-cell lymphoma expressing CD19 or CD20, has been demonstrated in several clinical trials (157–163). This holds promise for further use in hematological tumors and for treatment of solid tumors unresponsive to other immunotherapies.

Immune Checkpoint Blockade Immunotherapy

Targeting immune checkpoints with blocking monoclonal antibodies (mAb) such as anti-CTLA-4 and anti-PD-1 or anti-PD-L1 has provided clinical benefits for patients with advanced metastatic melanoma, NSCLC, RCC, and several other cancers (164, 165). While the CTLA-4 blockade reduces the activation threshold required for T-cell priming (166), the PD1/PD-L1 blockade in certain T-cell subpopulations (167) at least partly reverses immune alterations such as exhaustion (168). This allows synergy for combined treatments (169) and opens up new perspectives for combining these checkpoint blockers (i.e., anti-CTLA-4, -PD-1, or -PD-L1) with mAb toward additional inhibitory molecules, such as BTLA, TIM-3, or LAG-3. In this regard, synergistic antitumor effects were obtained in several preclinical models (170–172).

Accumulating evidence indicates that preexisting antitumor $CD8^+$ T cells predict the efficacy of ICI therapy (124, 173). Moreover, effective CTLA-4 and PD-1 blockade immunotherapy appears to be associated with the presence of T cells directed toward mutant cancer neoepitopes (48–50), and with the likelihood of MHC presentation of these neoantigens and subsequent recognition by specific T cells (174). Mutant neoantigens are highly immunogenic; they are not expressed by normal tissues and thus bypass thymic tolerance (175). Unfortunately, clinical trials demonstrated that only a fraction of cancer patients respond to such immunotherapy. Resistance to anti-PD-1 of tumors with a high mutational load was associated with defects in pathways involved in IFNγ-receptor signaling and antigen presentation by MHC-I molecules, concomitant with a truncating mutation in the gene encoding β2m (176, 177). Moreover, patients identified as non-responders to anti-CTLA-4 mAb had tumors with genomic defects in IFN-γ pathway genes (178). These findings demonstrate the importance of the IFN-γ signaling pathway and CD8 T-cell recognition of mutant neoantigens in response to checkpoint blockade immunotherapy.

Therapeutic Cancer Vaccines

The discovery of TAA has led to development of therapeutic cancer vaccines, based on either synthetic peptides, "naked" DNA, DC, or recombinant viruses, that attempt to strengthen the antitumor immune response, and particularly tumor antigen-specific CTL response (179, 180). Peptide vaccines have many advantages, including inexpensive, convenient acquisition of clinical-grade peptides, easy administration, higher specificity, and potency due to their higher compatibility with targeted proteins, the ability to penetrate the cell membrane and improved safety with few side effects (181, 182). Mechanisms underlying priming of anticancer immune responses by peptide-based vaccines, and hence their efficacy, is dependent, at least in part, on the size of the peptides. While short peptides (8–11 aa) bind directly to HLA-I molecules and mount MHC-I-restricted antigen-specific $CD8^+$ T-cell immunity (183–185), long synthetic peptides (25–50 aa) must be taken up, processed, and presented by APC to elicit a T-cell response. Vaccination with long peptides usually results in broader immunity than with short peptides, along with induction of both $CD8^+$ cytotoxic and $CD4^+$ helper T cells when conjugated with efficient adjuvants (186, 187). Indeed, $CD4^+$ T-cell help is required for generation of potent CTL and long-lived memory $CD8^+$ T cells (186).

First-generation cancer vaccines based on non-mutant TAA, also termed shared antigens because they are expressed by many patients' tumors, such as MART-1, gp100, tyrosinase, TRP-2, NY-ESO-1, MAGE-A3, and Her2/neu or telomerase proteins, were shown to be immunogenic and capable of inducing clinical responses in only a minority of patients with late-stage cancer (180, 188, 189). However, results showing that $CD4^+$ T cells directed toward NY-ESO-1 cancer-germline TAA and lymphocytes genetically engineered with a NY-ESO-1-reactive TCR display antitumor activity (40, 190) support the notion that T-cell responses to a subset of non-mutant antigens contribute to the effects of current cancer immunotherapies. The limited success of these active immunotherapy approaches might be due to the inability of effector T cells to overcome tolerance to self-antigens, expression of T-cell inhibitory receptors such as CTLA-4 and PD-1, and suboptimal activation of tumor-specific T cells in an immunosuppressive tumor microenvironment (191).

The current challenge in developing more efficient second-generation cancer vaccines is based on mutant epitopes that derive from tumor neoantigens (192, 193). Non-mutant tumor neoepitopes that emerge on the target cell surface upon alteration of TAP expression, such as the self-epitope derived from the human ppCT preprohormone (94, 95), are interesting targets

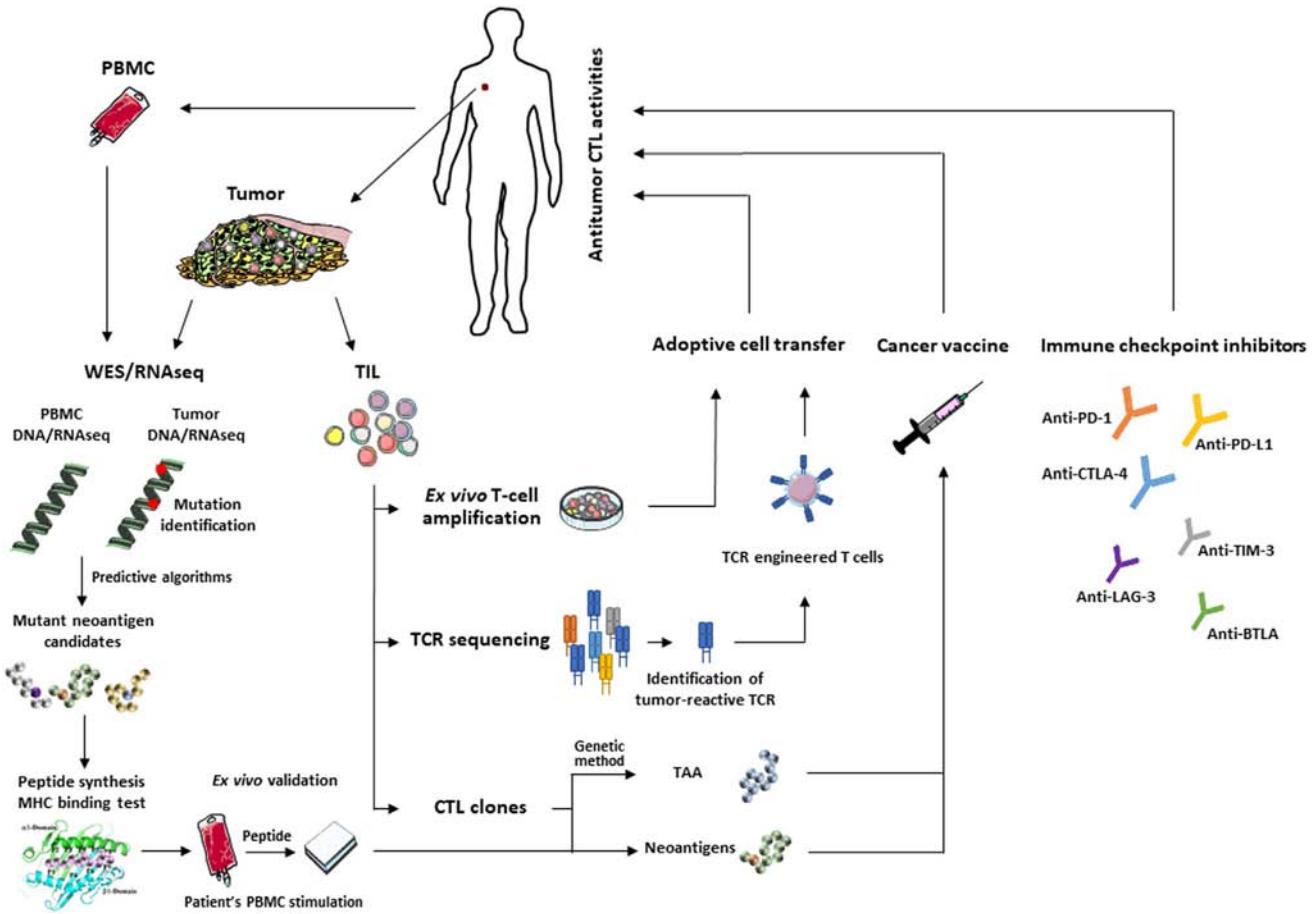

FIGURE 1 | Main approaches for T-cell-based cancer immunotherapy: identification of immunogenic tumor antigens using WES/RNAseq and predictive programs (left) or CTL and genetic approach for the development of effective therapeutic cancer vaccines. Adoptive transfer of selected tumor-infiltrating lymphocytes (TIL) or autologous T cells engineered to express tumor-reactive T-cell receptor (TCR) (middle). These approaches can be combined with immune checkpoint inhibitors (right) to reverse T-cell exhaustion and optimize antitumor T-cell response.

for peptide-based vaccination against immune-escaped tumors expressing low levels of pMHC-I complexes (194, 195). Recent technological advances in identifying mutation-derived tumor antigens have enabled development of patient-specific therapeutic vaccines, including peptides, proteins, DC, tumor cells, and viral vectors, that target individual cancer mutations (196). Over the past few years, examples of TSA-based personalized cancer immunotherapies have begun to emerge. For example, a durable clinical response to cancer vaccines with autologous melanoma-pulsed DC was obtained and correlated with the presence of effector memory T cells responding to mutant antigens (54). Moreover, DC-based vaccination directed at melanoma-neoepitope candidates resulted in an increase in clonal diversity of antitumor T-cell immunity and promoted a diverse neoantigen-specific TCR repertoire (55). Immunogenic personal neoantigen vaccines, based either on RNA or synthesized long peptides, have recently been developed for patients with melanoma. In this regard, personalized RNA-based mutanome vaccines, alone or in combination with anti-PD-1, induced effective T-cell responses against multiple vaccine neoepitopes and resulted in sustained progression-free survival (84). In another clinical trial, long peptide cancer vaccines that target predicted personal tumor neoantigens, administered alone or in combination with anti-PD-1, resulted in clinical benefits and induced polyfunctional $CD4^+$ and $CD8^+$ T cells, with expansion of the repertoire of neoantigen-specific T cells (132). Thus, a combination of neoepitope-based vaccines and ICI is promising for overcoming the anergic state of vaccine-induced T cells. These strategies open up new avenues for further development of personalized active immunotherapy, either alone or in combination with other therapies, for patients with different types of cancer (**Figure 1**). Personalized cancer immunotherapies offer promise of low toxicity and high specificity, and the opportunity to treat human malignancies resistant to current therapies.

CONCLUDING REMARKS

The success of cancer immunotherapy relies on the induction of immune effector mechanisms associated with generation of high-avidity tumor-specific CTL. To further improve their antitumor

effectiveness, and for more robust long-term disease control, a deeper understanding of host-tumor interactions and tumor immune escape strategies is required. Overcoming immune tolerance/suppression pathways within the tumor microenvironment, which may hinder the potency of immunotherapeutic approaches, is a major challenge in the field of tumor immunology and immunotherapy. In this context, optimizing the therapeutic potential of the immune system relies on a combination of different approaches, mainly cancer vaccines with ICI and/or ACT, which synergistically enhance antitumor T-cell responses. Selection of the right adjuvant or neoadjuvant, such as TLR agonists, is necessary to improve the immunogenicity of peptide-based vaccines, by targeting antigens to competent APC (and, in particular, DC, capable of cross-presentation and delivering of stimuli to activate both specific $CD4^+$ and $CD8^+$ T cells). Moreover, alternative routes of peptide administration for improved target delivery would help to induce strong long-lasting antitumor T-cell responses and thus improve clinical outcome. Therapeutic cancer vaccines combining both TAP-dependent and TAP-independent epitopes might also boost tumor-specific CD8 T-cell immunity, prevent immune escape mechanisms developed by malignant cells, and thereby potentiate current cancer immunotherapies. Remarkably, targeting of non-self tumor-specific neoantigens, generated by somatic mutations, has gained increasing interest over the past few years. Rising accessibility to NGS technologies, improved *in silico* prediction of truly immunogenic mutant peptides and easy peptide manufacturing are promising approaches to identifying patient-specific neoepitopes and evaluating their potential use in both prognosis and treatment. The utility of highly immunogenic neoantigens for personalizing therapeutic cancer vaccines will open up new perspectives for the refinement of current cancer immunotherapies.

AUTHOR CONTRIBUTIONS

FMC, AD, and SC: design and writing. YV: writing.

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REVIEW
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How to Hit Mesenchymal Stromal Cells and Make the Tumor Microenvironment Immunostimulant Rather Than Immunosuppressive

Alessandro Poggi[1], Serena Varesano[1] and Maria Raffaella Zocchi[2]*

[1] Molecular Oncology and Angiogenesis Unit, Policlinico San Martino, Genoa, Italy, [2] Division of Immunology, Transplants and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy

Experimental evidence indicates that mesenchymal stromal cells (MSCs) may regulate tumor microenvironment (TME). It is conceivable that the interaction with MSC can influence neoplastic cell functional behavior, remodeling TME and generating a tumor cell niche that supports tissue neovascularization, tumor invasion and metastasization. In addition, MSC can release transforming growth factor-beta that is involved in the epithelial–mesenchymal transition of carcinoma cells; this transition is essential to give rise to aggressive tumor cells and favor cancer progression. Also, MSC can both affect the anti-tumor immune response and limit drug availability surrounding tumor cells, thus creating a sort of barrier. This mechanism, in principle, should limit tumor expansion but, on the contrary, often leads to the impairment of the immune system-mediated recognition of tumor cells. Furthermore, the cross-talk between MSC and anti-tumor lymphocytes of the innate and adaptive arms of the immune system strongly drives TME to become immunosuppressive. Indeed, MSC can trigger the generation of several types of regulatory cells which block immune response and eventually impair the elimination of tumor cells. Based on these considerations, it should be possible to favor the anti-tumor immune response acting on TME. First, we will review the molecular mechanisms involved in MSC-mediated regulation of immune response. Second, we will focus on the experimental data supporting that it is possible to convert TME from immunosuppressive to immunostimulant, specifically targeting MSC.

Keywords: mesenchymal stromal cells, carcinoma-associated fibroblast, tumor-associated fibroblast, tumor microenvironment, immunosuppression

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Pin Wu,
Zhejiang University, China

****Correspondence:***
Alessandro Poggi
alessandro.poggi@hsanmartino.it

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are a key component of solid tumor microenvironment (TME) (1–4). They include fibroblasts, myofibroblasts, pericytes, vascular or lymphatic endothelial cells, and undifferentiated mesenchymal stem cells. These cells produce the large part of the extracellular matrix and are involved in the homeostasis of tissues in different organs. There is experimental evidence that MSC can be influenced by tumor cells and, in turn, regulate tumor cell growth and expansion (1–4). In many instances, MSCs are driven by tumor cells to modify the extracellular matrix components, allowing tumor cell adaptation to the surrounding microenvironment and eventually metastasization (1–4). In healthy tissues, MSCs represent the network on which epithelial cells,

blood, and lymphatic vasculature are organized and polarized. After receiving a danger signal, induced by biological, chemical, or physical injury, MSCs respond to maintain tissue homeostasis, favoring the repair of the tissue and reconstituting the healthy condition. During this process, MSCs come across the innate and adaptive arms of the immune system. This interaction should be highly regulated to avoid, on one hand, uncomplete repair and, on the other hand, an inefficient shut down of immune response leading to chronic inflammation (1–4). During this process, microenvironment is plenty of stimuli that, when out of control, can favor the overwhelming growth of epithelial cells with genetic alterations that are the basis of oncogenesis (1–4). Thus, the generation of a neoplasia can be dependent on the response of MSC to pathogenetic signals and to the cross-talk with immune and epithelial cells. Indeed, MSC can show immunosuppressive properties that are necessary during wound healing and repair process, but this feature is a drawback when a tumor is growing within the damaged tissue (1–4). Herein, we will briefly review the main features of MSC, from phenotype to functional properties, to clarify the molecular mechanisms whereby these cells can become immunosuppressive. Then, we will focus on the possible ways to modify MSC behavior and commute the TME from immunosuppressive to immunostimulant.

MSC: PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS

To talk about a cell type and its functional features, it is important to define their phenotypic and functional characteristics to avoid confusion among the different reports found in the literature (1–4). To simplify, a very comprehensive definition of MSC is that they are cells of mesodermal origin that are neither epithelial cells nor leukocytes (1–4). The term "MSCs" have been coined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (5, 6). These "MSCs" are defined as multipotent mesenchymal cells that can be found in several different tissues (1–6) and can differentiate, under appropriate culture conditions, into adipocytes, osteoblasts, and chondrocytes (5–10). It has been shown that adipocytes and osteoblasts can be obtained from cultures of fibroblast-like cells from skin biopsies (11). Thus, it is possible that the cultures set up to select fibroblasts contain residual stem cells that in turn differentiate to other stromal cells, such as adipocytes, chondrocytes, and osteoblasts (5–11). This implies that MSC is not a synonym of mesenchymal stem cell. Also, MSC can include fibroblasts, endothelial cells, pericytes, and mesenchymal stem cells (1–4); in turn, mesenchymal stem cells are precursors of osteoblasts, chondrocytes, and adipocytes, which can be considered as MSC. On this basis, the different cell types can be distinguished for their differentiation potential and preferential production of a given component of extracellular matrix, related to the grade of differentiation (1, 3). It is not clear whether all these kinds of cells can de-differentiate to give rise to different members of MSC, in other words, what is the degree of plasticity of a differentiated MSC (**Figure 1**). It is conceivable that the tissue microenvironment of a given organ leads a stem cell to differentiate into a given MSC with peculiar functional properties (1–4). If this is the case, any kind of cell derived from mesenchymal stem cells should share some phenotypic and functional characteristics (**Figure 1**). Although several phenotypic characteristics and functional activities of MSC have been well reviewed recently (1–10), we will briefly summarize the most relevant phenotypes, found in MSC cultured *in vitro*, related to their function in TME.

Collectively, MSC can be identified as cells that grow adherent to plastic, with elongated-diamond (fibroblast-like) shape, expressing a definite set of markers, including CD73, CD90, and CD105, but lacking the typical hematopoietic lineage and non-lineage-specific markers, such as CD34, CD45, CD14, CD11b, CD31, CD79, CD19, and HLA-DR (2–4). In some instances, some MSC cultures show peculiar markers, such as the fibroblast activation protein (FAP) found in tumor-associated fibroblasts (1–3), but it is hard to identify subpopulations of MSC on the basis of the bimodal expression of a given antigen. In other words, it is difficult to define a MSC-specific marker, as occurs in the case of $CD4^+$ or $CD8^+$ lymphocytes. Indeed, although distinct fibroblast subpopulations have been reported, based on the

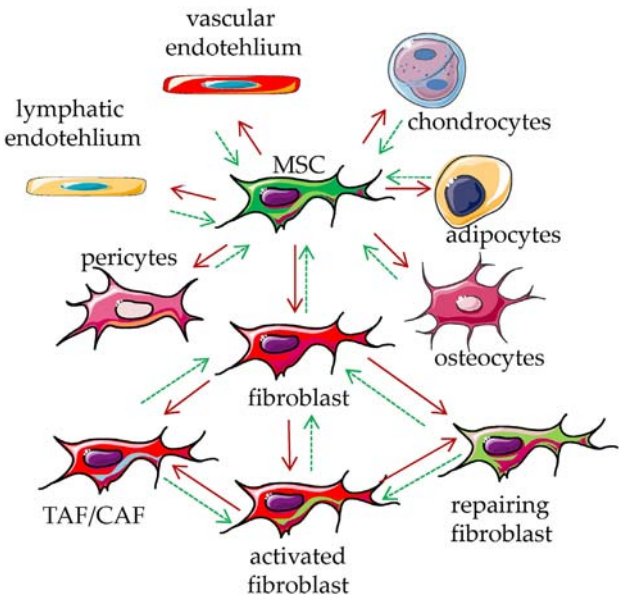

FIGURE 1 | Mesenchymal stromal cell (MSC) plasticity. MSCs are present in every tissue, where they represent a key component characterized by the ability to differentiate into several types of mesodermal cells, including osteocytes, adipocytes, chondrocytes, endothelial cells, pericytes, and fibroblasts (red arrows). It is not clear whether all these kinds of cells can in turn de-differentiate back to MSC (green dotted arrows). The function of these cells is to maintain the homeostasis of the tissue/organ where they are present, regulating the production of the extracellular matrix components. Upon stimulation with physical, chemical, or biological stimuli, they participate in the reconstitution of the equilibrium among cellular and matrix components of a given tissue, leading to damage repair. They can be considered as sensor of the tissue conditions which can coordinate the molecular mechanisms that maintain tissue integrity. Upon influence of microenvironment, fibroblasts can lead to tumor-associated/carcinoma-associated fibroblasts (TAF/CAF), activated fibroblast and fibroblast involved in repair of the tissue.

different intensity of expression of some cell surface molecules (1–4), it is not easy to distinguish these markers by immunofluorescence. In addition, MSC can produce a variety of cytokines, chemokines, and factors, such as basic fibroblast growth factor, heparin epidermal growth factor, insulin-like growth factor (IGF) 1, keratinocyte growth factor, platelet-derived growth factor-β chain (PDGF-β), vascular endothelial growth factor (VEGF), and angiopoietins, involved in tissue repair (1–3). Indeed, the main function of MSC is thought to be the repair of injuries: this process is triggered by both differentiation of MSC in specialized tissue elements, producing peculiar extracellular matrix proteins, and regeneration of the tissue and vessel architecture (1–4). In this context, the immunosuppressive properties of MSC have been demonstrated for differentiated mesenchymal stem cells (12–15) and fibroblasts (11). Unfortunately, several MSC properties have been discovered after *ex vivo* expansion upon culture *in vitro*, so that the resulting cell population may represent a selected subset of MSC. This can also explain why findings reported from different laboratories may be conflicting (16, 17). Another relevant point to be considered is the culture ratio between MSC and tumor cells or leukocytes. Several reports have shown that the maximal inhibiting effect exerted by MSC on lymphocyte functions is achieved at MSC-lymphocyte ratios ranging from 1:1 to 1:10 (16–28). While it is possible that these ratios can be found also *in situ*, it is evident that in *ex vivo* conventional cultures the microenvironment does not dynamically change as it occurs *in vivo*. Indeed, in the large majority of reports, the time points chosen to analyze an inhibiting effect were set up after several days of co-culture (16–30). This implies that the vitro culture microenvironment is composed of metabolites and factors not necessarily present *in situ*; indeed, *in vivo*, blood and lymphatic vessels are involved in the clearance and renewal of the tissue milieu (31). Experimental evidence has been reported to support that MSC can display immunosuppressive behavior *in vivo* (32–38). However, a direct demonstration of the immunosuppression exerted by MSC is far from to be demonstrated and even the potential relevance of these cells for regenerative medicine is not unequivocally proven (32).

To summarize, MSCs are present in both healthy and neoplastic tissues as undifferentiated and differentiated cells that maintain the homeostasis with a strong relevance in regulating epithelial cells growth and immune response.

MSC AND CARCINOMA-ASSOCIATED FIBROBLASTS

Mesenchymal stromal cells present in solid tumors are fibroblasts that are called carcinoma (or tumor)-associated fibroblasts (CAF or TAF) (1–4). These cells display characteristics different from MSC of healthy tissues, conceivably related to the surrounding milieu (1–4). Several factors produced by MSC, such as hepatocyte growth factor (HGF), IGF1, and FGF, in TME can interact with surface receptors on tumor cells influencing their growth (1–4). In addition, pro-angiogenic factors, such as VEGF and PDGF, produced by MSC can favor tumor cell growth indirectly, promoting the tumor niche neovascularization (1–4). Thus, it is evident the possibility of blocking tumor cell growth by inhibiting the VEGF and/or the PDGF signaling axis (39–41). Of course, also tumor and immune cells, including tumor-associated macrophages and tumor-infiltrating lymphocytes (of both the innate and the adaptive arm of the immune system) can produce these factors; thus, the block of angiogenesis can hit several components of the TME, besides MSC. MSCs are also able to release TGF-β; this cytokine can exert several opposite effects on tumor cells, depending on the type and stage of tumor (42). Indeed, TGF-β can act as a tumor promoter as well as a tumor suppressor (42); furthermore, this cytokine is a relevant factor in epithelial–mesenchymal transition (EMT), a phase of tumor life which is considered essential for the generation of cancer metastasis (42). Recently, molecular mechanisms underlining the cross-talk between MSC and carcinoma cells have been deeply reviewed (1–4, 43–47). It is of note that, besides the direct MSC–tumor cell interactions, exosomes released by MSC can contain factors, such as micro RNA (47–56), that may drive either solid tumor cell apoptosis or tumor growth and spreading.

MSC AS REGULATORS OF IMMUNE RESPONSE

There is experimental evidence that MSC, mainly the MSC from bone marrow, can suppress immune responses *in vivo* (1–4, 10, 23, 24). In particular, the ability of MSC to reduce graft-versus-host disease (GVHD) has been reported (32–38). *In vitro* experiments have shed a light on which leukocyte populations MSC can regulate (1–4). MSC can act on both the innate arm and the adaptive arm of the immune system, blocking the expression and function of activating surface receptors on effector cells, impairing the maturation of antigen-presenting cells (APC) and favoring the expansion of regulatory cells (1–4, 12, 26, 57–67). This evidence derives from experiments where, in well-defined settings, different cells of the immune system are cocultured with a feeder layer of MSC and triggered by a given stimulus (12, 26, 68–72). Usually, such stimuli can induce proliferation, secretion of pro-inflammatory cytokines, or acquisition of a potent cytolytic potential. Upon coculture with MSC, both lymphocytes and APC are impaired in the acquisition of functional features essential to evoke a "normal" immune response (12, 26). Indeed, APC do not differentiate adequately to permit a full response to antigen-dependent or -independent stimuli (12, 26) and do not express high amounts of accessory molecules, such as CD80 and CD86, essential to deliver an optimal second signal. On the other hand, T lymphocytes express low levels of receptors, including CD25, typical of an activation state and do not respond to IL2 (12, 22, 23). The generation, in cocultures with MSC, of T cells with regulatory activities is an additional mean through which MSC can indirectly deliver an inhibiting signal to immune response (57, 58). Several papers have pointed out that different types of MSC can exert different degrees of inhibition of immune responses (1–4). In addition, differentiated MSC can still act as potent regulators of immunity (12, 72, 73). However, depending on the type of fully differentiated mesenchymal cells,

pro-stimulating or pro-inhibiting effects have been described. For instance, it has been shown that mature adipocytes can trigger T cell proliferation and both HLA-DR and HLA-I appeared to be involved (74–76). Indeed, mature adipocytes express low levels of HLA-G, a surface structure responsible for the MSC-mediated T cell inhibition (76). It is of note that the ability of adipocytes to stimulate T cells was related with a stronger expression of HLA-DR and of the master transcriptional regulator CIITA factor, compared to de-differentiated adipocytes (76). On the other hand, fully differentiated chondrocytes can inhibit T cell proliferation triggered through the CD3–CD28 activating receptors, impairing CD25 expression. More importantly, chondrocytes can affect the differentiation of monocytes to dendritic cells (12). All these effects can in turn amplify each other, thus making the immunoregulatory activity of MSC really strong (**Figure 2**).

MOLECULAR MECHANISMS OF THE IMMUNOREGULATION MEDIATED BY MSC

Mesenchymal stromal cells regulate immune response by different means (1–4), shared with other components of the TME, such as myeloid-derived suppressor cells (MDSC), tumor cells, and infiltrating Treg lymphocytes (1–4, 77–84). Indeed, indoleamine 2,3 dioxygenase (IDO), hemeoxygenase (HO), arginase 1 and 2 (ARG1 and ARG2), nitric oxidase synthase 2 (NOS2), HGF, TGF-β, IL10, prostaglandin E_2 (PGE_2), and adenosine are all factors involved in the MSC-mediated regulation of innate and adaptive immunity (1–4, 23, 85–92) (**Figure 3**). It is of note that several of these factors are upregulated by inflammatory stimuli, such as IFN-γ (69). IDO and PGE_2, are strongly induced upon inflammation, conceivably to switch off the inflammatory response to danger signals. In the TME, IDO- and PGE_2-mediated immunosuppression can be the marker of a physiological response triggered to favor tissue repair, but undesired because it favors also tumor cell growth. Indeed, IDO induces kynurenine synthesis that can strongly inhibit both the innate and the adaptive immune response (93–98). Furthermore, TGF-β is not only relevant for tumor cell growth but can also directly inhibit the function of anti-tumor effector cells. This cytokine downregulates, at the surface of natural killer (NK) cells, $CD8^+$ cytolytic T cells and γδ T cells, the expression of the NKG2D activating receptor, which in turn cannot interact with the NKG2D ligands expressed by tumor cells. These events would limit the immunosurveillance to stress signals mediated by the growing tumor (1–4, 25). In addition, TGF-β is a critical factor to generate conventional $CD4^+CD25^{high+}$ Treg and regulatory γδ T cells (42, 99–104). Moreover, TAF expressing α-smooth muscle actin (SMA) can convert arginine in ornithine through the involvement of ARG2; this leads to the inhibition of TIL functional activities, especially in hypoxic conditions (88). PGE_2 derived from NK–MSC cocultures can impair the IL-2-dependent upregulation of activating NK-cell receptors, such as members of the natural cytotoxicity receptors and DNAM-1, thus inhibiting melanoma cell recognition (20).

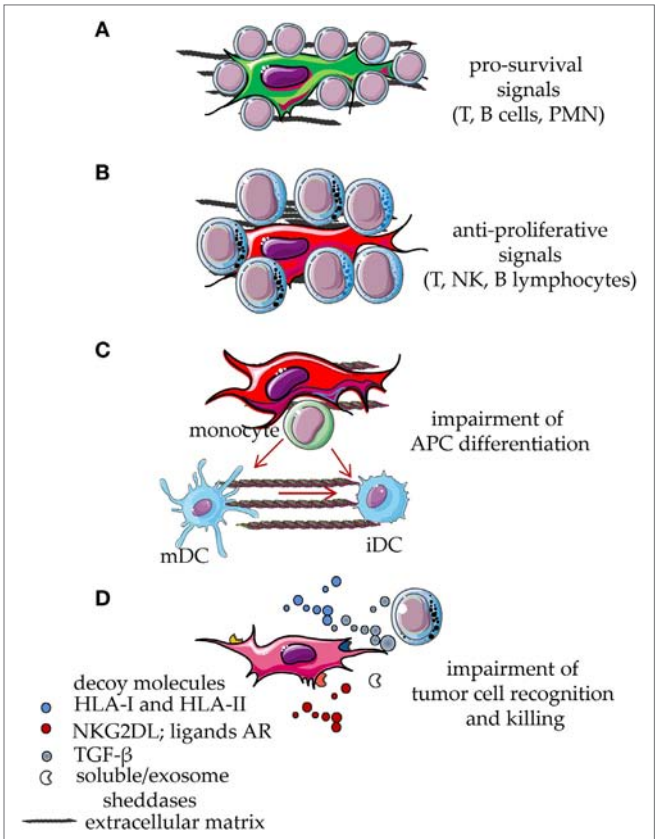

FIGURE 2 | Functional behavior of mesenchymal stromal cells (MSCs) interacting with cells of the immune system. MSCs display several functional abilities during the interaction with cell of the immune system. Resting leukocytes are typically supported by MSC through direct cell-to-cell contact involving different types of receptor on the leukocyte membrane and the corresponding ligands expressed on MSC or on differentiated MSC. Some soluble factors and interleukins are also involved, such as stromal-derived factor 1, IL-6, and IL-15. Due to this interaction, leukocytes receive an anti-apoptotic signal which leads to their survival **(A)**. This effect is also involved in the maintenance of the neoplastic counterpart of T, B lymphocytes and myeloid cells. After signals inducing proliferation, cytokine release, or activation of cytolytic machinery, MSCs exert a potent inhibitory effect that reduces leukocyte proliferation and effector functions **(B)**. In addition, activated MSCs interfere with the differentiation of monocytes to immature DC (iDC) or mature DC (mDC), thus blocking the generation of professional antigen-presenting cells (APC) **(C)**. The release *via* microvescicles, exosomes or in soluble form, of decoy molecules such as HLA-I, ligands for NKG2D or other activating receptors involved in tumor cell recognition and killing, hampers the anti-tumor activity of T and NK lymphocytes **(D)**. Furthermore, MSC can release TGF-β, sheddases, such as metalloproteinases, and a disintegrin and metalloproteinase members, which can induce the release of decoy receptors from MSC, tumor cells, and bystander cells in the microenvironment. TGF-β can inhibit tumor cell recognition reducing the activation-induced increase of NKG2D expression on anti-tumor effector lymphocytes **(D)**. All these events eventually lead to the impairment of both innate and adaptive immune responses.

Adenosine is an additional factor involved in MSC-mediated immunosuppression. Indeed, the ecto-5′-nucleotidase activity of CD73 expressed on MSC can catalyze the hydrolysis of the extracellular adenosine monophosphate (AMP) to adenosine. This metabolite can influence the activity of adenylyl cyclase,

the synthesis of cyclic AMP and the function of PKA exerting potent immunosuppressive effects (90–92).

TARGETING MSC WITH ANTI-TUMOR DRUGS

Tyrosine kinase inhibitors (TKi) are recent drugs that block the signaling cascade that follows the interaction of a growth factor with its specific receptor (105–107). It is not surprising that some TKi can affect MSC as well (**Figure 3**). Indeed, MSCs bear at the cell surface several receptors that can be considered as targets for tumor cell therapy with TKi. In particular, the expression on MSC of PDGFR-β and EGFR is well established; the effects of TKi such as imatinib, nilotinib, or gefitinib *in vitro* have pointed out that these drugs can affect both MSC proliferation and differentiation (108–122). These effects have been recently reviewed in very detail (122). It is clear from all these findings that, as expected, TKi can exert a strong inhibition on MSC growth and function, but their effects on MSC-mediated immunosuppression have not been studied. It is conceivable that the inhibition of MSC proliferation leads to the inhibition of MSC responsiveness to TME signals, but this is not determined yet. However, it has been recently shown that the encapsulated TKi sunitinib can work synergistically with vaccine therapy in an advanced mouse melanoma model, leading to the remodeling of TAF, collagen, and vessels of the tumor. Furthermore, TKi can induce a shift from Th2 to Th1 pattern of TIL, accompanied by an increment of these lymphocytes and a decrease of MDSC (123).

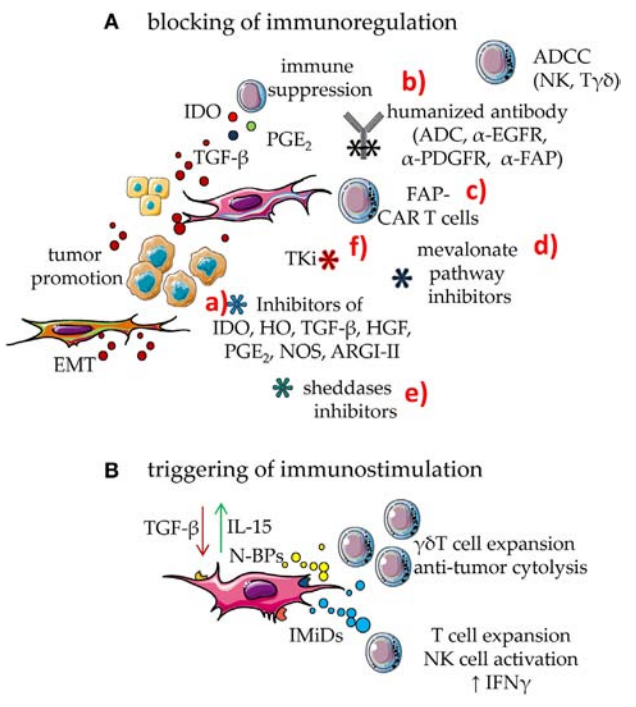

FIGURE 3 | Continued

FIGURE 3 | Means to enhance the immune response in the tumor microenvironment (TME). To counteract the mesenchymal stromal cell (MSC)-mediated downregulation of immune response, two main approaches can be utilized: **(A)** blocking of immunosuppressive effect; **(B)** triggering MSC to be immunostimulant rather than immunosuppressive. **(A)** MSC can downregulate immune response through several soluble factors such as indoleamine 2,3 dioxygenase (IDO) prostaglandin E_2 and TGF-β. In turn, TGF-β from MSC, tumor cells, and bystander cells in TME can support tumor cell growth and dissemination. This latter event is linked to epithelial–mesenchymal transition (EMT) that triggers the generation of metastasis. The blockade of MSC immunosuppression can be obtained by several means: (a) drugs that inhibit the activity or the generation of molecules involved in immunosuppression such as inhibitors of IDO, HO, TGF-β, hepatocyte growth factor (HGF), PGE_2, NOS, and ARGI–II; (b) antibodies directed either to MSC growth receptors, as the epidermal growth factor and platelet-derived growth factor (PDGF) or to the fibroblast activation protein (FAP). It is of note that some of these receptors are shared by tumor cells; thus, human or humanized antibodies-based therapy can target both MSC and cancer cells. These antibodies act inhibiting the effect of a given growth factor but also impairing the function of the target molecule. In addition, they trigger complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) elicited by Fcγ receptor-expressing cells, including natural killer (NK) cells and γδ T cells. These antibodies can be a portion of antibody–drug conjugates (ADC), which join the antibody-mediated effect to that of a cytotoxic drug, leading to a strong inhibition of tumor cell growth or MSC-mediated functions. (c) cytotoxic T cells equipped with chimeric antigen receptors (CARs) specific for FAP (FAP-CAR T cells) that can recognize FAP^+ cells; (d) drugs affecting the mevalonate pathway that is essential for both MSC and tumor cell metabolism; unfortunately, mevalonate is relevant also for the development of an optimal immune response; they should therefore be used carefully; (e) inhibitors of sheddases, as matrix metalloproteinase and a disintegrin and metalloproteinases, which can inhibit tumor cell growth limiting the generation of growth factors in a suitable form to trigger proliferation; furthermore, these inhibitors should impair the generation of decoy molecules, reducing the competition between membrane and soluble ligands for activating receptors on effector lymphocytes; (f) tyrosine kinase inhibitors (TKi) which block the activity of MSC besides hindering tumor cell growth. **(B)** Immunomodulatory drugs (IMiDs), among which thalidomide, pomalidomide, lenalidomide, and avadomide can trigger the innate and the adaptive immune responses, besides hampering angiogenesis in the tumor. Aminobiphosphonates (N-BPs), such as zoledronic acid, can interfere with the mevalonate pathway strongly enhancing the production of isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMPP). These small pyrophosphates can trigger the expansion of γδ T cells of the Vδ2 subset, a cell population with potent anti-tumoral capabilities. Furthermore, $V\delta2^+$ T cells express the FcγR involved in ADCC, reinforcing the anti-tumor effect of human/humanized antibodies.

TARGETING MSC ANTIGENS TO MODULATE TME

It is now evident that the immune system can have a significant role in limiting and controlling tumor cell growth (124–131). Indeed, both adoptive and immune check point inhibitor immunotherapies are based on the possibility of triggering, either passively or actively, the specific anti-tumor immune response (124–131). A third possibility of adoptive immunotherapy is the administration of tumor vaccines; however, tumor vaccination has led to contrasting results in clinical practice (132–138). In this setting, it is attractive to target not only tumor cells but also different components of the TME (40, 41, 139–162). Indeed, specific vaccines to tumor endothelial cells or blockers of the VEGF signaling have been used in preclinical studies, and clinical trials

are ongoing (40, 41). MSC can become a target for anti-tumor vaccines as well (141–162). For instance, the strong production of collagen type I by MSC can interfere with the uptake of anti-tumor drugs (149, 150); thus the targeting of MSC and the inhibition of extracellular matrix components can render more sensitive tumor cells to chemotherapy. Furthermore, antigens shared by tumor cells and TAF can be good targets for a vaccine. The fibroblast activation protein (FAP), a member of the serine protease family, can be expressed by TAF at higher levels than on resident fibroblast of healthy tissue. In addition, FAP can be also expressed by tumor cells; this would imply that an immune-based therapy focused on FAP can beat both tumor cells and TAF (140, 143–162). Indeed, it has been shown, in a murine model, that FAP^+ tumor cells can be used as a vaccine, leading to reduced vascular dissemination and elimination of different tumors. In the same model, tumor-infiltrating $CD8^+$ T cells increased and a net decrease of intratumor TAF, accompanied by a reduced recruitment of cells with immunosuppressive phenotype, was found in treated animals (144). In this context, the use of the humanized anti-FAP monoclonal antibody sibrotuzumab has been proposed in non-small cell lung and colorectal cancer (CRC), but the pilot study in CRC did not reach the minimal requirements for the continuation of the trial (163–166). However, FAP has been considered as a target for redirected T cells or chimeric antigen receptor (CAR) T cells (158, 159, 162) (**Figure 3**). It has been reported that transfer of murine T cells transduced with FAP-CAR construct can affect tumor cell growth increasing the $CD8^+$ T cell response. Also, the administration of anti-fibrotic agents, in several murine tumor models (E-G7 lymphoma, LLC1 Lewis lung cancer, or B16F1 melanoma) induced a strong increment of $CD8^+$ T cells, NK activity, and humoral immunity and a sharp decrease of MDSC, Treg, stromal-derived factor 1, TGF-β, and PGE_2 (162).

CAN MSC COUNTERACT CANCER DEVELOPMENT AND GROWTH?

Taking together the findings reported, it appears clear that MSC as TAF should be a mean by which tumor cells are facilitated in their growth and spreading. Thus, the higher is the content of TAF in a given tumor, the faster will be the expansion of that tumor. TAF elimination leads to an enhancement of immune response and, at the same time, to a lower support of tumor cell growth. By contrast, recent evidence in pancreatic ductal adenocarcinoma (PDAC) indicates, that the depletion of αSMA^+ myofibroblast, in a murine model can trigger tumor cell expansion and paradoxically accelerate disease progression (167). In addition, this depletion led to an increment of regulatory T cells without affecting NK cell infiltration. This was accompanied by a strong remodeling of the extracellular matrix composition and the therapy with CTLA-4 immune check point inhibitors could rescue the detrimental effect due to myofibroblast depletion. Furthermore, it appeared that the lower was the number of αSMA^+ myofibroblast in human PDAC, the worse was the prognosis of patients (167). How to explain this unexpected effect? The simplest explanation is that the reaction due to αSMA^+ myofibroblast represents a tool by which healthy MSC try to repair tissue and limit the expansion of PDAC, as suggested for other malign tumors (168–183). This phenomenon is known as desmoplastic reaction, which serves to repair tissue injury (175–182). It is conceivable that, at the onset of tumor growth, fibroblasts may function also as a physical barrier to tumor expansion. During tumor growth, due to the presence of subclones and/or cancer stem cells, this barrier can be modified by reciprocal cross-talk between tumor components and MSC. An additional explanation is that within αSMA^+ myofibroblast are present subsets of cells with different functional behaviors, with either positive or negative effects on tumor cell growth. After depletion of all αSMA^+ myofibroblast, these populations are lost and PDAC can grow without any brake (168, 173, 175, 177, 183). In such TME, immune system can receive misleading information with conflicting, undesired outcomes. Recently, it has been shown that NK cells can recognize and eliminate pancreatic stellate cells, *bona fide* myofibroblasts (171); this would suggest that innate immunity, in this case, can favor rather than inhibit tumor cell expansion by limiting stromal reaction.

RESEARCH GAPS AND FUTURE DEVELOPMENTS

At present, targeting MSC is complicated by the fact that a specific marker of these cells is missing (1–4). Indeed, MSCs have the property to differentiate and it is not clear whether there is also an intrinsic de-differentiation potential (1–4); these functional/plastic properties can impair the efficacy of a drug specific for a given MSC subpopulation. In addition, from data obtained in PDAC, it is clear that MSC can aid the host against cancer evolution. Finally, MSCs are present in each tissue and represent the key cell involved in the maintenance of the structural architecture of the whole body. Thus, therapeutic targeting of MSC should be made very carefully.

Targeting MSC with Antibodies

All the above reported matters render the targeting of MSC not as specific as desired and possibly accompanied by relevant drawbacks. By contrast, tumor cell targeting can be more specific, since the marker used as target is more expressed in tumor cells than in their healthy counterpart. For instance, in Hodgkin lymphoma and non-Hodgkin lymphomas (NHL), tumor targeting can be really efficient (184–187). Indeed, in these instances, administration of therapeutic antibodies to CD30 or CD20 molecules can spare the healthy counterpart of B cells, because the target antigen is not expressed or is expressed at low levels. Also, B lymphocyte precursors can substitute the bystander healthy B cells damaged by target therapy (184–187). An additional relevant question is whether therapies aimed to eliminate cancer cells have also an effect on MSC. Indeed, humanized monoclonal antibodies (huAb), directed to receptors involved in the proliferation of tumor cells, including EGFR or Her2b, may hit MSCs that share these molecules at the cell surface (**Figure 3**). MSC targeting might be useful, on the one hand, but the availability of the therapeutic huAb can be reduced. Moreover, it is conceivable that anti-EGFR and/or anti-HerB2 huAb can affect MSC-tumor cell

cross-talk due to the signal delivered upon huAb/receptor interaction (188, 189). The study of this interaction can shed new light on the reported unexpected effects observed with huAb therapy in some type of cancers, among which is CRC (190–193). As reported above, targeting FAP$^+$ TAF, or αSMA$^+$ myofibroblast has elicited unexpected drawbacks, since these cells can also function as negative regulators of cancer cell growth (167). The definition of subsets of MSC, myofibroblasts and even TAF, using a specific marker is a prerequisite to selectively hit the population that can favor the tumor cell growth and inhibit anti-tumor immune cell response. In this context, besides FAP, CD73, and CD105 (90, 91, 141–162), the finding that fibroblasts present in scar tissue and basal cell carcinoma express gremlin1, the secreted bone morphogenetic protein antagonist, would suggest that this can be a specific molecular target to distinguish TAF from healthy fibroblasts (194).

Interference with EMT and Role of MSC

It is well known that EMT is a key step of the spreading of cancer cells far from the primary tumor (100, 101, 104, 195) (**Figure 3**). TGF-β plays a relevant role in EMT (101, 104, 194, 196–204); thus, it is conceivable that the blockade of TGF-β production by MSC can impair EMT (197). Some evidence is reported on the prometastatic effect of CAF in different types of cancer (198, 199, 202, 203). It is of note that EMT can be also triggered by anti-EGFR huAb therapy in squamous cell carcinoma of the head and neck. Indeed, it has been reported that cetuximab therapy can induce modifications in the expression of genes and proteins implicated both in EMT and in the extracellular matrix production by CAF (201). Importantly, upregulation of CXCL12, ASPN, and OLFM3, factors secreted by CAF, has been observed; CXCL12, through the interaction with its receptor CXCR4, can lead to CXCL12 and TGFβ production and concur to myelofibrosis (205). One can speculate that EGFR signaling can drive TME to generate therapy resistance involving CAF. Targeting CAF to reduce production and release of TGFβ, CXCL12, and matrix metalloproteinases (MPP) can limit cancer cell spreading favored by TGFβ and MPP and the anti-apoptotic effect of CXCL12 on tumor cells. Unfortunately, the clinical use of inhibitors of TGFβ and MPP is far from to be well established, although the interference with CXCR4/CXCL12 axis, using AMD3100 or huAb, is already applied in several clinical trials (204–210).

Targeting Immunosuppressive Molecular Mechanisms of MSC with Inhibitory Drugs

The interference with MSC-mediated immunosuppressive molecular mechanisms, obtained using specific inhibitory drugs, is an additional mean by which the immune escape favored by tumor MSC can be avoided (1–4). In this context, all the inhibitors already used in therapeutic schemes to block IDO, HO, ARGI and II, NOS2, PGE_2, and TGF-β activity can be employed to reduce MSC influence on tumor cell growth (211–217) (**Figure 3**). In this context, the immune check point inhibitors anti-PD1 and/or PDL-1 huAb can have an important role (132, 137, 218–220). Indeed, it has been shown that PD1 is involved in MSC immunoregulation of T and B cell proliferation (18, 221, 222). The striking therapeutic effect observed upon blockade of PD1–PDL-1 with huAb can be dependent not only on the direct effect on tumor cell–effector lymphocyte interaction, but also on the switch off of the inhibiting signal elicited by PD1–PDL-1 binding during lymphocyte–MSC interaction. PDL-1 expression is upregulated on MSC by IFNγ and this cytokine can upregulate IDO as well (223); this suggests that the combination of IDO and immune check point inhibitors can concur to overcome TME immunosuppression (224). Some drugs, such as hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, can influence both immunosuppressive effects and cancer pro-survival signals delivered by MSC (28, 225) (**Figure 3**). Furthermore, it is clear that mevalonate, the metabolic product of the HMG-CoA reductase activity, is a key molecule for tumor cell fate (226). However, limiting mevalonate production can influence the functional behavior of macrophages and lead to regulatory T cell expansion, thus favoring tumor cell spreading (227). In addition, anti-tumor effector cell-mediated lytic activity is strongly reduced by HMG-CoA reductase inhibition (228–232). This can be related to the decrease of cholesterol content in lymphocyte membrane that limits the formation of rafts; these rafts are essential in the delivery of the activating signals that lead to granzyme and perforin release, upon effector–target interaction (232, 233), and consequent target cell killing. Thus, it is relevant to design inhibitors of mevalonate pathway that can be delivered specifically to MSC in order to limit tumor cell growth sparing immune surveillance.

Drugs to Transform MSC from Immunosuppressive to Immunostimulant

Another approach to downregulate the inhibitory effect of MSC on immune system is to convert their behavior from immunosuppressive to immunostimulant. Recently, it has been demonstrated, both in NHL and CRC, that priming of MSC, derived from lymph nodes or colon mucosa, with the aminobisphosphonate (N-BP) zoledronic acid can trigger Vδ2 T cell proliferation (25, 234, 235). In NHL, zoledronate-pulsed MSC are impaired in the secretion of TGF-β, whereas there is an increment in the production of IL-15 (234) (N-BPs in **Figure 3**). It should be defined whether priming with zoledronate can favor the expansion of other anti-tumor effector cells that are inhibited by MSC and whether MSC can become a target of Vδ2 T cells. If this is the case, the specific delivery of zoledronic acid to the lymph node TME would trigger anti-tumor immunity. It is well known that N-BPs have a strong tropism to bone (236); for this reason they are commonly used to treat neoplasias primarily localized in the bone, such as multiple myeloma, or bone metastases of different carcinomas (237–239). In these instances, N-BPs have a dual effect: support the deposition of bone matrix to repair the osteolytic damage induced by tumor cells and trigger γδ T cell-mediated anti-tumor immune response (237–241). When tumors are localized in other tissues, a major issue for the administration of N-BPs is to efficiently target the tumor outside the bone. It can be hypothesized that the generation of antibody–drug conjugates (ADC) (242), made of huAb linked to N-BPs, can be a good tool to deliver N-BPs to a specific tumor site. So far, ADC have been developed with huAb specific

for a tumor marker linked to cytotoxic drugs, the specificity of the antibody being the key parameter to maximize anti-tumor effect. It is conceivable that also the linkage of immunostimulant drugs to huAb specific for tumor cells and MSC can combine the specifity for the target with the triggering of anti-tumor γδ T cell immune response.

Immunomodulatory drugs (IMiDs), from the first described thalidomide to the recent reported avadomide (CC-122) (**Figure 3**), can affect both directly and indirectly tumor cell growth (243–251). Indeed, it has been reported that IMiDs can impair cereblon, a ubiquitin ligase constitutive in every cell type but crucial for cancer cell survival, causing mis-regulation of developmental signaling molecules and generation of reactive oxygen species, which in turn kill tumor cells. Furthermore, IMiDs inhibit tumor neoangiogenesis leading to the reduction of tumor cell growth. IMiDs can also modulate NK cell number and function, besides co-stimulate T cell proliferation; these effects have led to their use in multiple myeloma and several types of lymphomas. IMiDs administration has been proven to be effective in clinical trials, because these compounds can hit different components of the TME, including MSC (124, 139, 239, 243–248). It is conceivable that a progressively larger application to several kinds of solid tumors, since these drugs have shown remarkable effects in CRC and sarcomas (249–272). Importantly, in the bone marrow microenvironment, IMiDs inhibit the production of IL-6, essential for myeloma cell growth, by regulating SOCS1 (273). In addition, these compounds affect osteoblast differentiation, indicating that bone anabolic therapeutics are needed in myeloma to counteract the negative effect on bone metabolism of IMiD exposure. In this instance, the use of N-BPs can favor the deposition of bone matrix, thus limiting the damage induced by IMiDs.

Drugs to Interfere with the Generation of Decoy Receptors from MSC and Tumor Cells

MSC can release the MHC-class-I related molecules MIC-A, MIC-B, and the UL16-binding proteins (ULBPs) into TME, through the enzymatic activity of members of the a disintegrin and metalloproteinases (ADAMs) family (274–279). These released NKG2D-L can function as decoy ligands blocking the NKG2D-mediated recognition of cancer cells that usually express them on the cell membrane (277–281) (**Figure 3**). It is reasonable that ADAM10 and ADAM17 in MSC can act on such stressed molecules expressed not only by MSC but also by other cells present in TME. In addition, ADAMs can be released in exosomes and microvescicles by MSC, thus spreading their enzymatic activity. This would imply that ADAMs inhibitors can reduce the MSC-mediated release of stress molecules, allowing cancer cell recognition by immune cells and eventually leading to an increment of tumor cell killing (280). In this context, it is becoming evident that the analysis of MSC secretome is highly relevant to understand the physiological and pathological behavior of these cells (282). The targeting of ADAMs inhibitors to TME could be achieved again, using ADC which recognize MSC and/or tumor cells. Importantly, the delivery to MSC of drugs, such as N-BPs and ADAMs inhibitors, either alone or in combination with huAb as ADC, can take advantage of nanotechnology (283–285). Nanovectors can be artificially built with different morphology and physico-chemical properties (283–285). The choice of these parameters is relevant to design the optimal combination and obtain the maximal effect (283–285).

The New Frontier of Three-Dimensional (3D) Models: To Study the Interactions among MSC, Tumor Cell, and the Immune System

The study of the functional cross-talk among MSC, tumor cells, and the immune system can be more reliable using 3D models instead of classical *in vitro* culture systems (272, 286–299). Indeed, in these 3D models, the control of cell culture conditions and the regulation of biomechanical stimuli can give relevant insight on how biophysical cues can influence stromal cell phenotype and function; this can clarify how these modifications impact on tumor drug sensitivity. In addition, the cross-talk of tumor and stromal cells with immune cells can be studied in detail, varying the experimental conditions in a setting that reproduces tissue architecture; this can spare time, limit the costs of animal experimentation and reduce the environmental impact of animal breeding farms (290, 295, 297, 299). These culture systems, validated by the EU Reference Laboratories (EURL-ECVAM) as preclinical models, are reproducible 3D culture microenvironments useful for studying pharmaceuticals or biological pathways (300, 301). Among them, the hydrogels of matrix components, such as collagen, fibronectin, or cell derivates such as Matrigel or amorphic scaffold have been used (286, 293, 295). More recently, in multiple myeloma a model that recapitulates the interactions among MSC, myeloma cells, endothelial cells, and bone remodeling has been set up in order to analyze dynamically the cross-talk among all these cell populations (273). Indeed, this 3D model uses silk protein-based scaffolds that allow active cell attachment and growth on the scaffolds, rather than passive encapsulation in 3D hydrogel cultures. This represents a unique model to analyze under mechanical stress, similar to the bone tissue, the interactions of cancer cells and bone in a 3D microenvironment. The interaction among tumor cells, anti-tumor lymphocytes and MSC can be achieved in different 3D experimental setting as tumor spheroids, organoids and 3D on-chip cell cultures (291, 297, 301, 302). The 3D models where metabolic microenvironment is dynamically changed are essential to confirm the findings obtained in the murine system regarding the role of PDL-1 blocking in tumor metabolism (303, 304). Infact, in a mouse sarcoma model, it has been shown that glucose consumption by tumors can metabollically inhibit T cell responses, impairing glycolytic activity and IFNγ production. More importantly, anti-PDL-1 antibodies can block tumor glucose utilization favoring T cell glycolysis and IFNγ release (304). To validate these findings and further analyze the mechanisms of regulation of metabolism of immune cells humanized mice can be employed (305). However, these mice are engrafted with human hematopoietic stem cells and, for this reason, should be immunodeficient. Although this model can aid in mimicking the pathophysiological conditions

of human beings, it is evident that the large majority of TME is composed of murine cells. On the contrary, organoids of tumors from patients' specimens can be obtained and analyzed in detail (306–309). For instance, it has been recently shown that human intestinal organoids can be generated and used, not only for research purposes but even to treat intestinal injury (310). In addition, bioprinting techniques have led to the biofabrication of accurate models that can recreate the biophysical and biochemical characteristics of a given tissue (292). Thus, in the near future, the cross-talk among the different components of the TME will be analyzed using more and more precise 3D models and organoids from a given patient to test the sensitivity to selected targeted therapy (289, 294, 302, 306, 311–313).

CONCLUDING REMARKS

It is now clear that MSC represent a key player in regulating TME through direct cell-to-cell interactions, producing several cytokines and releasing exosomes (314–322). The secretome of MSC can play an important role in immunosuppression (319, 320): its modification with drugs can represent a new tool for drug delivery and cell-free regeneration after tumor injury (314–318, 321, 322). Because of the lack of specific markers that identify subsets of MSC, the specific targeting of these cells appears to be difficult, to achieve selective inhibition of immunosuppression. Furthermore, it is still to be elucidated whether different subsets of MSC, due to their plasticity, can represent functional subsets of cancer-associated fibroblasts (323–328). This would imply that a specific marker for the immunosuppressive MSC will be still elusive for a long time. Nevertheless, it is conceivable that drug combination therapies of cancer, which limit, on the one hand, tumor cell proliferation and, on the other hand, trigger immune responses, which already involve MSC. The *in situ* analysis of MSC functional features, together with their study in 3D tumor culture systems, would allow to clarify the existence in humans of MSC subsets and to assess the effects of drug treatment in order to choose the right combination of therapeutic means for each patient.

AUTHOR CONTRIBUTIONS

AP, SV, and MZ wrote, edited, and revised the paper. AP takes primary responsibility of the manuscript content.

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Corrigendum: How to Hit Mesenchymal Stromal Cells and Make the Tumor Microenvironment Immunostimulant Rather Than Immunosuppressive

Alessandro Poggi[1], Serena Varesano[1] and Maria Raffaella Zocchi[2]*

[1] Molecular Oncology and Angiogenesis Unit, Policlinico San Martino, Genoa, Italy, [2] Division of Immunology, Transplants and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy

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****Correspondence:***
Alessandro Poggi
alessandro.poggi@hsanmartino.it

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Targeting Tumor Metabolism: A New Challenge to Improve Immunotherapy

Soumaya Kouidhi[1,2], Farhat Ben Ayed[3] and Amel Benammar Elgaaied[2]*

[1] Laboratory BVBGR, LR11ES31, Higher Institute of Biotechnology of Sidi Thabet (ISBST), Department of Biotechnology, University of Manouba, Sidi Thabet, Tunisia, [2] Laboratory of Genetics, Immunology and Human Pathology, Faculty of Sciences of Tunis, Department of Biology, University Tunis El Manar, Tunis, Tunisia, [3] Association Tunisienne de Lutte contre le Cancer (ATCC), Tunis, Tunisia

Currently, a marked number of clinical trials on cancer treatment have revealed the success of immunomodulatory therapies based on immune checkpoint inhibitors that activate tumor-specific T cells. However, the therapeutic efficacy of cancer immunotherapies is only restricted to a small fraction of patients. A deeper understanding of key mechanisms generating an immunosuppressive tumor microenvironment (TME) remains a major challenge for more effective antitumor immunity. There is a growing evidence that the TME supports inappropriate metabolic reprogramming that dampens T cell function, and therefore impacts the antitumor immune response and tumor progression. Notably, the immunosuppressive TME is characterized by a lack of crucial carbon sources critical for T cell function and increased inhibitory signals. Here, we summarize the basics of intrinsic and extrinsic metabolic remodeling and metabolic checkpoints underlying the competition between cancer and infiltrating immune cells for nutrients and metabolites. Intriguingly, the upregulation of tumor programmed death-L1 and cytotoxic T lymphocyte-associated antigen 4 alters the metabolic programme of T cells and drives their exhaustion. In this context, targeting both tumor and T cell metabolism can beneficially enhance or temper immunity in an inhospitable microenvironment and markedly improve the success of immunotherapies.

Keywords: T-lymphocyte metabolism, tumor cell metabolism, tumor microenvironment, immunotherapy, immune checkpoints, metabolic checkpoints

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****Correspondence:***
Soumaya Kouidhi
soumayakouidhi@gmail.com

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INTRODUCTION

Over the past decades, huge efforts have focused on refinement of conventional cancer therapeutic strategies of chemotherapy, radiation, surgery, or targeted therapies. Although all these advances have displayed clear improvement of clinical outcomes for many types of cancers (1–3), their therapeutic efficacy remains unsatisfactory. Since the cells and the molecules of the immune system

Abbreviations: 2DG, 2-deoxyglucose; ACT, adoptive cell transfer; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; CAR, chimeric-antigen receptor; CTLs, cytolytic T cells; CTLA-4, cytotoxic T-lymphocyte antigen; FAO, fatty acid oxidation; HIF, hypoxia-inducible factor; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; mTOR, mammalian target of rapamycin; OXPHOS, oxidative phosphorylation; PD-1, programmed death-1; PD-L1, programmed death ligand-1; PI3K, phosphatidylinositol-3 kinase; PPP, pentose phosphate pathway; TCA, tricarboxylic acid; Teff, effector T cells; Th, helper T cells; TIL, tumor-infiltrating lymphocytes; TME, Tumor microenvironment; Treg, regulatory T cells.

are a fundamental component of the tumor microenvironment (TME), cancer immunotherapy has emerged as a powerful new therapeutic approach to boost antitumor immunity response (4). Collectively, the immunotherapy principle consists in the modulation of the immune cells activity, predominantly T cells, using adoptive cell transfer, chimeric-antigen receptor T-cells, or monoclonal antibodies (mAbs) (5, 6). The "Checkpoint blockade" that utilizes mAbs specific to cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and the programmed cell death protein 1 pathway (PD-1/PD-L1), is arising as a newer strategy used to fight cancer and one of the most promising immunotherapies (7, 8). Indeed, encouraging results demonstrate unprecedented responses in patients with several types of metastatic tumors that were previously resistant to available treatment options (9–11). While these clinical successes have dramatically harnessed host antitumor immunity and clinical outcomes for patients, there are several limitations for immunotherapy (12). In fact, this approach is confronting a highly immunosuppressive TME and low immunogenicity of cancer cells (13). Moreover, despite the success of immunotherapy, mechanisms that govern anticancer immunity and their relevant biomarkers are still being elucidated. Therefore, the development of new methods to overcome such challenge and to improve the efficacy of this therapy is needed in cancer therapy.

Tumor-infiltrating lymphocytes (TIL) reflect tumor biology and prognostic significance. However, they are challenged with a hostile microenvironment that dampens their function and produces antitumor effects (14). Nevertheless, in the setting of malignancy, multiple mechanisms of immune suppression may exist that prevent effective antitumor immunity (15, 16). Along with negative immunologic regulators called "immune checkpoints," TIL function is also negatively impacted by a variety of "metabolic checkpoints" (17). Increasing evidence suggests that the deregulation of energy metabolism plays a pivotal role in the inhibition of the antitumor immune response and thereby in tumor progression and metastasis (18). Under a suppressive microenvironment, TIL operate with a metabolic disadvantage since they are subjected to a lack of crucial carbon sources and increased inhibitory signals (19). This may be mainly due to the competition between T cells and tumor cells with deregulated metabolic activities, for limiting nutrients (20). Rapidly dividing tumor cells exhibit complex and dynamic metabolic reprogramming and highly glycolytic level, a phenomenon called the "Warburg effect" and recognized as one of the hallmarks of cancer (21, 22). Thus, tumor cells impede T cell access to nutrients necessary for their activation and generate high levels of lactate. The resulting nutrient scarceness and metabolic waste products accumulation in the TME lead to TIL metabolic switch that impairs their appropriate proliferation and function (23).

Collectively, the cancer cell energetics dictates the metabolic landscape of the TME. Abnormal metabolic activities of cancer cells lead to intratumoral heterogeneity and immunosuppression that could be responsible for the failure of immunotherapy (24, 25). Therefore, a deeper understanding of the metabolic challenges within the TME and their impacts on metabolic fitness of immune cells might contribute the discovery of novel promising approaches to rewire metabolic fitness of TILs that boost existing immunotherapies.

OVERLAPPING METABOLIC PROFILES OF CANCER CELLS AND T LYMPHOCYTES

Metabolism Impacts T Cell Fate and Activation

T cells fate and activation is closely linked to metabolic reprogramming to acquire effector functions (26). Briefly, Naive $CD4^+$ T cells can differentiate into T helper (Th) subsets or into regulatory T cells Treg, while $CD8^+$ T cells differentiate into effector cytotoxic T lymphocytes (CTLs). Importantly, each T cell functional subset utilizes a distinct metabolic program (27, 28).

Highly proliferative cells increase glucose uptake and undergo upregulated aerobic glycolysis, a critical metabolic pathway for activated T cells (29). In parallel to glucose metabolism, T cell activation also enhances mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) and drives mitochondrial membrane hyperpolarization, amino acid uptake, and glutaminolysis (30). There are several signaling pathways that govern the metabolic reprogramming of activated T cells. The critical checkpoint pathways known to regulate the metabolic switch are mammalian target of rapamycin (mTOR) (31) and adenosine monophosphate-activated protein (AMPK) pathways (32). The phosphoinositide-3-kinase (PI3-kinase)-Akt-mTOR pathway is a central integrator of T cell metabolism to sense and require nutrient availability in order to support high glycolytic rate in proliferating T cells (33). Notably, both activated mTOR complexes mTORC1 and mTORC2 play a role in driving glycolysis (34). Additionally, glycolysis activation is concomitant with the pentose phosphate pathway (PPP) upregulation, necessary to build-up of biochemical intermediates that are necessary for nucleotide, amino acid and fatty acid synthesis. Hypoxia-inducible factor-1α (HIF1α) is a master transcription factor enhanced by mTORC activity, which is monitoring and promoting glycolytic enzymes expression (35).

Also, in response to metabolic stress, AMPK inhibits mTOR signaling and increases catabolic metabolism (36). This results in glycolysis suppression and upregulation of oxidative metabolism and mitochondrial complex 1 activity. AMPK activation promotes generation of Treg, Th1, and Th17 subsets (37).

Metabolism Impacts Tumor Proliferation and Progression

Cancer progression has been recognized for a long time as consequence of multiple genetic events that imply activation of oncogenes and function loss of specific tumor suppressor genes (38, 39). Increasing data point out that this is directly linked to an altered tumor metabolism. Cancer cells exhibit increased glycolysis despite the presence of oxygen, because they must divide rapidly to ensure malignant transformation and tumor development (40, 41). This phenomenon of metabolic reprogramming called "the Warburg effect," has been recognized as one of the 10 hallmarks of cancer (42). The rate of glycolysis is largely faster than OXPHOS, providing competitive advantages to cancer cells to consume more glucose than surrounding slow-dividing cells and to grow under hypoxia and nutrient deprivation conditions over the TME (43, 44).

Furthermore, glycolysis is an effective metabolic pathway for highly proliferative cancer cells to supply nucleotide, lipid, and amino acid synthesis (45). For instance, increased levels of the glycolysis intermediates provide essential precursors for pivotal anabolic pathways such as the PPP and the serine pathway (46).

It is well established that hypoxia is as a key process supporting glycolysis in tumorigenesis (47). HIF-1α, a transcription factor induced by hypoxia, induces glucose transport by increasing expression of glucose transporters 1–3 along with the transcription of pyruvate dehydrogenase kinase (48). As a result, the tricarboxylic acid cycle is inhibited and several glycolytic enzymes activities are enhanced, including hexokinase 2 (HK2) (49) and lactate dehydrogenase A that converts pyruvate to lactate (50, 51). Therefore, intensive aerobic glycolysis generates high rate of lactate. For instance, the accumulation of lactate in TME results in acidic pH that promotes tumor progression and metastasis and contributes to cancer therapy resistance (52).

While aerobic glycolysis is considered as a key feature in cancer metabolism, clear evidence suggest that mitochondrial metabolism remains functional in most glycolytic cancer cells.

Although most cancer cells rely on aerobic glycolysis, it is clear that a tumor displays considerable heterogeneity in metabolic phenotypes (53). Such intratumorally metabolic heterogeneity may be critical for the failure of therapeutic effects. In fact, recent data has shown that cancer stem-like cells (CSCs) exhibit a distinct metabolism from the rest of tumor cells (54). This CSC metabolism depends on mitochondria function (55, 56). Moreover, the particular metabolic phenotype of CSCs may probably render them resistant to conventional antitumor therapies and explain minimal residual disease (57). Interestingly, encouraging results showed that targeting CSC metabolism (by inhibiting mitochondrial biogenesis) could be an attractive approach to reduce drug resistance (58, 59).

T CELL IMPAIRED FUNCTION UNDER HOSTILE TME

Metabolic Interplay between Cancer Cells and TIL in TME

The tumor tissue consists of complex sets of cell populations including tumor cells, endothelial cells, T cells, natural killer (NK) cells, macrophages, dendritic cells, fibroblasts, and adipocytes. Regarding its genetic and metabolic diversity, this intricate network of cells contributes to the intratumoral heterogeneity. Tumors exhibit a metabolic shift and shape the TME in such a way to support cancer proliferation and metastasis (17, 60). Yet, this milieu is very hostile for T cells to mediate their antitumor effects because of hypoxia, reduced pH and acidosis, inhibitory signals, competition for nutrients, and waste products accumulation (61, 62).

It is well known that tumor cells like effector T cells (Teff), exhibit intensive aerobic glycolysis that improve their metabolic fitness and provide cell-extrinsic advantage, resulting in competition for vital metabolites such as glucose and amino acids. Therefore, tumor-infiltrating T cells are exposed to nutrient depletion in TME and become dysfunctional (62, 63). Nutrient competition has emerged as one of the major axis of tumor immunosuppression due to the anergy and exhaustion of TILs. Indeed, resources scarceness alters T cell activation and antitumor effector functions tumors through several ways (64). Rapidly dividing tumor cells impede T cell access to glucose essential for T cell metabolic switch and activation. Therefore, glucose depletion enhances AMPK pathways and decreases mTORC1 activity, glycolytic capacity, interferon-γ (IFN-γ) production, and cytolytic activity of T cells (65). This may favor Treg subsets instead of Teff and promote tumor progression. Furthermore, decreased levels of amino acids critical for efficient T cell activation and proliferative responses, can modulate the activity of TILs. Glutamine, arginine, and tryptophan deficiency in TME is immunosuppressive and dampens the proliferation of Teff subset (66).

Moreover, it has been recognized that in addition to consumption of key nutrients, tumors produce large amounts of waste products: lactate, arginine and tryptophan by-products, and phosphoenolpyruvate, that impair T cell metabolism and function and confer worse prognosis for patients (67, 68).

Lactate accumulation due to the use of aerobic glycolysis by cancer cells has been described in TME, accompanied by consequent low pH and acidification of the milieu. In mouse models, lactate levels negatively correlate with markers of T cell activation in melanoma (69). The tumor-derived lactate has positive effects on promoting survival, migration and invasion of cancer cells (70). However, lactate negatively impacts T-cell proliferation and function (71). Such acidic condition increases the expression of proangiogenic factors IL-8 and VEGF, both important involved in cancer metastasis (72). Yet, lactate inhibits the phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR pathway and thus glycolytic metabolism in T cells by abolishing their cytokine production (73). Lactate also impairs the migration of T cells by reducing the chemokine receptors expression. Added to that, lactate has been demonstrated to be preferentially utilized by Tregs since they prefer oxidative metabolism, resulting in T-cell polarization toward a Treg phenotype. Excess of lactate may also regulate macrophage polarization and represses NK cells functions through a restriction of IFN-γ, IL-10, and TGF-beta (74, 75). Hence, the acidic TME has been contemplated as an attractive target for cancer therapy. Interesting results showed that buffering the tumor pH with bicarbonate improved immunotherapy outcomes.

Proliferative cancer cells create a state of tryptophan deprivation in the TME because of their increased demand for tryptophan (76). Indoleamine 2,3-dioxygenase (IDO) is a pivotal enzyme involved in tryptophan catabolism. IDO is also the first enzyme involved in the production of nicotinamid adenine nucleotide. Upregulation of IDO has been demonstrated to be correlated with an increased malignancy (77). In such context, cancer cells express high levels of IDO that deplete tryptophan availability in the TME and consequently impede T cell responses. In addition to its role in cancer cells, expression of IDO has been shown in other cells: endothelial, tumor-associated macrophages, and dendritic cells and was associated with suppression of antitumor Teff response. IDO contributes to tryptophan deprivation and degradation to kynurenine (78). Accumulation of kynurenine in TME has been described in several tumors leading to immunosuppression (79). Moreover, kynurenine is endogenously able to promote Treg cells and to reduce proliferation of Teff (40). Currently, several trials

targeting IDO in combination with checkpoint inhibition are under investigation (80).

Crosstalk between Immunologic Checkpoints and T Cell Metabolism

Immune checkpoint regulators are critical to coordinate effective and efficient immune response, to maintain self-tolerance and to prevent the onset of autoimmunity (81). Nevertheless, T cell effector function is correlated with the expression patterns of coinhibitory and costimulatory immune checkpoint receptors (82). The most described checkpoint proteins playing a central role in maintain immune self-tolerance belong to the TNFR superfamily (83) and B7 family (84).

Tumors can evade immune surveillance through defective immune-checkpoint signaling pathways (81, 85). It is now clear that under tumoral context, aberrantly expressed inhibitory checkpoint proteins are described to disrupt antitumor immune response. CTLA-4 and PD1 are critical coinhibitory receptors highly expressed in T cells under TME (86). Moreover, PD-1 ligands PD-L1 and PD-L2 are upregulated by cancer cells and thus disrupt T cells mediated antitumor response (87). Accordingly, immune checkpoints ligand–receptor interactions were proven to be effective targets to enhance antitumor immunity moving immunotherapy into a new era (88). In fact, immune checkpoints blocking antibodies have achieved an outstanding benefit in cancer treatment enabling patients to produce an effective and durable antitumor response. Currently, three checkpoint inhibitors are approved for the treatment of advanced melanomas: ipilimumab, a CTLA-4-specific mAb (89), and pembrolizumab and nivolumab, which are PD-1-specific mAbs (11). Furthermore, remarkable clinical effectiveness has been reported in other cancers such as, ovarian (90) non–small cell lung carcinoma (91), breast (92), prostate (93), and lymphoma (94).

Although the effectiveness of the immune checkpoint blockade in enhancing antitumor immunity by reducing the number and/or the suppressive activity of Tregs and by restoring the activity of Teff has been reported, little is known about mechanisms underlying T-cell activation. Recent evidence suggest that both checkpoint ligation and inhibition may directly modify metabolism of T cells and cancer cells and alter their metabolic feature. Emerging data have shown that PD-1 binding to its ligands impairs the metabolic phenotype of TIL, by inhibiting glycolysis and upregulating fatty acid oxidation (FAO) (95, 96). CTLA-4 ligation to B7 inhibits glycolysis without augmenting FAO, which suggests that CTLA-4 would not affect the metabolic profile of non-stimulated cells (95). Hence, this abrogation of energy generation impacts antitumor response and leads to reduced cytokine secretion and Teff exhaustion (97). Moreover, immune checkpoints also have an impact on cancer cell metabolic reprogramming. Ligation of PD-L1 directly upregulate glycolysis in cancer cells by promoting glucose uptake and production of lactate (98). Hence, signaling through PD-L1benefits cancer cell metabolism, leading to their expansion and survival (61).

Interestingly, the immune checkpoint blockade appears to differentially impact the metabolic profile in TME by favoring T cell activation and in contrast inhibiting cancer cells. Blocking PD-1 and PD-L1 may reduce glycolysis level in cancer cells by inhibiting mTOR pathway (61). Consequently cancer glucose uptake and lactate secretion decrease which restore glucose availability in TME. Besides, the immune checkpoint blockade has a benefit on T cell metabolism and function. A melanoma mice model study showed that tumor treatment with immune checkpoint inhibitors increases glucose rates in TME and enhances T-cell glycolysis and cytotoxic function (99).

In conclusion, clear evidences demonstrated that tumor cell metabolism deeply affects TME differentiation and functions. By modulating tumor cell metabolism, one can control nutrient availability for T cells, thus promoting either their antitumor or immunosuppressive functions.

TARGETING METABOLISM FOR EFFICIENT IMMUNOTHERAPY

Targeting Glucose Metabolism

In tumors, T cell activation and proliferation could be impaired by metabolic disruption, therefore cell metabolism becomes an attractive target to restore anti tumor immunity and to develop anticancer therapy (100). However, in tumoral context, it is wise to consider the overlapping metabolic requirements of tumor and immune cells.

Several drugs have been proposed to target tumor glucose metabolism for cancer treatment. For instance, inhibition of glycolytic enzymes that catalyze several steps of glucose metabolism has been known to support anticancer effects (101). 2-Deoxyglucose (2DG) is a non-metabolizable glucose analog and inhibitor of HK used to shut down glycolysis since the first steps. Despite the safety of this drug in cancer patients and its efficiency beyond glycolysis inhibition in cancer cells (102–104), 2DG has also been shown to impair the metabolism of T cells, which results in decreased secretion of cytokines and reduced T cell antitumor function that may be critical for therapeutic success (105). Dichloroacetate (DCA) is another drug targeting cancer cell metabolism which showed conflicting results. DCA is a metabolic disruptor inducing a shift from glycolysis to OXPHOS and inhibiting growth of tumor cells *in vitro* (106, 107) and in mouse models (108). Similar to 2DG, DCA is not specific to tumor cell metabolism, therefore, it mediates the same metabolic shift in T cells, favoring Treg formation (109).

The TME is particularly immunosuppressive because of lactic acid production in the extracellular milieu that may stand against the therapeutic efficacy (110). To overcome the "Warburg effect" in cancer cells, some therapeutic approaches target lactate with lactate dehydrogenase (LDH) and monocarboxylate transporter (MCT) inhibitors or oral bicarbonate supplementation to tamper the acidic microenvironment (111). Importantly, the inhibition of LDH, the enzyme that catalyzes the conversion of pyruvate into lactate, shows impaired glycolysis and growth arrest in cancer cells (51, 112). Moreover, lactate blockade improves the response to 5-fluorouracil treatment in colorectal cancer (113). However, LDH inhibition demonstrates contradictory results in proliferating T cells response. While it has been reported that deletion of LDH using small-molecule FX11 or Galloflavin ameliorates lactate levels (114, 115), other studies demonstrate that such inhibition leads to a decrease in T cells IFN-γ production (116). Therefore,

the differential impact of LDH inhibitors on cancer and immune cells should be considered when administrated for tumor therapy.

Beside the inhibition of the enzyme LDH, the lactate transporters MCT-1–4 may also be targeted to avoid acidic milieu (117). MCT of the *SLC16A* gene family influences substrate availability, the metabolic path of lactate and pH balance within the tumor (118). Recent studies have described new MCT disruptors, thalidomide, lenalidomide, and pomalidomide that act on cancer cells to impair the CD147–MCT-1 ligation (119, 120). In addition, the treatment with lenalidomide has been reported to enhance IL-2 and IFN-γ secretion in T cells (121), suggesting that lenalidomide could suppress tumor cell proliferation while favoring T cells activation. Although these drugs cause a loss of cell surface expression of MCT-1, the efficacy may be limited as cancer cells express not only MCT-1 but also MCT-4. Further, AZD3965 another lactate transporter inhibitor, is currently in phase I clinical trials for advanced solid tumors and diffuse large B cell lymphomas (http://www.clinicaltrials. gov/ct2/show/NCT01791595). AZD3965 is targeting MCT-1/MCT-2. Yet, the inhibitory effect has also been observed in T cells (122). Recently, the effect of diclofenac, a nonsteroidal anti-inflammatory drug, has been investigated on lactate transport and secretion. Diclofenac has been reported to reduce tumor growth, the number of infiltrating Tregs and the lactate rate in the microenvironment in glioma model (123, 124). Therefore, this result raises the possibility that the application of diclofenac should be feasible to improve the efficacy of immunotherapies.

Further, lactic acid production and resulting low-pH TME are shown to dampen CTLs proliferation and cytotoxic response (125–127). Hence, neutralization of TME may have a meaningful impact on improving the efficacy and outcomes of anticancer immunotherapy therapeutics (128). Emerging data show that buffering lactic acid with bicarbonate or proton pump inhibitor, Esomeprazole improves the pH of TME (129, 130). More importantly, neutralization of TME pH improves outcomes in CTLs and in NK cell mediated anticancer as well. Notably, buffering TME with oral bicarbonate inhibits tumor growth when combined with anti-PD-1 immunotherapy in a melanoma model, and improves survival when combined with adoptive T-cell transfer (131). Altogether, these data indicate that targeting TME acidification by buffering provide a new perspective for immunotherapy outcomes.

The PI3K-AKT-mTOR is an important pathway well known to play a critical role in cancer and immune cell metabolism (31, 132). Further, this pathway has been extensively studied in various cancers showing inappropriate activation supporting tumor growth and survival. Over the last decades, several therapies were developed against mTOR signaling in several solid malignancies (133, 134). Analogs of rapamycin, a drug that inhibits the mTOR signaling, have been approved for the treatment of breast (135), renal (136), and pancreatic cancers (137). An increasing number of studies have reported that inhibition of the mTOR pathway suppresses the glycolytic metabolism and sensitizes tumor cells to chemotherapy (138, 139). Yet, it has been reported that rapamycin can mediate opposite effects on T cells since it broadens Tregs and cytotoxic memory T cells but at the same time decreases Teff proliferation (140). Interestingly, recent evidence suggest that treatment with rapamycin combined with immunotherapy augments cytotoxic and memory T-cell functions in glioblastoma cancer (141). Therefore, rapamycin could be an attractive adjuvant to be used in combination with immunotherapy.

Besides glycolysis, OXPHOS is also a possible target structure in cancer cells. Several reports have described the potential effects of metformin, which is commonly used to treat type II diabetes, as an anticancer drug. Indeed, a large number of retrospective clinical studies and randomized control trials show that metformin prevents tumor growth and improves clinical prognosis in various cancers including lung and prostate cancers (142, 143).

Interestingly, those effects seem to be partially immune-mediated as metformin improved T cell function *in vivo* (144). Further, metformin has been proposed as a treatment for melanomas due to the limitations of current therapies (145). Metformin is known to target the mitochondrial respiratory complex I and to activate AMPK pathway signal transduction (146, 147). Several reports have demonstrated that AMPK plays pleiotropic and conflicting effects at the interface of cellular metabolism and function (37). In fact, activated AMPK may engender both antitumor and protumor effects in a manner not yet understood (148, 149). Notably, activated AMPK pathway impedes mTOR signaling, and shuts down glycolytic gene expression leading to antiproliferative effects in cancer (150, 151). However, AMPK activation on another side helps cancer cells accommodation to metabolic stresses, which raises their survival (152). Metformin's AMPK activating effects could also impact T cells behavior mainly by enhancing memory T cells (105, 153) and Treg expansion (154). Therefore, metformin treatment may improve secondary responses. Yet it could favor immunosuppressive Treg cells in TME.

Targeting Amino Acid Catabolism

In the context of the TME, cancer cells require a continuous and high rate of supply of energy to take advantage of their metabolic reprogramming and to avoid immune surveillance. In fact, cancer cells create a state of nutrient deprivation for the T cells and redirect glucose and amino acids for their own advantage. It is well known that L-arginine, tryptophan and glutamine are fundamental in tumor progression and immunity (155). Therefore, targeting theses amino acids in cancer therapy becomes a promising strategy for the development of novel therapeutic agents (156). In fact, many clinical trials are actually testing specific drugs inhibiting amino acid metabolism in cancer cells. Depletion of arginine was assessed using ADI-PEG20 inhibitor (157). It can inhibit cell proliferation *in vitro* and tumor growth *in vivo and* decrease Treg accumulation (158). However, it would be more pertinent to prevent amino acid depletion by tumor cells or myeloid cells rather than decreasing amino acid rates in the TME. This approach is currently tested in a clinical trial with CB-1158, an ARG inhibitor, in combination with checkpoint therapy (159).

Furthermore, increasing evidence suggest that tryptophan is critical in supporting oncogenic signature and in maintaining the immunosuppressive phenotype in several cancers (160). Interestingly, it has been reported that the silencing of IDO boosted antitumor immunity in metastatic liver tumor model (161), improved cytotoxic T cell function and decreased Treg numbers (162). Accordingly, it is well established that IDO is a key target of drug discovery in cancer immunotherapy (80, 163). Imatinib is another drug displaying improved anti tumor

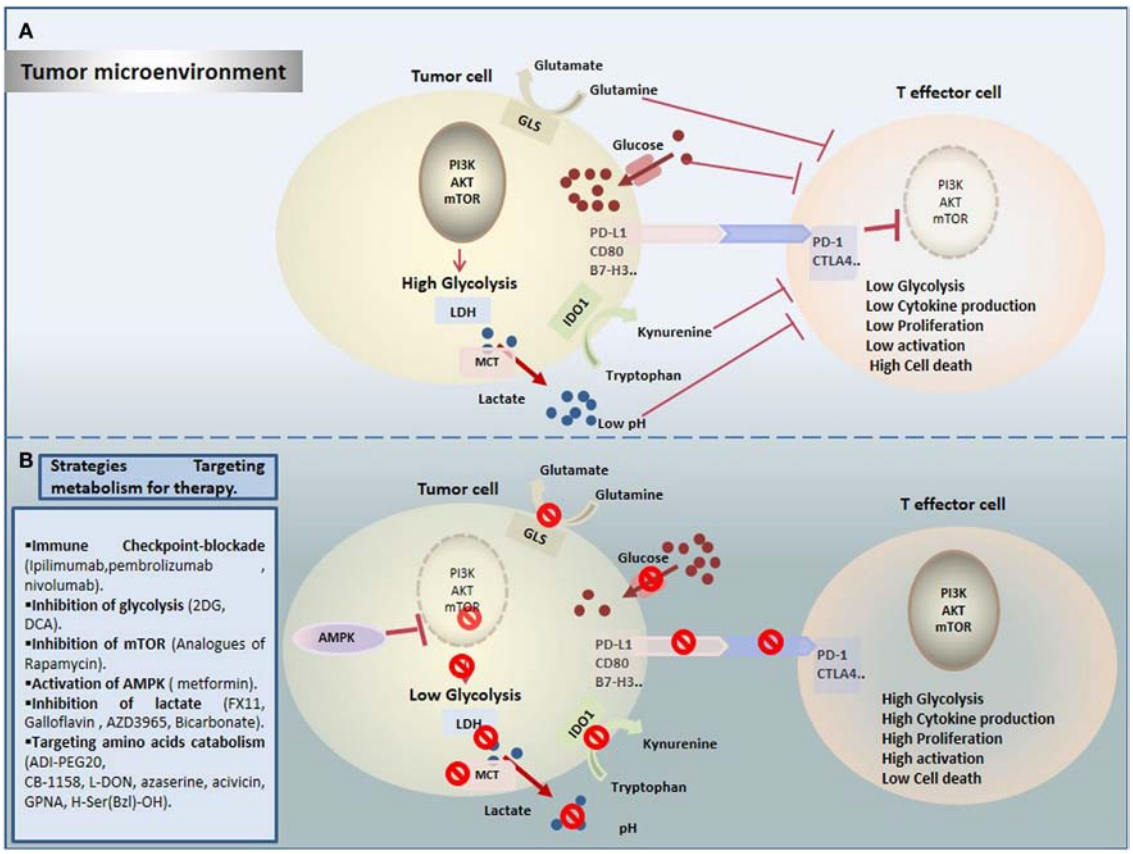

FIGURE 1 | Therapeutic targeting cell metabolism in the tumor microenvironment (TME). **(A)** Tumor cells create a hostile TME that affects metabolic fitness of T cells through multiple ways. T cells are challenged by different immunologic and metabolic checkpoints: Glucose and amino acid depletion, high acidity and lactate, and upregulation of immune checkpoints influence T cell metabolism to suppress glycolysis thereby reducing their activation and proliferation. **(B)** Currently, several novel promising approaches are proposed to rewire metabolic fitness of T cells in the TME and to boost existing immunotherapies.

immunity by activating T effector cells and suppressing Tregs, in a manner dependent on IDO pathway (164). For instance, a current clinical trial is assessing the combination between imatinib and anti-CTLA4 approach in GIST (165).

Glutamine is considered as a critical amino acid for cancer cell metabolism as well as for rapidly dividing T cells. To overcome the high glutamine consumption rates of cancer cells, several therapeutic agents targeting glutamine metabolism have been explored in preclinical studies (166). Three compounds were assessed as glutamine analogs, 6-diazo-5-oxo-L-norleucine, azaserine, and acivicin. These agents showed impaired activity of enzymes utilizing glutamine in many tumor models (167, 168). Moreover, testing glutamine transporter inhibitors gamma-l-glutamyl-*p*-nitroanilide and benzylserine [H-Ser(Bzl)-OH], showed reduced glutamine uptake and cell growth in lung and prostate cancers (169, 170). Yet, glutamine plays also a key role in normal Teff. Therefore, it is conceivable to consider better tumor-targeting options under the TME.

CONCLUDING REMARKS

Cancer immunotherapy provides successful and powerful opportunity in cancer treatment. However, it is important to get comprehensive understanding of mechanisms leading to reduced antitumor immunity under hostile TME. Importantly, TILs have to surpass not only immune checkpoints but also a wide range of metabolic checkpoints that fate their energetic behavior defects and dampen their function. In fact, cancer cells upregulate nutrients uptake and waste metabolites production to generate an immunosuppressive TME that allows their evasion and growth, and that dictates immune cell fate (**Figure 1A**). Increasing emerging data point out the modulation of cellular metabolism, using combinational approaches of metabolic disruptors with immune checkpoint blockade (**Figure 1B**). However, a special attention should be devoted to target specific tumor site, in order to avoid systemic toxicity and innumerable other side effects. In summary, by operating through distinct and complementary mechanisms, these new therapeutic strategies might reinvigorate TILs by restoring their metabolic properties and improving the efficacy of immunotherapies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Alteration of the Antitumor Immune Response by Cancer-Associated Fibroblasts

Linda Ziani[1,2,3], *Salem Chouaib*[1,2,3] *and Jerome Thiery*[1,2,3]*

[1] INSERM, UMR 1186, Villejuif, France, [2] Gustave Roussy Cancer Campus, Villejuif, France, [3] Faculty of Medicine, University Paris Sud, Le Kremlin Bicêtre, France

Among cells present in the tumor microenvironment, activated fibroblasts termed cancer-associated fibroblasts (CAFs), play a critical role in the complex process of tumor-stroma interaction. CAFs, one of the prominent stromal cell populations in most types of human carcinomas, have been involved in tumor growth, angiogenesis, cancer stemness, extracellular matrix remodeling, tissue invasion, metastasis, and even chemoresistance. During the past decade, these activated tumor-associated fibroblasts have also been involved in the modulation of the anti-tumor immune response on various levels. In this review, we describe our current understanding of how CAFs accomplish this task as well as their potential therapeutic implications.

Keywords: cancer, tumor microenvironment, cancer-associated fibroblasts, immune suppression, immunotherapy

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***Correspondence:**
Jerome Thiery
jerome.thiery@gustaveroussy.fr

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INTRODUCTION

It is now well admitted that tumor progression and metastasis formation do not only depend on cancer cell genetic and epigenetic defects but are also controlled by the tumor microenvironment (TME) (1, 2). The TME or stroma is composed of cells from endothelial, mesenchymal, and hematopoietic origins embedded in a complex extracellular matrix (ECM), which enter into a dynamic crosstalk with tumor cells, suitable for tumor growth. Consequently, different elements such as angiogenesis, hypoxia, ECM remodeling, interstitial pressure, metabolism changes have received recent attention as key determinants of the TME modifying cancer cell behavior and disease progression, with potential clinical applications (2, 3). Moreover, the TME is also clearly involved in shaping the cellular fate of tumor-infiltrating lymphocytes and the efficacy of the anti-tumor immune response. Indeed, during tumor progression, tumor cells proliferate under adverse host conditions and use several survival strategies to block the action of key regulators/effectors of the immune response and to circumvent anti-tumor defenses (4–6). Besides the several known classical strategies used by tumor cells to escape immune surveillance (such as down regulation of antigen expression, resistance to cell-mediated lysis or expression/secretion of immunosuppressive molecules), it should be noted that tumor cell evasion from immunosurveillance is also under the control of the TME complexity (7–9). The ability of tumors to orchestrate an immunosuppressive microenvironment is dependent on several mechanisms ultimately leading to the inhibition of various immune effector cells [such as cytotoxic T cell (CTL) or natural killer (NK) cells] or to the recruitment and stimulation in the TME of immunosuppressive cells [such as regulatory T cells (Tregs), type II macrophages or myeloid-derived suppressor cells (MDSCs)]. In particular, among the stromal cells, activated fibroblasts that share similarities with fibroblasts stimulated by acute or chronic inflammatory signals, activated during a wound healing process and observed during tissue fibrosis, also known as myofibroblasts, play a critical role in the complex process of tumor cell-stroma interaction (10–13) and have emerged

as important regulators of the anti-tumor immune response (14–16). Here, we will discuss the different mechanisms involved in the immuno-suppressive capabilities of activated fibroblasts in the TME, as well as their potential application for therapeutic intervention, especially in the field of cancer immunotherapy.

ORIGIN OF ACTIVATED FIBROBLASTS IN THE TME AND ROLE IN CANCER PROGRESSION

Fibroblasts are spindle-shaped, non epithelial (cytokeratin$^-$, E-cadherin$^-$), non endothelial (CD31$^-$) and non-immune (CD45$^-$) cells of a mesenchymal lineage origin (vimentin$^+$). In normal tissue, fibroblasts are usually considered as resting/quiescent cells with negligible metabolic and transcriptional activities (11), but with the ability to respond to growth factors to become activated. During this activation process, fibroblasts exhibit contractile activity, exert physical forces to modify tissue architecture, acquire proliferation and migration properties and become transcriptionally active leading to the secretion of several factors (cytokines, chemokines, etc.) and ECM components (17–19). The ability of resting fibroblasts to become activated was first observed in the context of wound healing (20) and subsequently in pathologic conditions such as acute or chronic inflammation or tissue fibrosis (a chronic wound healing response) (17, 21). This chronic tissue repair response also occurs in the context of cancer, considered as a "wound that never heals" (22). Indeed, emergence and/or accumulation of cancer cells in a given tissue represent a tissue injury, imitating a chronic wound healing response toward the tumor cells, also known as tumor fibrosis or desmoplastic reaction (23). Consequently, major players in tumor fibrotic microenvironment include activated fibroblasts, termed cancer-associated fibroblasts (CAFs), which represent one of the most abundant stromal cell types of several carcinomas including breast, prostate, pancreatic, esophageal, and colon cancers while CAFs are less abundant, but still present, in other neoplasias including ovarian, melanoma, or renal tumors (24). For example, in pancreatic cancer, 60–70% of the tumor tissue is composed of a desmoplastic stroma characterized by extensive collagen deposition and activated CAFs (25).

Several studies have clearly demonstrated that cancer cells can recruit and activate tissue resident fibroblasts in the stroma (26, 27). This phenomenon is mainly dependent on growth factors released by the cancer cells and also by infiltrating immune cells. In particular, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) secreted by tumor cells are key determinants of fibroblast activation and proliferation within the TME (28–31). Moreover, the secretion of interleukin (IL)-1β (IL-1β) by immune cells in early neoplasia has emerged as an initiator of nuclear factor-κB signaling in fibroblasts involved in their education and production of pro-tumorogenic and pro-inflammatory factors (32). Furthermore, emerging data suggest that the irreversible activation of CAFs might be driven by epigenetic alterations (33–36). Nevertheless, CAFs can also originate from other cell populations than resident fibroblasts through different mechanisms and depending on the tissue analyzed. Several other local sources of CAFs have been thus suggested. In breast, kidney, lung, and liver carcinomas, a portion of CAFs have been shown to potentially differentiate from epithelial cells *via* an epithelial-to-mesenchymal transition (EMT) (37, 38). A related process, termed endothelial-to-mesenchymal transition has been involved in the trans-differentiation of endothelial cells to a cell population with a CAF-like phenotype (39). Other cells linked to blood vessels, named pericytes, can trans-differentiate into CAFs in a PDGF-dependent manner (40). Moreover, in breast cancer, adipocytes were shown to differentiate in CAFs (41, 42). Finally, in liver and pancreas tumors, stellate cells, normally involved in organ regeneration, are involved in fibrosis preceding the occurrence of tumors, making them a possible source of CAFs (43, 44). Beyond these local sources, more distant one can be involved in CAFs recruitment/differentiation in the TME. In particular, mesenchymal stem cells, normally residing in the bone marrow, can be attracted in the TME to become an important source of CAFs (42, 45–48). Similarly, fibrocytes, a circulating mesenchymal cell population arising from monocytes precursors which are recruited to sites of chronic inflammation, can differentiate into CAFs after their recruitment into the TME (46, 49).

These various sources represent an important determinant that contributes to the heterogeneity of CAFs (**Figure 1**) and makes them difficult to distinguish from other cell types present in TME. In this context, morphology and spatial distribution are key determinants in order to identify fibroblasts in a resting or activated state (11). Different markers, which are lower or not expressed by their normal counterparts, can also be used to identify activated fibroblasts such as α-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP-1; also called S100A4), fibroblast-activation protein (FAP), PDGF receptors (PDGFR) α or β, neuron-glial antigen-2 (NG2), periostin (POSTN), podoplanin (PDPN), tenascin-C (TNC), desmin, CD90/THY1, or discoidin domain-containing receptor 2 (DDR2) (24, 50–57). However, it is crucial to note that none of these markers is specific for normal or activated fibroblasts, and that many activated fibroblasts may not express all of these markers at the same time, most likely reflecting the high degree of heterogeneity of CAFs in the TME, as well as possible different and opposite functions in the context of specific TMEs (24). It is indeed conceivable that, depending of the context, quiescent fibroblasts or the other cell types mentioned above might be capable of differentiating into distinct subsets of functional CAFs, with possible diverse functions, either pro- or anti-tumorigenic, as observed for type I and type II macrophages (11, 58). In other words, even if a large body of literature currently supports the tumor-promoting effect of CAFs, some evidence also suggests that CAFs might also restrain tumor growth. For example, the depletion of α-SMA$^+$ CAFs in pancreatic cancer accelerates tumor growth, induces immunosuppression by increasing the number of CD4$^+$Foxp3$^+$ Tregs in tumors and reduces survival (59). Similarly, the deletion of sonic hedgehog, a soluble ligand overexpressed by neoplastic cells in pancreatic ductal adenocarcinoma which drives the formation of a fibroblast-rich desmoplastic stroma, increases the aggressiveness of tumors (60). Nevertheless, for simplicity, we will focus the following part of this review on the

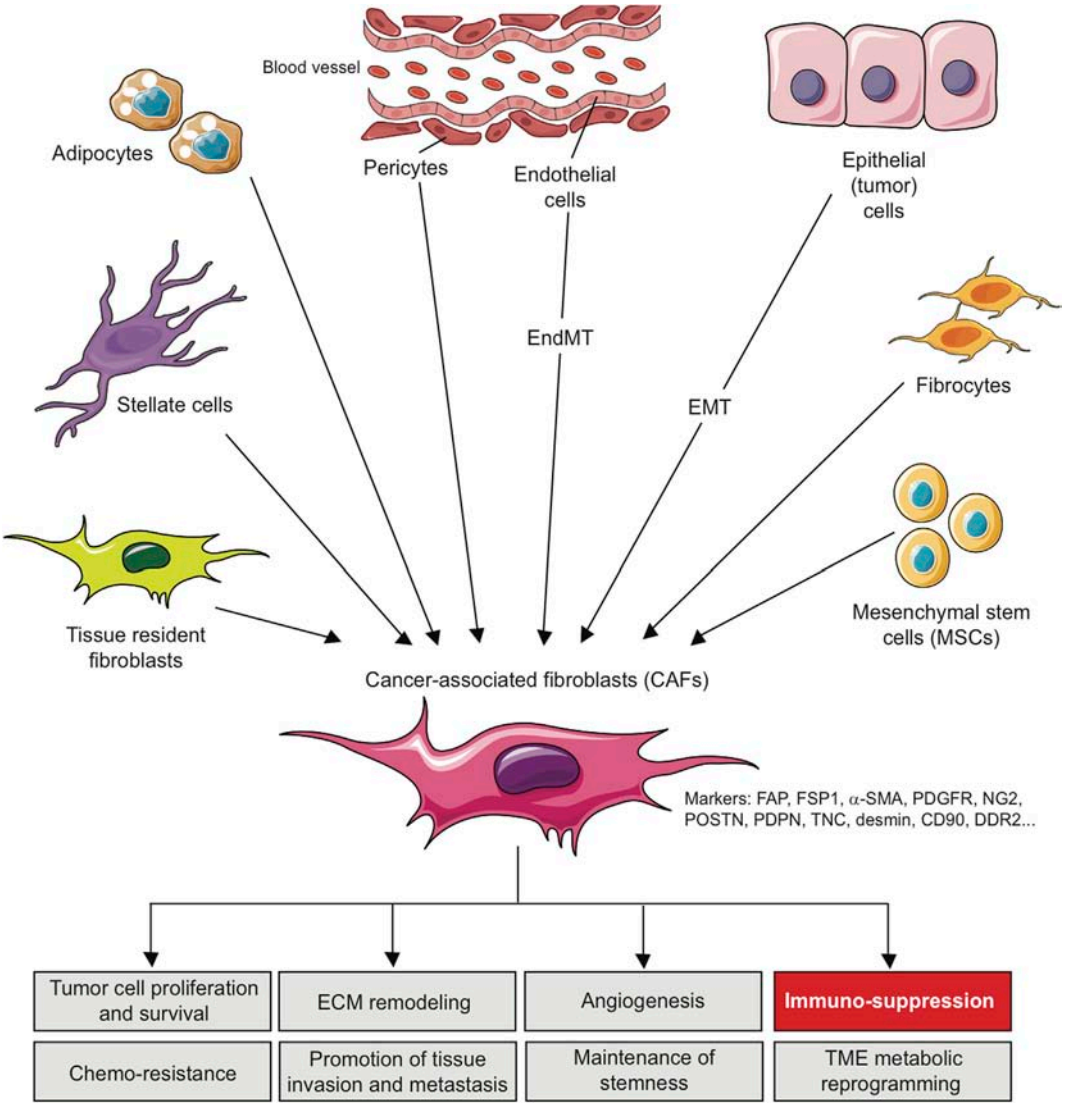

FIGURE 1 | Origins of cancer-associated fibroblasts in the tumor microenvironment (TME) and role in cancer progression. CAFs can originate from diverse cell populations through different mechanisms and depending on the tissue analyzed. Local sources of CAFs include activated tissue resident fibroblasts, trans-differentiated epithelial or endothelial cells resulting from an epithelial-to-mesenchymal transition (EMT) or an endothelial-to-mesenchymal transition (EndMT), trans-differentiated pericytes, adipocytes or stellate cells. Beyond those local sources, more distant one can be involved in CAFs recruitment/differentiation in the TME, including mesenchymal stem cells, normally residing in the bone marrow, and fibrocytes. The acquisition of a CAF phenotype is associated with the potential expression of a variety of CAF-related markers as indicated. In the TME, CAFs can affect several processes leading to tumor growth, as indicated, including immuno-suppression.

tumor-promoting and immunosuppressive capabilities of CAFs, unless otherwise stated.

In the tumor stroma, CAFs interact with tumor cells and other cell types and as a sign of their activation secrete several factors such as ECM proteins (e.g., collagens), ECM-remodeling enzymes such matrix metallo-proteinases (MMPs), proteoglycans (e.g., laminin, fibronectin), chemokines [e.g., C-X-C motif chemokine ligand 2 (CXCL2), CXCL12/SDF1, chemokine ligand 2 (CCL2/MCP-1), and CCL5/Rantes], vascularization promoting factors [e.g., vascular endothelial growth factor (VEGF)] and other factors/proteins which affect tumor cells proliferation, invasiveness, survival, cancer cell metabolism, and stemness [e.g., TGF-β, EGF, FGF, hepatocyte growth factor (HGF)]. Consequently, CAFs have been involved in tumor growth, cancer cell survival, angiogenesis, maintenance of cancer stemness, ECM remodeling, tissue invasion, metastasis, metabolic reprograming of the TME and even chemoresistance [see Ref. (10–13, 24, 61) for review] (**Figure 1**). During the past few years, these activated tumor-associated fibroblasts have also been involved in the modulation of the anti-tumor immune response by the secretion of immunosuppressive and pro-inflammatory factors, chemokines, and chemical mediators in the TME. As such, CAFs can potentially affect both innate and adaptive antitumor immune response and consequently tumor progression.

CAF-MEDIATED REGULATION OF THE INNATE ANTI-TUMOR IMMUNE RESPONSE

As mentioned above, several studies including gene signature or mass spectrometry analysis (62–66) have shown that CAFs exhibit a particular immunomodulatory secretome including, but not limited to, CXCL1, CXCL2, CXCL5, CXCL6/GCP-2, CXCL8, CXCL9, CXCL10, CXCL12/SDF1, CCL2/MCP-1, CCL3, CCL5/Rantes, CCL7, CCL20, CCL26, IL-1β, IL-6, IL-10, VEGF, TGF-β, indoleamine-2,3-dioxygenase (IDO), prostaglandin (PG) E2 (PGE2), tumor necrosis factor (TNF) or nitric oxide (NO). This secretion profile is thought to be a major player in shaping the TME, with multiple roles in tumor progression, but beyond its role on tumor cells, this CAFs-related secretome can potentially regulate the innate immune response in several ways (**Figure 2**).

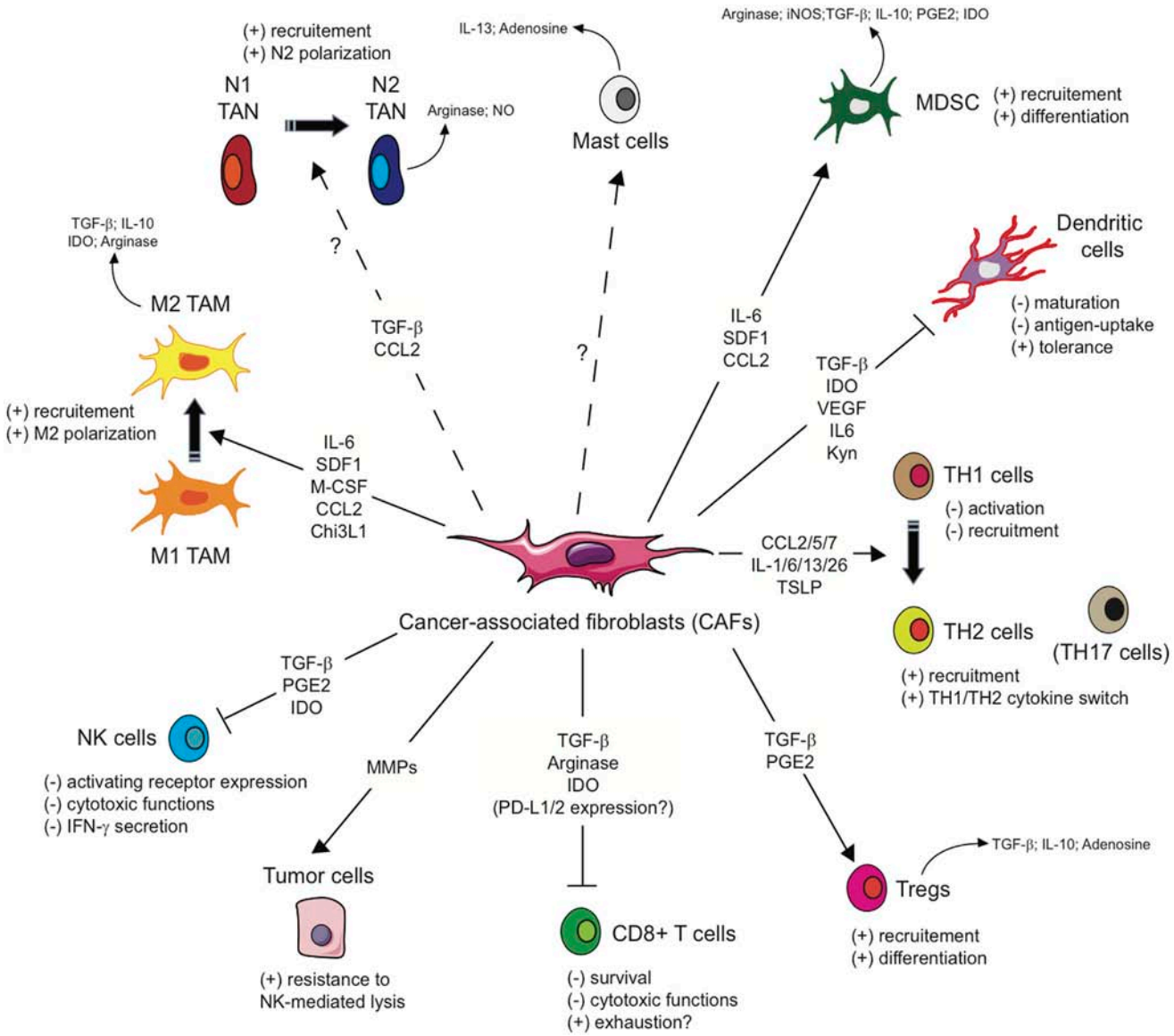

FIGURE 2 | Influence of cancer-associated fibroblasts on the regulation and function of immune cells involved in the antitumor immune response. Due to their secretion of the indicated cytokines, chemokines, or other soluble factors, cancer-associated fibroblasts (CAFs) shape the tumor microenvironment and influence both the innate and adaptive anti-tumor immune response. CAFs favor the recruitment of innate immune cells, such as tumor-associated macrophages (TAM) or potentially tumor-associated neutrophils (TAN), and their acquisition of an immunosuppressive phenotype (M2 and N2, respectively), affect cytotoxic function and cytokine production of natural killer (NK) cells, as well as the susceptibility of tumor cells to NK-mediated lysis, and activate mast cells with a potential immunosuppressive phenotype. CAFs favor the recruitment and differentiation of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) and interfere with the maturation and function or dendritic cells. CAFs have also the potential ability to influence $CD4^+$ Helper T (T_H) lymphocytes, favoring tumor-promoting TH2 and TH17 responses, and reduce the activation, functions, and survival of $CD8^+$ cytotoxic T cells.

In particular, CAFs are important players affecting another major stromal component within tumors, known as tumor-associated macrophages (TAMs) (67). Macrophages are mainly classified into two distinct types: "classically" activated (M1 or type I) and "alternatively" activated (M2 or type II) macrophages. M1 macrophages produce high amounts of pro-inflammatory cytokines and reactive oxygen species and have the capacity to orchestrate a T_H1 anti-tumor immune response. On the opposite, M2 macrophages play a significant role in tumor progression, promote tissue repair and angiogenesis, and are characterized by the production of immuno-suppressive factors such as IL10, Arginase, IDO and TGF-β, which inhibit cytotoxic $CD8^+$ T cell-mediated immune response in the TME (67). At least in some settings, CAFs actively promote the recruitment of monocytes to the TME and their differentiation toward M2 macrophages (68). In particular, the secretion of CXCL12/SDF1, macrophage colony-stimulating factor (M-CSF also known as CSF-1), IL-6, and CCL2/MCP-1 by CAFs actively promotes the recruitment of monocytes to the TME and their differentiation into a M2 immunosuppressive phenotype (69–74). It was also recently shown that Chitinase-3-like-1 (Chi3L1; YKL-40 in humans), a secreted glycoprotein involved in several diseases including chronic inflammatory conditions, fibrotic disorders and various types of cancer, is highly expressed in CAFs isolated from mammary tumors and pulmonary metastases in mice, and in the stromal compartment of human breast carcinomas, and enhances macrophage migration in the TME and their expression of an M2-like gene signature (75). Finally, the expression of both CAF (α-SMA^+, $FSP1^+$, and FAP^+) and M2 macrophages ($CD163^+$ and DC-$SIGN^+$) markers is associated with the poor clinical outcome of colorectal cancer and oral squamous cell carcinoma patients (76, 77), suggesting an association between these two cell types.

Cancer-associated fibroblasts are also potentially involved in the recruitment of neutrophils into the TME, notably through the secretion of CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, and CCL2. Tumor-associated neutrophils (TANs) have been linked to a poorer prognosis for patients with renal and pancreatic cancer; gastric, hepatocellular, colorectal, head and neck carcinomas, and melanoma (78). TAN-derived factors promote tumor cell proliferation, migration, and invasion, and also induce tumor vascularization by the production of pro-angiogenic factors. Moreover, the production of Arginase 1 (Arg 1) and NO by TANs in response to CXCL8 signaling has been linked to the inhibition of T cell functions (79, 80). Nevertheless, recent studies have suggested that TANs can be polarized to an N1 anti-tumoral or N2 pro-tumoral phenotype in the TME, as observed for TAMs. N1 neutrophils are induced upon TGF-β blockade and express immuno-activating cytokines and chemokines, low levels of Arg 1, and are able to kill cancer cells. On the opposite, N2 neutrophils are characterized by expression of CXCR4, VEGF, and MMP9 and are induced following exposure to high TGF-β levels (81) and inhibit $CD8^+$ T cell function by several mechanisms (82). At this point, it is thus uncertain whether CAFs can recruit TANs and drive them to an N2 phenotype in the TME, and whether this recruitment/polarization of TANs participates to the immunosuppressive activity of CAFs.

Another cell population has also been implicated in the complex CAFs-TME interaction. Mast cells, derived from $CD34^+$/$CD117^+$ pluripotent hematopoietic stem cells, are tissue resident sentinel cells that, upon activation, release a wide spectrum of chemokines and cytokines (83). Interestingly, it was demonstrated in pancreatic tumors that a complex interaction between mast cells and stellate cells (often described as CAF precursors) is able to activate mast cells, which in turn enhance CAF proliferation by their secretion of IL-13 and tryptase, favoring tumor growth (84). Of note, activated mast cells could not only increase tumor progression but might also alter the anti-tumor immune response. For example the release of free adenosine (85) or IL-13 by mast cells might, respectively, inhibit T cell function and promote M2 polarization (83, 86, 87). Mast cells can also promote the generation of highly suppressive MDSCs and Tregs in the TME (88, 89). However, whether CAF-mast cell interactions are linked to the immuno-suppressive capabilities of CAFs is also not clearly established and requires further investigations.

Finally, CAFs can also affect the activity of major innate effector cells, NK cells, which participate to the early immune response through their cytotoxic activity and contribute to the adaptive immune response by the secretion of cytokines and by the promotion of antigen-presenting cell maturation. As previously mentioned, CAFs are thought to be an important source of TGF-β in the TME (90, 91). TGF-β has been involved in the decrease of NK cell activation and cytotoxic activity (92). In this regard, TGF-β-induced miR-183 inhibits DAP12 transcription (a key accessory protein for relaying signals by NK cell receptors) and reduces the expression of the NK-activating receptor NKp30 and NK Group 2D (NKG2D) (93–95), resulting in a weak NK cell cytotoxic activity in the TME. TGF-β also reduces IFN-γ secretion by NK cells, which is important for stimulating effector $CD4^+$ T_H1 cells that are required for clearing tumors, notably by repressing T-bet expression through Smad 3 (96–98). Moreover, studies involving melanoma, hepatocellular, and colorectal carcinoma-derived fibroblasts have shown that CAFs can decrease the expression of several NK activating receptors (including NKp30, NKp44, and NKG2D) on the NK cell surface, as well as perforin and granzyme B expression, through the secretion of PGE2 and/or IDO (99–101) leading to an attenuated cytotoxic activity of NK cells against their tumor target cells. We also recently demonstrated that CAFs isolated from melanoma decrease the susceptibility of melanoma tumor cells to NK cell-mediated lysis through the secretion of active MMPs which cleave two ligands of the NK-activating receptor NKG2D, MHC class I-related chain (MIC)-A and MIC-B, at the surface of the tumor cells and consequently decrease the NKG2D-dependent cytotoxic activity of NK cells against melanoma tumor cells, as well as their secretion of IFN-γ (102).

In conclusion, due to their secretion of cytokines, chemokines, or other soluble factors, CAFs shape the TME and favor the recruitment of innate immune cells, such as monocytes or neutrophils, and their acquisition of an immunosuppressive phenotype, but also affect cytotoxic function and cytokine production of NK cells.

CAF-MEDIATED REGULATION OF THE ADAPTIVE ANTI-TUMOR IMMUNE RESPONSE

Based on the immunomodulatory secretome mentioned above, CAFs might also interfere with the adaptive anti-tumor immune response at different levels, leading to a disruption of T cell function in the TME (**Figure 2**).

In the TME, dendritic cells (DCs), the most important antigen-presenting cell population, have a pivotal role for the activation of T cell-mediated anti-tumor immunity (103). DC biology can potentially be affected by the CAF secretome in several ways. In particular, CAF-derived TGF-β can affect DC function (96). In response to TGF-β, DCs downregulate the expression of MHC class II molecules and of the co-stimulatory molecules CD40, CD80, and CD86, which are necessary for efficient antigen presentation, and of TNF-α, IFN-γ, and IL-12, that promote T cell recruitment and survival. The resulting immature or tolerogenic DCs alter $CD8^+$ cytotoxic T cell activation and the T_H1 polarization of $CD4^+$ helper T (T_H) cell populations and also promote the formation of $CD4^+FoxP3^+$ Treg cells that potently inhibit the function of other T cells (104, 105). CAFs can also secrete IL-6 and could affect DC functions through this way. Indeed, IL-6-mediated activation of the STAT3 pathway has been involved in the alteration of the DC maturation, disabling T cell activation and inducing T cell anergy and immune tolerance (106–108). Fibroblast-produced IL-6 was also reported to favor the emergence of TAMs from monocytes at the expense of DCs (69). Expression of tryptophan 2,3-dioxygenase (TDO2) by CAFs isolated from lung cancer also promotes tryptophan degradation in kynurenines (Kyn) that inhibits DCs differentiation and functions (109). Finally, CAF-derived VEGF, in addition to its pro-angiogenic effect, has multiple immunoregulatory roles (110). In particular, VEGF inhibits DC generation and maturation (111–114), notably by reducing their MHC class II expression and their ability to take up antigens.

The role of CAFs in regulating T cell activity and function in the TME has also been suggested by several studies. As mentioned earlier, CAFs can be an important source of TGF-β in the TME, which may act on both $CD8^+$ and $CD4^+$ T cells (96, 105). For example, TGF-β promotes cell death of effector $CD8^+$ T cells by inhibiting expression of the pro-survival protein Bcl-2 (115). TGF-β also directly alters cytotoxic $CD8^+$ T cell function by inhibiting the expression of key genes involved in their cytototoxic activity, including perforin, granzymes A and B, Fas ligand, and IFN-γ (116, 117). Furthermore, CAFs could also impair T cell proliferation and effector function through other mechanisms (118), notably depending on their production of metabolic reprogramming factors. The secretion by CAFs of IDO1 (119, 120), an immuno-regulatory enzyme, might contribute to immuno-suppression, tolerance, and tumor escape by catabolizing tryptophan degradation into kynurenines (Kyn), creating an immunosuppressive TME resulting in T-cell anergy and apoptosis through depletion of tryptophan and accumulation of immunosuppressive tryptophan catabolites (121, 122). Similarly, the secretion by CAFs of Arginase 2 (Arg 2), an enzyme metabolizing L-Arginine to L-Ornithine and urea, might participate to the deprivation of Arginine in the TME, which is in normal conditions important for T cell proliferation and functions (123). In this regard, pancreatic cancer suffering patients with CAFs expressing high levels of Arg 2, especially in hypoxia-inducible factor (HIF)-1α positive hypoxic zones, demonstrate a poor clinical outcome (124). CAFs can also secrete galectins, a class of carbohydrate binding proteins that have a high affinity for β galactosides (125, 126), which possess immunoregulatory properties (127) such as, for Galectin-1, induction of apoptosis of activated T cells by binding the glycoprotein receptors CD7, CD43, and CD45 on the cell surface (128, 129). Finally, the secretion of CXCL12/SDF-1 by CAFs from lung and pancreatic tumors can contribute to the exclusion of T cells from the cancer cell proximity (130).

Cancer-associated fibroblasts have also the potential ability to influence $CD4^+$ Helper T (T_H) lymphocytes, switching them from anti-tumor to pro-tumor cells. $CD4^+$ T_H cells can differentiate into multiple sublineages with different functions and cytokine secretion profiles, which in turn can induce, maintain or regulate antitumor immune responses (131). Schematically, naïve $CD4^+$ T cells can differentiate into T_{H1} cells mainly secreting IFN-γ and promoting $CD8^+$ T cell-dependent immune response, or into T_{H2} cells mainly secreting IL-4 and orchestrating humoral immunity. In terms of antitumor immune responses, the superior effects of T_H1 cells are thought to be the result of the production of large amounts of IFN-γ, as well as chemokines, which enhance the priming and expansion of antitumor $CD8^+$ cells and help to recruit NK cells and type I macrophages to tumor sites. A third major effector population of $CD4^+$ T cells that could be derived from naïve $CD4^+$ T cells was also shown to exist. These cells, designated T_H17 cells (132, 133), are characterized by the production of IL-17 and IL-22 and might have, at least under some circumstances, pro-tumor and immunosuppressive functions in the TME (134), even if this particular point remains highly controversial. Finally, under tolerogenic conditions, naïve $CD4^+$ T cell precursors can differentiate into inducible Tregs that upregulate the expression of the FoxP3 transcription factor (135). Depending on the tumor type, Tregs can be highly enriched in the TME, limiting antitumor immune responses and promoting immunological ignorance of cancer cells, especially through the secretion of immunosuppressive cytokines (TGF-β, IL-10…) (136). In the TME, the presence of CAFs and their secretion of CCL2, CCL5, and CCL17 as well as the polarizing cytokines IL-1, IL-6, IL-13, and IL-26 can favor a tumor promoting T_H2 and T_H17 immune response, as the expense of tumor protective T_H1 response (32, 137–139). For example, in a murine model of breast tumor, the elimination of CAFs *in vivo* by a DNA vaccine targeting FAP resulted in a shift of the immune TME from a T_H2 to a T_H1 polarization. This shift was characterized by an increased expression of IL-2 and IL-7, an increased of $CD8^+$ T cell population, and a diminished recruitment of TAM, MDSC, and Tregs (139). Moreover, in pancreatic cancer, the secretion of thymic stromal lymphopoietin (TSLP) by CAFs has been associated with a T_H2 cell polarization through myeloid DC conditioning (140). As a main source of TGF-β in the TME, CAFs can also promote Tregs recruitment and differentiation (141). Of note, it has been suggested that CAFs and Tregs enter to

a cross-talk *via* their reciprocal expression of TGF-β, increasing both CAFs activation and Tregs activity. In this regard, FoxP3$^+$ Tregs coexisting with CAFs are correlated with a poor outcome in lung adenocarcinoma (142). Moreover, it was shown that the expression of cyclo-oxygenase-2 (COX-2) by CAFs in lung or pancreatic cancers leads to their secretion of PGE-2, which plays an essential role in Tregs functionality by inducing FoxP3 expression (143, 144).

Cancer-associated fibroblasts in the TME can also interfere with the T cell-dependent immune response by modulating MDSCs. MDSCs are a heterogeneous population of immature myeloid cells that accumulate during pathologic conditions, such as cancer (145, 146). The main factors involved in MDSC-mediated immune suppression include the secretion of Arginase, iNOS, TGF-β, IL-10, PGE2 and IDO, regulating DC and T cell functions, as well as NK cells and macrophages. It has been demonstrated that CAFs isolated from pancreatic tumors drive monocyte precursors toward an MDSC phenotype, in a STAT3-dependent manner, through their secretion of IL-6 (72, 147). Similarly, CAFs from hepatic carcinomas attract monocytes to the TME by their secretion of CXCL12/SDF1 and induce their differentiation into MDSCs through IL-6-mediated STAT3 activation (148), thus altering T cell proliferation and functions, as well as the patients overall survival. Pancreatic stellate cells (described as CAFs precursors) also produce MDSC-promoting cytokines (IL-6, VEGF, M-CSF) and chemokines (CXCL12/SDF1, CCL2/MCP-1) and similarly promote differentiation of MDSCs in a STAT3-dependent manner (72). In a murine liver tumor model, it was also shown that FAP$^+$ CAFs are a major source of CCL2 and that fibroblastic STAT3-CCL2 signaling promotes tumor growth by enhancing the recruitment of MDSCs, which also predicts poor prognosis of patients with intrahepatic cholangiocarcinoma (149).

Finally, an interesting but still controversial point was recently raised based on the observation that CAFs from colon and lung cancers or from melanoma might express programmed death-ligand-1 (PD-L1) and/or PD-L2 (150–152). PD-L1 and PD-L2 are members of the B7 family of co-stimulatory/co-inhibitory molecules expressed by a wide range of cancer cells and engage their receptor programmed death receptor 1 (PD1) expressed on T-cells, strongly counteracting TCR signaling and CD28-co-stimulation (153), resulting in the inhibition of T cell activation, proliferation, and functions. As such, therapeutic antibodies that block PD-L1/PD1 interactions between cancer cells and T cells have recently received great attention because of their capacity to reverse T cell exhaustion in response to persistent antigen stimulation and to improve the immune control of cancer in a variety of tumor types, including melanoma, lung, and renal cell carcinomas (154). As mentioned above, it was shown that myofibroblasts/CAFs from colon cancer expressed PD-L1 and PD-L2 and negatively regulate CD4$^+$ T_H cell proliferative response (152). Similarly, CAFs isolated from lung carcinoma were shown to constitutively express PD-L1 and PD-L2, which can be upregulated by IFN-γ, and negatively regulate tumor-associated CD8$^+$ T cell activation (151). In melanoma, PD-L1 expression on CAFs seems to be dependent of IL-1α/β secreted by melanoma tumor cells and melanocytes and could participate to the suppression of melanoma-specific CD8$^+$ T cells (150). However, most of these discoveries rely on CAFs isolation and *in vitro* experiments, with potential artifacts (155), and clearly require further investigations to determine the physiological relevance of potential PD-L1/L2 expression by CAFs on their immunosuppressive capabilities *in vivo*.

In conclusion, the CAF secretome can shape the T cell-dependent antitumor immune response by affecting several populations such as DCs, MDSCs, by switching CD4$^+$ T_H lymphocytes from a T_{H1} to a T_{H2} phenotype, by affecting Tregs and T_H17 cells, by affecting CD8$^+$ T cell functions or eventually by expressing some ligands of immune checkpoint receptors.

INDIRECT EFFECT OF CAFs ON ANTI-TUMOR IMMUNE RESPONSE

As mentioned earlier, CAF activation in the TME results in a remodeling of the ECM through deposition of several components and by proteolytic degradation, which in turn affect tumor behavior (18, 156, 157). For example, increased ECM rigidity resulting from thickening and linearization of collagen fibers has been shown to regulate tumor growth and metastasis (158, 159). This modified ECM protein network is also presumed to restrict access of immune cells to cancer cells, serving as a physical barrier at least in some models (160, 161). As such, CAF-modified ECM might be involved in T cell exclusion from the proximity of cancer cells, which has been shown as a dominant immunosuppressive mechanism in multiple cancers and a predictor of patient clinical outcome (160). In this regard, in pancreatic tumor models, it has been proposed that when fibrosis is extensive, the "scar-like" ECM may act as a barrier for CTL infiltration into tumors (162). It was also found that focal adhesion kinase [FAK; a crucial signaling protein that is activated by numerous stimuli and functions as a biosensor to control cell motility (163)] activity is elevated in human pancreatic ductal adenocarcinoma tissues and correlates with high levels of fibrosis and poor CD8$^+$ CTL infiltration (164). Similarly, in lung cancers, CAFs could restrict CD4$^+$ and CD8$^+$ T cells motility. Indeed, it was observed an active T cell motility in loose fibronectin and collagen regions, whereas T cells poorly migrate in dense matrix areas. Furthermore, aligned fibers in perivascular regions and around tumor epithelial cell regions dictate the migratory trajectory of T cells and restricted them from entering tumor islets (165, 166). Finally, interactions between tumor cells and the surrounding modified ECM have been involved as primary forces driving the EMT process. Consequently, the imbalanced biomechanical force at the tumor-stroma interface is an important player initiating EMT (167), which can subsequently lead to tumor cells escaping from T cell-mediated lysis after their acquisition of a mesenchymal-like phenotype (168–170). Thus, in the region where the ECM has been extensively modified by CAFs, an EMT process could protect tumor cells from T cell-mediated destruction.

The CAF-mediated remodeling of the ECM might also affect other immune population than T cells. For example, CAFs have been identified as an important source of hyaluronan, also called hyaluronic acid, a component of the ECM which promotes TAM recruitment, as the genetic ablation of the hyaluronan synthase strongly diminishes their presence within the TME (171). In

pancreatic and breast cancers, it was also found that extensive deposition of type I collagen, which can be highly secreted by CAFs, improves TAM infiltration (172), with a potential effect of the ECM composition on their M2 polarization (173, 174). The high levels of CAF-secreted collagen I in tumors could also activate leukocyte-associated Ig-like receptor (LAIR)-1, a collagen-receptor that inhibits immune cell function upon collagen binding (175). Nevertheless, the regulation of macrophages polarization by the ECM composition, as well as its effect on, but not limited to, MDSC, neutrophils, or DCs is still poorly understood.

In addition to the extensive remodeling of the ECM, CAFs might also indirectly regulate the anti-tumor immune response by participating in the emergence of hypoxic stress within the TME. Indeed, in tumors with a high level of fibrosis, tumor tissues are often poorly oxygenated, with a limited number of functional blood vessels, resulting in the presence of zones with a low oxygen pressure called "hypoxic zones" (16, 176, 177). Even if, as mentioned above, CAFs are described as regulators of angiogenesis through the secretion of pro-angiogenic factors, such as VEGF or through the recruitment of endothelial progenitors in the tumor through the release of SDF-1 in the TME (178), the blood vessels present in the TME are poorly functional and leaky. The resulting leaky vessels not only trigger a high interstitial fluid pressure in the TME which affect immune cell transmigration from the vessels to the TME (179), but also affect oxygen availability and acidification of the TME (180). In other words, by their global action on the TME, the presence of CAFs might participate to abnormal angiogenesis and to the creation of hypoxic zones that contribute to the immunosuppressive network within the TME. Indeed, hypoxia has been found to impair the antitumor immune response by several mechanisms (181–184), such as alteration of NK and T cell activation and effector functions, induction of PD-L1 expression on MDSCs *via* HIF-1α transcription factor, and attraction of TAMs or Tregs to the tumor bed. Furthermore, hypoxic tumor cells secrete factors including TGF-β and PDGF that promote conversion of precursor cell types into CAFs (185), and it was also shown that stromal fibroblasts synergize with hypoxic stress to enhance melanoma aggressiveness (186). This indicates a potential role of hypoxia in the CAFs activation, either by directly acting on CAFs or indirectly by acting on tumor cells, or in their function in the TME. Thus, one may consider that hypoxia not only promotes CAFs activation but might also increase their immunosuppressive properties, even if this last particular point needs to be clarified.

Overall, CAFs might indirectly affect the anti-tumor immune response, with many described and not yet elucidated distinct possibilities, such as the modification of the ECM, vasculature or architecture of the tumors, which make this field very challenging.

TARGETING CAFs TO IMPROVE ANTI-TUMOR IMMUNE RESPONSE AND IMMUNOTHERAPY

Given the fact that CAFs impair the anti-tumor immunity (and more generally exert pro-tumorigenic effects) by several mechanisms, the design of pre-clinical or clinical studies in order to target these cells in the TME is very seductive to amplify the antitumor immune response and to develop "anti-CAF"-based immunotherapeutic approaches. Such studies can be envisioned based on agents directly targeting CAF specific proteins (e.g., FAP…) and signaling pathways involved in CAF activation (e.g., TGF-β, PDGF, FGF…) or less specifically targeting CAF-secreted factors. Potential therapies aiming at targeting CAFs or reversing the CAF "state," as well as the ongoing clinical trials have been extensively reviewed in Ref. (18).

Recently, anti-CAF therapies have been mainly focused on FAP (187). A pioneer study has shown, in a transgenic mouse model in which FAP-expressing cells can be ablated, that the depletion of FAP-expressing cells cause rapid hypoxic necrosis of both Lewis lung carcinoma and stromal cells in immunogenic tumors by a process involving IFN-γ and TNF-α, which have previously been shown to be involved in $CD8^+$ T cell-dependent killing of tumor cells (188). The development of chimeric antigen receptor (CAR) T cells targeting FAP has also shown promising results in murine models (189–191) and in malignant pleural mesothelioma patient derived xenograft models (192). A recent study has also demonstrated in two murine melanoma models that depleting FAP^+ stromal cells from the TME upon vaccination with an adenoviral-vector reduced the frequencies and functions of immunosuppressive cells, resulting in prolonged survival of melanoma-bearing mice associated with a robust $CD8^+$ T cell response (193). Similarly, in LL2 (murine lung cancer), CT26 (murine colon cancer), and B16F10 (murine melanoma) models, a whole-tumor cell vaccine modified to express FAP seems to induce antitumor immunity against both tumor cells and CAFs and enhances the infiltration of $CD8^+$ T lymphocytes and decreases the accumulation of immunosuppressive cells in the TME (194). Nevertheless, it should be noted that, in addition to CAFs, FAP can be expressed by cells present in several tissues, including multipotent bone marrow stem cells or skeletal muscles. As such, another study has shown that adoptive transfer of FAP-reactive CAR-T cells into mice bearing a variety of subcutaneous tumors mediated limited antitumor effects and induced significant cachexia (a syndrome of progressive weight loss, anorexia, and persistent erosion of body muscle mass) and lethal bone toxicities in two murine strains (195). Thus, these lethal bone toxicity and cachexia observed after CAR T cell-based immunotherapy targeting FAP highlight cautions against its use as a universal target.

As such, targeting the CAF "secretome" or activation pathways, in order to revert the CAF "state," might be a safer alternative to abrogate, at least partly and probably less specifically, their immunosuppressive role in the TME. In this regard, a recent publication demonstrated that targeting CXCL12 from FAP-expressing CAFs with AMD3100 (Plerixafor) synergizes with anti-PD-L1 immunotherapy in pancreatic cancer (130). Similarly, other proteins secreted by CAFs could be also targeted in order to restrain the immunosuppressive capabilities of these cells, such as IL-6 or TGF-β, using multiple inhibitors (18). For example, trihydroxyphenolic compounds were identified as potent blockers of TGF-β1 in the presence of active lysyl oxidase-like 2 (LOXL2; a member of mammalian copper-dependent LOX enzymes only

expressed by fibroblasts or cancer cells and involved in intra- and intermolecular covalent collagen cross-links), and induce potent blockade of pathological collagen accumulation *in vivo* (196). Thus, these compounds might interfere with the T cell exclusion mediated by the CAF-dependent ECM remodeling previously mentioned, even if this particular point is still hypothetical. The use of Tranilast (Rizaben) (a known suppressor of fibroblast proliferation and TGF-β secretion) has also demonstrated a synergistic effect with a DC-based vaccine in C57BL/6 mice bearing syngeneic E-G7 lymphoma, LLC1 Lewis lung cancer or B16F1 melanoma (197). Another example is retinoic acid, a small molecular derivative of vitamin A, which inhibits IL-6 and ECM production by CAFs (198), potentially affecting their immunosuppressive properties. Nevertheless, more studies are clearly needed to identify other potential therapeutic agents targeting CAFs and/or their immunosuppressive network, which might be use in combination with the current or future anti-tumor immunotherapeutic approaches.

CONCLUDING REMARKS

Despite their relative abundance in tumors, fibroblasts have been ignored over decades, but their crucial role has now emerged in the fields of tumor biology and oncology. CAFs have pleiotropic functions in tumor growth and participate to the inflammatory phenotype of the TME by releasing a variety of chemokines, cytokines, and other factors leading to the alteration of the antitumor immune response. Nevertheless, this complex immunosuppressive network related to the "secretome" of CAFs is still poorly understood, even if extensive efforts allowed apprehending their role in both the innate and the adaptive immune response. Of note, the notion that the CAF-specific secretome modulates the anti-tumor immune response often relies on studies limited to cells expanded *in vitro*. Future challenging studies using preclinical models will be thus needed in order to define more precisely the functional list of CAF-derived factors that exert an immunomodulatory role in the context of the TME complexity *in vivo*. This is crucial in order to fully understand the global regulation of the antitumor immune response and might also lead to the identification of novel potential therapeutic targets with the ability to increase the efficiency of anti-tumor immunotherapeutic approaches. In particular, targeting the CAFs or their secretome may probably not induce a complete tumor cell death by itself, but it will help to reduce immune effector cell dysfunctions as well as the recruitment of immunosuppressive cells, thus releasing the "brake" for a more effective immune response in combination with therapy targeting immune checkpoints (e.g., anti-CTLA4, anti-PD1/PD-L1 antibodies) or other mechanisms impairing the anti-tumor immune response in patients (199).

AUTHOR CONTRIBUTIONS

JT wrote the manuscript. LZ and SC participate to helpful discussion and edited the manuscript.

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REVIEW
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Targeting Myeloid-Derived Suppressor Cells to Bypass Tumor-Induced Immunosuppression

*Viktor Fleming[1,2], Xiaoying Hu[1,2], Rebekka Weber[1,2], Vasyl Nagibin[1,2], Christopher Groth[1,2], Peter Altevogt[1,2], Jochen Utikal[1,2] and Viktor Umansky[1,2]**

[1] Skin Cancer Unit, German Cancer Research Center (DKFZ), Heidelberg, Germany, [2] Department of Dermatology, Venereology and Allergology, University Medical Center Mannheim, Heidelberg University, Mannheim, Germany

The immune system has many sophisticated mechanisms to balance an extensive immune response. Distinct immunosuppressive cells could protect from excessive tissue damage and autoimmune disorders. Tumor cells take an advantage of those immunosuppressive mechanisms and establish a strongly immunosuppressive tumor microenvironment (TME), which inhibits antitumor immune responses, supporting the disease progression. Myeloid-derived suppressor cells (MDSC) play a crucial role in this immunosuppressive TME. Those cells represent a heterogeneous population of immature myeloid cells with a strong immunosuppressive potential. They inhibit an antitumor reactivity of T cells and NK cells. Furthermore, they promote angiogenesis, establish pre-metastatic niches, and recruit other immunosuppressive cells such as regulatory T cells. Accumulating evidences demonstrated that the enrichment and activation of MDSC correlated with tumor progression, recurrence, and negative clinical outcome. In the last few years, various preclinical studies and clinical trials targeting MDSC showed promising results. In this review, we discuss different therapeutic approaches on MDSC targeting to overcome immunosuppressive TME and enhance the efficiency of current tumor immunotherapies.

Keywords: myeloid-derived suppressor cells, immunosuppression, cancer immunotherapy, tumor microenvironment, therapeutic targeting

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****Correspondence:***
Viktor Umansky
v.umansky@dkfz.de

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INTRODUCTION

Immunosuppression is a hallmark of most cancer entities and is pivotal for cancer growth and progression (1, 2). In recent years, accumulating data highlighted myeloid-derived suppressor cells (MDSC) as one of the main driver of an immunosuppressive tumor microenvironment (TME) (3). Their accumulation and activation correlated with tumor progression, metastasis, and recurrence of many types of tumors. In addition, the efficacy of immunotherapy was negatively

Abbreviations: ARG1, arginase-1; ATRA, all-trans retinoic acid; bFGF, basic fibroblast growth factor; CD, cluster of differentiation; CCL, C-C motif chemokine ligand; CCR, C-C motif receptor; COX, cyclooxygenase; CXCL, C-X-C motif ligand; CXCR, C-X-C motif receptor; DC, dendritic cell; ERK, extracellular-signal regulated kinase; EV, extracellular vesicles; HSP, heat shock protein; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LLC, Lewis lung carcinoma; LOX, lectin-type oxidized low-density lipoprotein receptor; IMC, immature myeloid cells; M, monocytic; MCP, monocyte chemoattractant protein; MDSC, myeloid-derived suppressor cells; MMP, matrix metalloproteinases; NO, nitric oxide; NSCLC, non-small cell lung cancer; PD-1, programmed death receptor; PD-L1, programmed death ligand 1; PMN, polymorphonuclear; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TGF, transforming growth factor; TLR, toll-like receptor; TME, tumor microenvironment; TNF, tumor necrosis factor; Treg, regulatory T cells; VEGF, vascular endothelial growth factor.

correlated with an increased MDSC frequency and activity (4, 5). Therefore, targeting MDSC becomes a promising treatment approach to overcome tumor progression and tumor-mediated immunosuppression.

Myeloid-derived suppressor cells represent a heterogeneous population of immature myeloid cells (IMC) that fail to terminally differentiate and exhibit a strong capacity to suppress the functions of T and NK cells (6–9). Under healthy conditions, IMC differentiate into macrophages, dendritic cells (DCs), or granulocytes. During an acute inflammation, IMC expand and differentiate mainly into monocytes and activated neutrophils (7). This process, known as myelopoiesis, is essential to protect the host from pathological conditions. In contrast to acute inflammation, chronic inflammation and cancer are characterized by a persistent release of signals of low stimulatory intensity (10–12). Although these stimuli still activate myelopoiesis, the accumulating IMC fail to completely differentiate into activated neutrophils and monocytes. Instead, the long-term inflammatory signals create conditions for the expansion and activation of MDSC (13, 14). They migrate to the site of inflammation, lymphoid organs, and pre-metastatic niches and promote tumor progression by immunological and non-immunological mechanisms (15). **Figure 1** illustrates the biology and functions of MDSC during tumor progression.

PHENOTYPE OF MDSC

Myeloid-derived suppressor cells consist of two major subpopulations, which are traditionally described by their phenotypical and morphological characteristics. The first population is called monocytic MDSC (M-MDSC), whereas the second is polymorphonuclear MDSC (PMN-MDSC) (8), which was previously known as granulocytic MDSC (6). Both MDSC subsets can be found under pathological conditions in the bone marrow, spleen, lung, peripheral blood, and tumor tissue; in most cancer entities, PMN-MDSC represent more than 80% of all MDSC (16). In mice, M-MDSC are defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$ and share phenotypical and morphological characteristics with monocytes. PMN-MDSC are described as $CD11b^{+}Ly6G^{high}Ly6C^{low}$ cells and resemble neutrophils (16, 17). In human, M-MDSC are defined as $CD11b^{+}CD14^{+}CD15^{-}HLA\text{-}DR^{low/-}$ cells. Due to the low or absence of the HLA-DR expression, M-MDSC they can be distinguished from monocytes. Human PMN-MDSC are characterized as $CD11b^{+}CD14^{-}CD15^{+}HLA\text{-}DR^{-}$ or

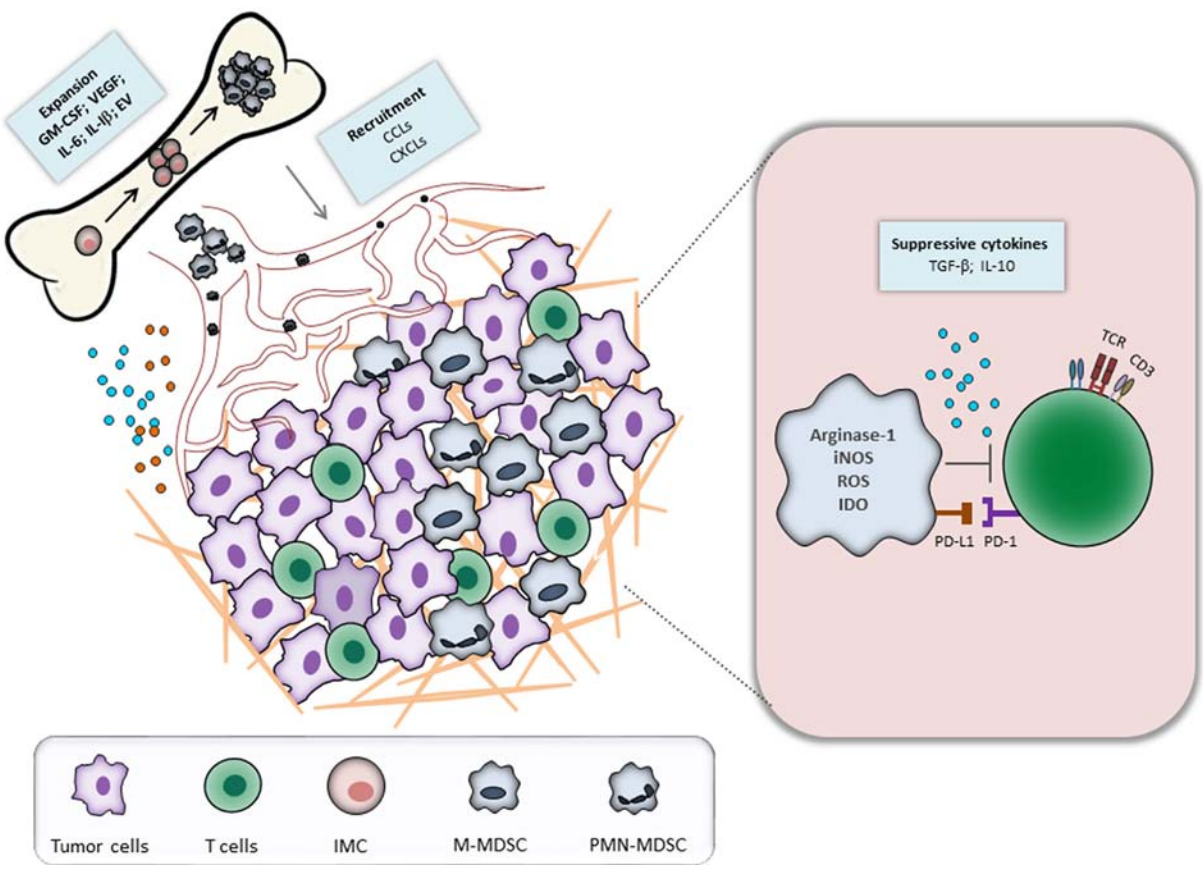

FIGURE 1 | Myeloid-derived suppressor cells (MDSC) recruitment and activation during tumor progression. Tumor and immune cells constantly release inflammatory mediators, leading to the dysregulation of normal myelopoiesis and to the conversion of immature myeloid cells (IMC) into MDSC in the bone marrow. The latter cells expand and migrate to the tumor site through the interaction between CCR and respective chemokines (CCL). In the tumor microenvironment, MDSC are activated and strongly inhibit an antitumor reactivity of T cells *via* various mechanisms.

$CD11b^+CD14^-CD66b^+$ (17, 18). In addition, a subset of more immature human MDSC characterized as Lin^- (including CD3, CD14, CD15, CD19, CD56) HLA-DR^-CD33^+ cells was defined as early-stage MDSC (eMDSC) (17). At the moment, the mouse equivalent of eMDSC is not clearly determined. Recently, a new marker for human PMN-MDSC has been proposed; they were found to express lectin-type oxidized LDL receptor-1 (LOX-1) that can discriminate them from neutrophils (19).

CONVERSION OF IMC INTO MDSC BY TUMOR-DERIVED EXTRACELLULAR VESICLES (EV)

Expansion and activation of MDSC could be stimulated by many soluble factors, which are predominately released within the TME by tumor and immune cells (20). Specifically, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF, macrophage CSF, stem cell factor, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), prostaglandin E2, cyclooxygenase 2, S100A9, S100A8, interleukin (IL)-1β, IL-6, and IL-10 are considered to be crucial for MDSC expansion (6, 8, 21–23). Furthermore, tumor cells can stimulate the secretion of these inflammatory mediators by cancer-associated fibroblasts and vice versa leading to an autocrine loop, which promotes tumor growth by converting myeloid cells into MDSC (20).

In addition to soluble inflammatory factors, tumor-derived EV could contribute to the generation of MDSC. EV consist of microvesicles that are created by the outward budding of the plasma membrane and exosomes, which are generated through the endosomal system (24). Due to their phospholipid bilayer, EV are stable vehicles to carry biological active molecules (25). It was shown that tumor-derived EV are predominately taken up by MDSC (26). After the uptake of EV derived from a Lewis lung carcinoma (LLC) and glioma, MDSC displayed an increased expression of immunosuppressive molecules like arginase-1 (ARG1) and programmed death ligand 1 (PD-L1) (26). Filipazzi et al. (27) demonstrated that $CD14^+$ monocytes lost the expression of HLA-DR and acquired an immunosuppressive activity upon EV uptake. In contract, EV from healthy donors were not able to convert monocytes into MDSC-like cells (27). Several studies showed that EV trigger toll-like receptor (TLR) signaling in myeloid cells. THP-1 monocytic cell line showed increased production of inflammatory molecules like IL-1β, IL-6, and TNF-α upon the EV treatment, which was due to TLR2 and TLR4 signaling (28, 29). Chalmin et al. (30) demonstrated that tumor-derived EV triggered the expansion and activation of murine and human MDSC *via* HSP72 that stimulated TLR2 signaling. Furthermore, by using the B16 transplantable melanoma model, it was shown that tumor EV could facilitate formation of metastasis through the transfer of the Met receptor tyrosine kinase to bone marrow cells (31). As the bone marrow cells were not further characterized, it is conceivable that such melanoma-derived EV converted bone marrow-derived IMC into potent MDSC.

IMMUNOSUPPRESSION INDUCED BY MDSC

Myeloid-derived suppressor cells use a broad range of suppressive molecules to inhibit antitumor reactivity of immune cells, supporting thereby tumor growth and metastasis. By inhibiting the activity of tumor-infiltrating lymphocytes, MDSC show their extraordinary potential of silencing the immune response (6–11, 16–18, 32, 33). One of the main immunosuppressive mediators is ARG1, which is an essential enzyme for the urea cycle (34, 35). It converts L-arginine into L-ornithine and urea, leading to the depletion of L-arginine. The lack of L-arginine causes a translational blockade in infiltrating T cells leading to cell cycle arrest in G_0-G_1 (36). Furthermore, T cells become anergic due to the downregulation of the T cell receptor (TCR) ζ-chain, which is essential for TCR signaling (37). Besides ARG1, MDSC express also of inducible nitric oxide synthase (iNOS), which also catabolize L-arginine. The main product of the reaction is nitric oxide (NO) that could induce T cell anergy (16) and nitrosylate important mediators of the IL-2 pathway (38). MDSC express also elevated levels of indoleamine 2,3-dioxygenase (IDO) that degrade L-tryptophan into *N*-formylkynurenine. The lack of tryptophan results in the cell cycle arrest in T cells and induces anergy (39). Moreover, tryptophan starvation is known to drive the differentiation of $CD4^+$ T cells into immunosuppressive regulatory T cells (Treg) (40). Kynurenine and 3-hydroxykynurenine, the products of IDO activity, exert also immunosuppressive functions, inhibiting effector T cell survival and proliferation (41). In addition, kynurenine drives the differentiation of $CD4^+$ T cells into Treg and induces apoptosis in thymocytes (42, 43). Kynurenine was also reported to dampen NK cell function and proliferation (44). Furthermore, reactive oxygen species (ROS) produced by MDSC in high concentrations were shown to induce T cell apoptosis (9, 11, 16) In addition, ROS was demonstrated to downregulate the expression of TCR ζ-chain, leading to impaired TCR signaling (10, 16, 17). Reacting with NO, ROS form peroxynitrite, which nitrosylates the TCR, resulting in T cell anergy (45). MDSC also secrete immunosuppressive cytokines and growth factors such as TGF-β and IL-10 that reduce antitumor activity of effector T cells and recruit Treg (46, 47).

It has been recently described that MDSC could exert their immunosuppressive effects *via* upregulation of PD-L1 (48, 49). Upon the binding of PD-L1 to the PD-1 receptor expressed on T cells, they become anergic, losing their ability to produce interferon (IFN)-γ and IL-2 (48). Moreover, MDSC were shown to express the death receptor CD95 and induce T cell apoptosis *via* CD95 ligand expressed on activated T cells (50).

NON-IMMUNOLOGICAL WAYS OF PROMOTING TUMOR PROGRESSION

In addition to the establishment of an immunosuppressive TME, MDSC could promote tumor progression by non-immunological mechanisms (51). In particular, MDSC produce large amounts of matrix metalloproteinases (MMP), especially

MMP9, which process the extracellular matrix and basal membrane and enable the tumor to leave the tissue, to enter the blood stream, and migrate to the site of later metastasis (52). It was shown that the pre-metastatic niche is performed before the tumor cells enter the blood stream (53). This process is still not fully understood but studies have confirmed that MDSC play an essential role (9, 54). It was found that MDSC accumulated in pre-metastatic niches with the help of monocyte chemoattractant protein-1 that dampens the activity of NK cells, which are also preferably found in the pre-metastatic niche (55). In addition, it was reported that MDSC produce MMP9 within the pre-metastatic niche, facilitating the penetration of metastatic cells (56). A further hallmark of tumor progression is angiogenesis that is crucial for the nutrition, vasculature, and dissemination of the tumor (57). MDSC promote angiogenesis by secreting elevated levels of VEGF and basic fibroblast growth factor (bFGF) (58). It was reported that blocking of angiogenesis resulted in the inhibition of tumor migration and formation of metastasis (59).

CORRELATION BETWEEN TUMOR BURDEN, RESISTANCE TO IMMUNOTHERAPY, AND MDSC

The expansion of MDSC has been demonstrated in many types of human tumors (6, 7). Moreover, elevated levels of MDSC were found not only in solid tumors but also in blood of non-Hodgkin lymphoma and multiple myeloma patients (18). Importantly, the frequency of circulating MDSC was found to correlate with the disease stage. It was reported that patients with stage III and IV hepatocellular carcinoma, melanoma, non-small cell lung cancer, pancreatic, esophageal, gastric, and bladder cancer had higher frequencies of MDSC in the peripheral blood as compared to stage I and II patients (60–63). In addition, an association between MDSC numbers and clinical response to radio-, chemo-, and immunotherapy was reported (64). Several recent studies described that in melanoma patients treated with the immune checkpoint inhibitor, ipilimumab, decreased amounts and immunosuppressive functionality of both M- and PMN-MDSC correlated with beneficial therapeutic effects (65–68). Altogether, these studies show that MDSC could be not only promising biomarkers for the survival of patients and the treatment efficacy but also could serve as a valuable target in combined immunotherapy of cancer patients.

MDSC TARGETING IN CANCER

In recent years, increasing numbers of preclinical and clinical studies were performed to target MDSC with beneficial effects, resulting in the tumor growth inhibition and the survival prolongation. The MDSC modulation was achieved by (i) the inhibition of their immunosuppressive activity; (ii) the blockade of MDSC recruitment to the tumor site; and (iii) the regulation of myelopoiesis and/or depletion of MDSC in the tumor-bearing hosts (**Figure 2**). Ongoing clinical trials are summarized in **Table 1**.

INHIBITION OF MDSC-MEDIATED IMMUNOSUPPRESSION

In preclinical mouse models, it has been demonstrated that inhibitors of phosphodiesterase-5, sildenafil, and tadalafil significantly inhibited the MDSC functions by the downregulation of iNOS and ARG1 activities, leading to the activation of antitumor immunity and the prolongation of survival of tumor-bearing mice (69–71). Recent clinical trials with tadalafil in patients with head and neck squamous cell carcinoma and melanoma confirmed this positive effect (72–74). It was shown that decreased amounts of MDSC and their immunosuppressive pattern correlated with an increased T cell reactivity and improved clinical outcome of advanced cancer patients.

A class I histone deacetylase inhibitor, entinostat, has been recently evaluated in several preclinical tumor models for its ability to affect MDSC functions (75, 76). The authors demonstrated that entinostat reduced the expression of ARG1, iNOS, and COX2 in both M- and PMN-MDSC subsets. In addition, they observed a strong reduction of tumor-infiltrating macrophages, suggesting a strong effect of this drug on the innate immunity. Interestingly, the combination of entinostat with anti-PD-1 antibodies significantly increased survival and delayed tumor growth in mice with LLC and renal cell carcinoma as compared to the treatment with anti-PD-1 antibodies alone. A combined therapy with nivolumab and entinostat in renal cell carcinoma patients is now planned.

A further promising way to target MDSC is the blockade of the activation of STAT3, which is a main transcription factor for immunosuppressive activity in myeloid cells (77). In the past, a number of clinical trials have been performed to target STAT3 with small molecular inhibitors with a limited efficacy and broad side effects (78). Recently, a new possibility to target STAT3 has been tested, in which STAT3 siRNA or decoy oligonucleotides were used to interfere with STAT3 mRNA (78). At the moment, several STAT3 oligonucleotide inhibitors, in particular AZD9150, were applied in the combination with immune checkpoint inhibitors in the frame of the phase I/II clinical trial (**Table 1**). In another approach, STAT3 siRNA or decoy oligonucleotides were coupled to CpG oligonucleotides, which are well-known agonists of TLR9 (79, 80). By this technique, a selective delivery of the drugs to TLR9-positive cells was ensured. Upon the treatment, TLR9-expressing myeloid cells (in particular, PMN-MDSC) displayed a decreased immunosuppressive activity, whereas TLR9-positive tumor cells lost the resistance to apoptosis *via* the STAT3 signaling (79, 80).

A further possibility to target MDSC is the modulation their metabolic pathways (81, 82). It was shown that tumor-infiltrating MDSC displayed an upregulation of the fatty acid translocase, CD36, which resulted in an increased uptake and oxidation of fatty acids. Accumulated lipids were reported to further increase an immunosuppressive capacity of MDSC in a STAT3- and STAT5-dependent manner (83). Pharmacological inhibition of the fatty acid oxidation decreased the immunosuppressive capacity of MDSC and in combination with low-dose chemotherapy and adoptive cellular therapy resulted in antitumor effect in LLC and colon adenocarcinoma mouse models (81).

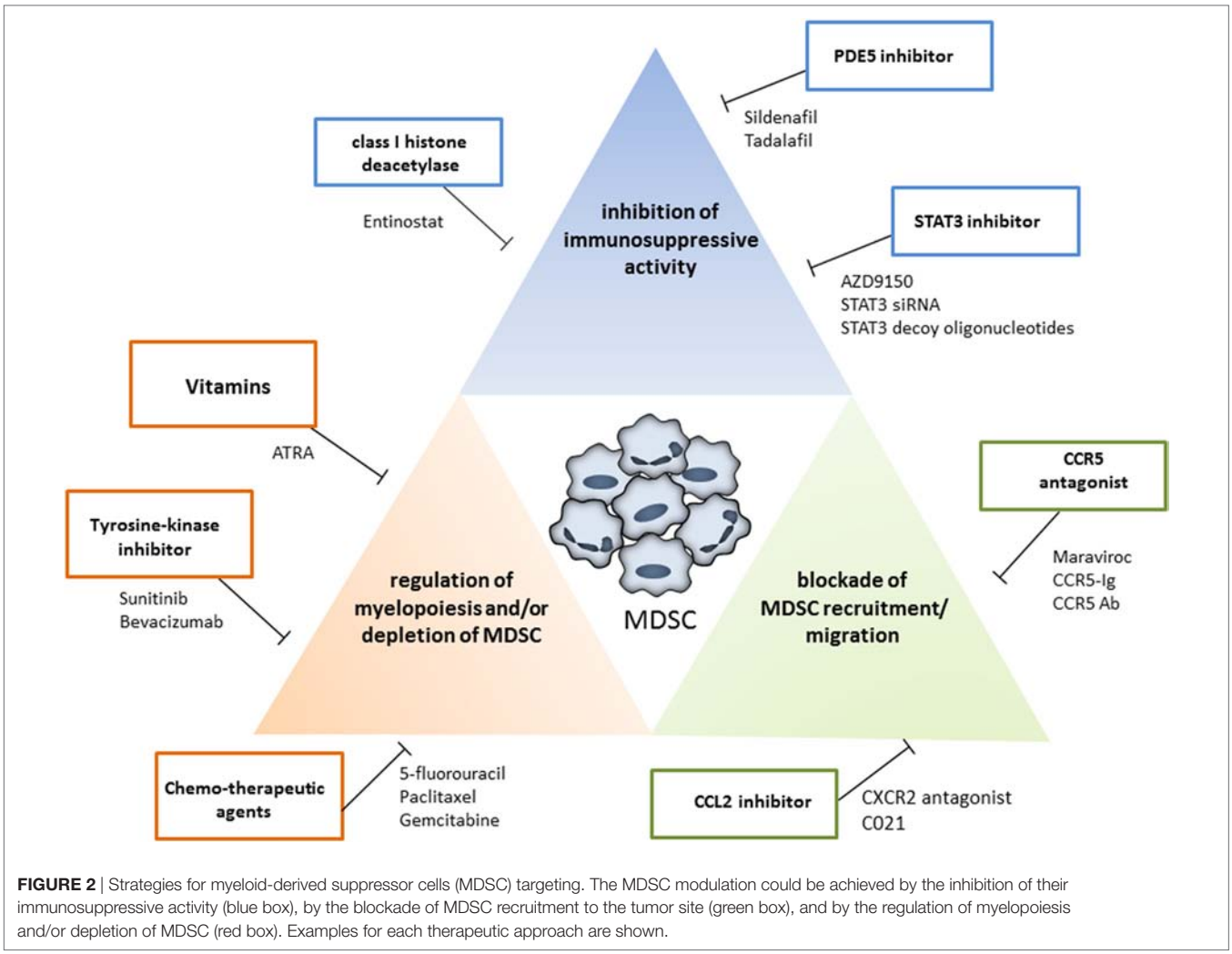

FIGURE 2 | Strategies for myeloid-derived suppressor cells (MDSC) targeting. The MDSC modulation could be achieved by the inhibition of their immunosuppressive activity (blue box), by the blockade of MDSC recruitment to the tumor site (green box), and by the regulation of myelopoiesis and/or depletion of MDSC (red box). Examples for each therapeutic approach are shown.

BLOCKING MDSC TRAFFICKING

Myeloid-derived suppressor cells exhibit their main immunosuppressive activity within the TME. Therefore, intensive investigations were performed to block the migration of MDSC to the tumor site. Chemokine receptors are a key driving force for the migration of immune cells (84). Myeloid cells (in particular, MDSC) express C-X-C motif chemokine receptor (CXCR) 2 (85). The main ligands for CXCR2 are C-C motif chemokine ligand (CCL)2 and CCL5, which are elevated in the TME (86, 87). To block the CXCR2-CCL2 interaction, tumor-bearing mice were treated with the chemotherapeutic drug docetaxel combination with a CXCR2 antagonist, showing a significant therapeutic effect (88).

Another chemokine receptor CCR5, which is expressed on a broad spectrum of immune cells (84), interacts with its ligands CCL3, CCL4, and CCL5 (89). Interestingly, the patients with a mutated CCR5 variant were reported to be resistant to the prostate cancer development (90). Furthermore, CCR5 has a critical role in tumor progression since it has been shown that the CCR5–CCL5 axis supported tumor growth, invasion, and migration of MDSC to the tumor site (87, 91). By targeting the CCR5-CCR5 ligand interaction, tumor growth and invasiveness could be suppressed in pancreatic, colorectal, prostate, and breast cancer (92–94).

In a spontaneous *Ret* transgenic mouse melanoma model, we have demonstrated that the tumor progression correlated with the accumulation of $CCR5^+$ MDSC in the TME that displayed significantly stronger immunosuppressive capacity than their $CCR5^-$ counterpart (87). By blocking the CCR5–CCR5 ligand interaction with a mCCR5-Ig fusion protein, the survival of melanoma bearing mice was significantly improved as compared to the control group. Importantly, it was also shown that the frequency of $CCR5^+$ M-MDSC and $CCR5^+$ PMN-MDSC was increased in the peripheral blood of melanoma patients and that $CCR5^+$ M-MDSC accumulated in melanoma lesions (87). Similar to the situation in melanoma bearing mice, $CCR5^+$ MDSC from melanoma patients displayed an increased immunosuppressive pattern compared to the $CCR5^-$ MDSC subset. Taken together, targeting CCR5 on MDSC could be applied not only to prevent the MDSC migration and accumulation in the TME but also to reduce MDSC immunosuppressive functions in cancer patients (87, 91).

TABLE 1 | Ongoing clinical trials to target myeloid-derived suppressor cells (MDSC) in cancer patients.

No.	Title	Disease or conditions	Interventions	Trial number
1	MDSC and chronic myeloid leukemia	Chronic myeloid leukemia	Imatinib	NCT03214718
2	Depletion of MDSC to enhance anti-PD-1 therapy	Non-small cell lung cancer (NSCLC), stage IIIB	Nivolumab Nivolumab + Gemcitabine	NCT03302247
3	MDSC and checkpoint immune regulators' expression in allogeneic SCT Using Flu-Bu-ATG	Leukemia, myelodysplastic syndromes	Fludarabine, Busulfan Methotrexate	NCT02916979
4	MDSC control by signal regulatory protein-alpha: investigation in hepatocellular carcinoma	Hepatocellular carcinoma	Therapy-independent collection of human samples	NCT02868255
5	Myeloid-derived suppressor cells clinical assay in finding kidney cancer	Metastatic and recurrent renal cell cancer	Computed tomography, cytology specimen collection, laboratory biomarker analysis, magnetic resonance imaging	NCT02664883
6	Capecitabine + bevacizumab in patients with recurrent glioblastoma	Glioblastoma	Capecitabine Bevacizumab	NCT02669173
7	Dendritic cell (DC) vaccine with or without gemcitabine. pre-treatment for adults and children with sarcoma	Sarcoma Soft tissue sarcoma Bone sarcoma	Gemcitabine DCs vaccine	NCT01803152
8	SX-682 treatment in subjects with metastatic melanoma concurrently treated with pembrolizumab	Melanoma stage III Melanoma stage IV	SX-682 Pembrolizumab	NCT03161431
9	PDE5 inhibition *via* tadalafil to enhance antitumor Mucin 1 vaccine efficacy in patients with HNSCC	Head and neck squamous cell carcinoma	Tadalafil Anti-MUC1 vaccine Anti-influenza vaccine	NCT02544880
10	Phase II trial of EP4 receptor antagonist, AAT-007 (RQ-07; CJ-023,423) in advanced solid tumors	Prostate cancer NSCLC Breast cancer	RQ-00000007 Gemcitabine	NCT02538432
11	MDSC clinical assay in finding and monitoring cancer cells in blood and urine samples from patients with or without localized or metastatic bladder cancer	Stage II bladder cancer Stage III bladder cancer	Cytology specimen collection procedure, laboratory biomarker analysis	NCT02735512
12	RTA 408 capsules in patients with melanoma—REVEAL	Melanoma Unresectable (stage III) melanoma Metastatic (stage IV)	Omaveloxolone Ipilimumab Nivolumab	NCT02259231
13	PDL-1 expression on circulating tumor cells in NSCLC	Lung cancer	Blood sample collection for CTC and MDSC analysis	NCT02827344
14	Effect of Astragalus-based formula: Qingshu-Yiqi-Tang on modulating immune alterations in lung cancer patients	Non-small-cell lung carcinoma	Astagalus-based formula: Qingshu-Yiqi-Tang	NCT01802021
16	A phase II trial of tadalafil in patients with squamous cell carcinoma of the upper aero-digestive tract	Head and neck squamous cell carcinoma	Tadalafil	NCT01697800
17	Relevance of peripheral cells in the pathophysiology of chronic myelomonocytic leukemia	Chronic myelomonocytic leukemia	Clinical data collection	NCT03280888
18	Histamine receptor 2 antagonists as enhancers of antitumor immunity	Cancer	Ranitidine	NCT03145012
19	Preoperative nutrition with immune enhancing nutritional supplement (immunomodulation)	Pancreatic adenocarcinoma	Dietary supplement: Nestle IMPACT advanced recovery and Nestle Boost high protein drink	NCT02838966
20	A study of RGX-104 in patients with advanced solid malignancies and lymphoma	Malignant neoplasms	RGX-104	NCT02922764
21	Determination of immune phenotype in glioblastoma patients	Glioblastoma multiforme	Surgery	NCT02751138
22	Academia Sinica Investigator Award 2010	Breast cancer	Unknown	NCT01287468
23	The "Fuzzing" therapy of TCM to improve the survival quality of early-stage NSCLC by intervening the CTCs	NSCLC	JinFuKang Cisplatin Pemetrexed	NCT02603003
24	Antibody DS-8273a administered in combination with nivolumab in subjects with advanced colorectal cancer	Colorectal neoplasm	DS-8273a + nivolumab	NCT02991196
25	Study to assess safety and immune response of stage IIB-IV resected melanoma after treatment with MAGE-A3 ASCI	Melanoma	recMAGE-A3 + AS15 ASCI	NCT01425749

(*Continued*)

TABLE 1 | Continued

No.	Title	Disease or conditions	Interventions	Trial number
26	Potentiation of cetuximab by regulatory T cells depletion with CSA in advanced head and neck cancer	Head and neck squamous cell carcinoma	Cyclophosphamide Cetuximab	NCT01581970
27	IMA970A plus CV8102 in very early, early and intermediate stage hepatocellular carcinoma patients	Hepatocellular carcinoma	IMA970A, CV8102, Cyclophosphamide	NCT03203005
28	Intensive locoregional chemoimmunotherapy for recurrent ovarian cancer plus intranodal DC vaccines	Cancer of ovary	Cisplatin + celecoxib + DC vaccine, cisplatin + CKM + celecoxib + DC vaccine	NCT02432378
29	Trial of SBRT with concurrent ipilimumab in metastatic melanoma	Melanoma	Stereotactic body radiotherapy, ipilimumab	NCT02406183
30	Lenalidomide maintenance therapy for multiple myeloma	Multiple myeloma	Lenalidomide	NCT01675141
31	Ipilimumab and all-trans retinoic acid combination treatment of stage IV melanoma	Melanoma	All-trans retinoic acid ipilimumab	NCT02403778
32	Study evaluating the influence of LV5FU2 bevacizumab plus ANAKINRA Association on Metastatic Colorectal Cancer	Metastatic colorectal cancer	ANAKINRA	NCT02090101
33	A phase I/Ib study of AZD9150 (ISIS-STAT3Rx) in patients with advanced/metastatic hepatocellular carcinoma	Advanced adult hepatocellular carcinoma Hepatocellular carcinoma metastatic	AZD9150	NCT01839604
34	AZD9150 with MEDI4736 in patients with advanced pancreatic, non-small lung and colorectal cancer	Malignant neoplasm of digestive organs intestinal tract; primary malignant neoplasm of respiratory and intrathoracic organ carcinoma	MEDI4736 AZD9150	NCT02983578
35	Study to assess MEDI4736 with either AZD9150 or AZD5069 in advanced solid tumors and relapsed metastatic squamous cell carcinoma of head and neck	Advanced solid tumors and metastatic squamous cell carcinoma of the head and neck	MEDI4736 AZD9150 AZD5069 Tremelimumab	NCT02499328

DEPLETION OF MDSC

The number of MDSC in tumor-bearing hosts could be reduced by (i) the normalization of myelopoiesis, (ii) the inhibition of the conversion of IMC into MDSC, and (iii) the differentiation of MDSC into mature myeloid cells like DC or macrophages. All-trans retinoic acid (ATRA) seems to be a very promising agent for these approaches. ATRA is a vitamin A derivative binding to the retinoic acid receptor. By blocking the retinoic acid signal transduction, MDSC could differentiate into DC and macrophages (95). In addition, it was described that administration of ATRA led to the downregulation of ROS production in MDSC by activating the extracellular-signal regulated kinase (ERK)1/2 pathway (96). In a completed clinical trial, ATRA was applied in metastatic renal carcinoma patients in combination with the IL-2 administration (97). The frequency of MDSC was significantly decreased, and the ratio between DC and MDSC was much higher than in the untreated group. In a second clinical trial with late stage small cell lung cancer patients, ATRA was used together with a DC vaccine against p53 (98). The outcome confirmed the inhibitory effect of ATRA on the frequency of circulating MDSC. The combination of the DC vaccine and ATRA resulted in the development of p53-specific $CD8^+$ T cells. It should be mentioned that ATRA was used in many other clinical trials with inhibitory effects on tumor progression; however, MDSC were not evaluated in these trials, and the positive effect was linked to other mechanisms.

Since tumor-derived EV were reported to induce the conversion of non-immunosuppressive IMC into MDSC and further activated their immunosuppressive functions (26, 27), the inhibitors of the EV release from tumor cells were tested in mice-bearing CT26 colon carcinoma (30). It was demonstrated that the treatment of these mice with dimethyl amiloride or omeprazole reduced EV content in serum that was associated with the reduction of MDSC expansion and immunosuppressive activity (30).

Clinical trials with tyrosine kinase inhibitors (such as sunitinib) revealed that these agents could target MDSC. Since sunitinib could block VEGF and c-kit signaling, which are involved in the generation of MDSC (99), its effect on MDSC from cancer patients was evaluated. Sunitinib treatment of metastatic renal cell carcinoma patients was reported to decrease the number of circulating MDSC (100, 101). Interestingly, M-MDSC from treated patients displayed a reduced STAT3 activation and ARG1 expression that was accompanied with an elevated activity and proliferation of CD8 T cells. However, no significant prolongation of the overall survival was observed.

Other chemotherapeutics such as gemcitabine and 5-fluorouracil were shown to induce selectively apoptosis of MDSC in the spleen and TME in several mouse tumor models (102–104). Interestingly, both chemotherapeutic agents displayed no significant effect on the frequencies of T cells, NK cells, DC, and B cells. It was also shown that gemcitabine reduced the frequency MDSC and Treg as well TGF-β1 level in the peripheral blood of pancreatic cancer patients (103). Similar to the preclinical observation, gemcitabine has no effect in effector T cells. In a clinical trial, gemcitabine treatment of pancreatic cancer patients resulted in a dramatic decrease in PMN-MDSC (103). An application of 5-fluorouracil in the preclinical mouse model and colorectal

cancer patients affected MDSC, leading to the immune recovery and tumor regression (104). Administration of another chemotherapeutic, docetaxel, induced a decrease of tumor burden in a preclinical mouse model of mammary carcinoma (105). This beneficial effect was accompanied by the conversion of MDSC into a M1-like cells characterized by the upregulation of CCR7 (105). The effect of doxorubicin on MDSC in mammary cancer models was also investigated (106). The treatment of these mice with doxorubicin led to the reduction of MDSC frequencies in the spleen, peripheral blood, and tumors. Furthermore, the immunosuppressive activity of residual MDSC was impaired. The depletion of MDSC resulted in the enhancement of granzyme B and IFN-γ production by effector T and NK cells (106). Moreover, this study demonstrated that MDSC isolated from patients were also sensitive to doxorubicin treatment *in vitro* (106).

Using *Ret* transgenic melanoma mouse model, we demonstrated that the administration of ultra-low, non-cytotoxic doses of paclitaxel induced the reduction of MDSC numbers and immunosuppressive functions (107). This effect was associated with an inhibition of the p38 MAPK pathway as well as the production of TNF-α and S100A9 in MDSC. Treated mice showed elevated activity of CD8 T cells, which correlated with the prolongation of mouse survival (107). In addition, it was reported that the treatment of MDSC *in vitro* with ultra-low concentrations of paclitaxel stimulated their differentiation into DC (108).

FUTURE PERSPECTIVES

Tumor cells developed multiple mechanisms to evade the immune system and to progress. One of the key mechanisms is the establishment of an immunosuppressive TME, where MDSC play a crucial role. By altering MDSC function and biology, various preclinical and clinical studies showed a beneficial effect. This suggests that MDSC targeting could be a promising strategy to apply together with existing immunotherapeutic strategies such as boosting the immune system by vaccination or negative immune checkpoint inhibitors. Thus, combining gemcitabine with a DNA vaccination induced a strong antitumor immune response accompanied by a reduced self-tolerance in a preclinical HER2-expressing mouse tumor model (109). Furthermore, another preclinical study showed that the administration of sunitinib with an HPV vaccination resulted in a tumor-free survival in 75% mice in the HPV-expressing tumor model (110). In addition, a clinical trial was initiated in stage IV melanoma patients, by whom ATRA was applied together with ipilimumab (111). This trial and many other starting combinatorial approaches will help to develop an efficient strategy for the treatment of cancer patients.

AUTHOR CONTRIBUTIONS

VF: writing, review, and revision of the manuscript and revision of the figures. XH: preparing the figures. RW, PA, and JU: review and revision of the manuscript. VN: preparing the table. CG: revision of the manuscript. VU: writing, review, and revision of the manuscript and revision of the table and figures.

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REVIEW
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Contribution to Tumor Angiogenesis From Innate Immune Cells Within the Tumor Microenvironment: Implications for Immunotherapy

Adriana Albini[1,2†], Antonino Bruno[1†], Douglas M. Noonan[1,3‡] and Lorenzo Mortara[3‡]*

[1] Scientific and Technology Pole, IRCCS MultiMedica, Milano, Italy, [2] Department of Medicine and Surgery, University Milano-Bicocca, Monza, Italy, [3] Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy

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***Correspondence:**
Adriana Albini
albini.adriana@gmail.com

[†]These authors have contributed equally to this work.

[‡]These authors share senior authorship.

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The critical role of angiogenesis in promoting tumor growth and metastasis is strongly established. However, tumors show considerable variation in angiogenic characteristics and in their sensitivity to antiangiogenic therapy. Tumor angiogenesis involves not only cancer cells but also various tumor-associated leukocytes (TALs) and stromal cells. TALs produce chemokines, cytokines, proteases, structural proteins, and microvescicles. Vascular endothelial growth factor (VEGF) and inflammatory chemokines are not only major proangiogenic factors but are also immune modulators, which increase angiogenesis and lead to immune suppression. In our review, we discuss the regulation of angiogenesis by innate immune cells in the tumor microenvironment, specific features, and roles of major players: macrophages, neutrophils, myeloid-derived suppressor and dendritic cells, mast cells, γδT cells, innate lymphoid cells, and natural killer cells. Anti-VEGF or anti-inflammatory drugs could balance an immunosuppressive microenvironment to an immune permissive one. Anti-VEGF as well as anti-inflammatory drugs could therefore represent partners for combinations with immune checkpoint inhibitors, enhancing the effects of immune therapy.

Keywords: angiogenesis, chemoprevention, tumor microenvironment, immune cells, immunotherapy

INTRODUCTION

The "gradient" of phenotype, genetic, and epigenetic features of transformed cells inside the tumor gives rise to the most known and studied tumor heterogeneity, the "intrinsic" one. However, increasing attention is devoted to "extrinsic" heterogeneity, i.e., all those cellular and molecular "players" that include the non-cancerous hosting environment. Cancers develop in complex tissue environments, both in the primary and in the target organs of metastasis. A "hostile" setting is elicited, such as low oxygen, acidity, and altered metabolic conditions. Cancer cells adapt more rapidly than healthy ones to the adverse conditions that paradoxically sustain growth, invasion, and metastasis. In such an "infernal" environment, interactions between tumor cells and the associated stroma represent a dangerous relationship that reciprocally influences disease initiation, progression and, in the end, determines patient prognosis (1).

The confirmed theory that the presence of inflammatory cells plays a crucial role within the tumor microenvironment (TME) is a very old one (2). "Evading immune destruction" and "tumor-promoting inflammation" are recognized host-dependent tumor hallmarks as defined by Hanahan and Weinberg (3). Among the tumor-friendly phenomena generated through the activity of the

inflammatory cells in the microenvironment, there is the orchestration of angiogenesis, a biological phenomenon necessary to bring oxygen, nutrition to the tumors, and last but not least, to transport the cancer cell to metastatic sites (4–7). Innate immune cells, as a consequence of their plasticity, have been reported to acquire an altered phenotype that can be proangiogenic. For many immune cells, both from innate and adaptive immunity, the release of proangiogenic cytokines is accompanied by a switch to a tolerogenic/immunosuppressive behavior (4, 7–9). In this review, we choose to describe the role in angiogenesis of selected major classes of inflammatory cells: macrophages, neutrophils, myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), mast cells (MCs), gammadelta (γδ)T type 17 cells (γδT17), innate lymphoid cells (ILCs), and natural killer (NK) cells (**Figure 1**). We also sustain the rationale behind using antiangiogenic drugs before the onset of immunotherapy and we propose as an innovative, low-cost strategy the use of "repurposed" anti-inflammatory/chemopreventive drugs to assist immunotherapies.

MACROPHAGES

Macrophages constitute professional phagocytes of the innate immune cell compartment with different specialized functions, depending on the type of danger signals and endogenous molecules to which they are exposed (10). They act as sentinels in all tissues of the body against invading pathogens, are able to

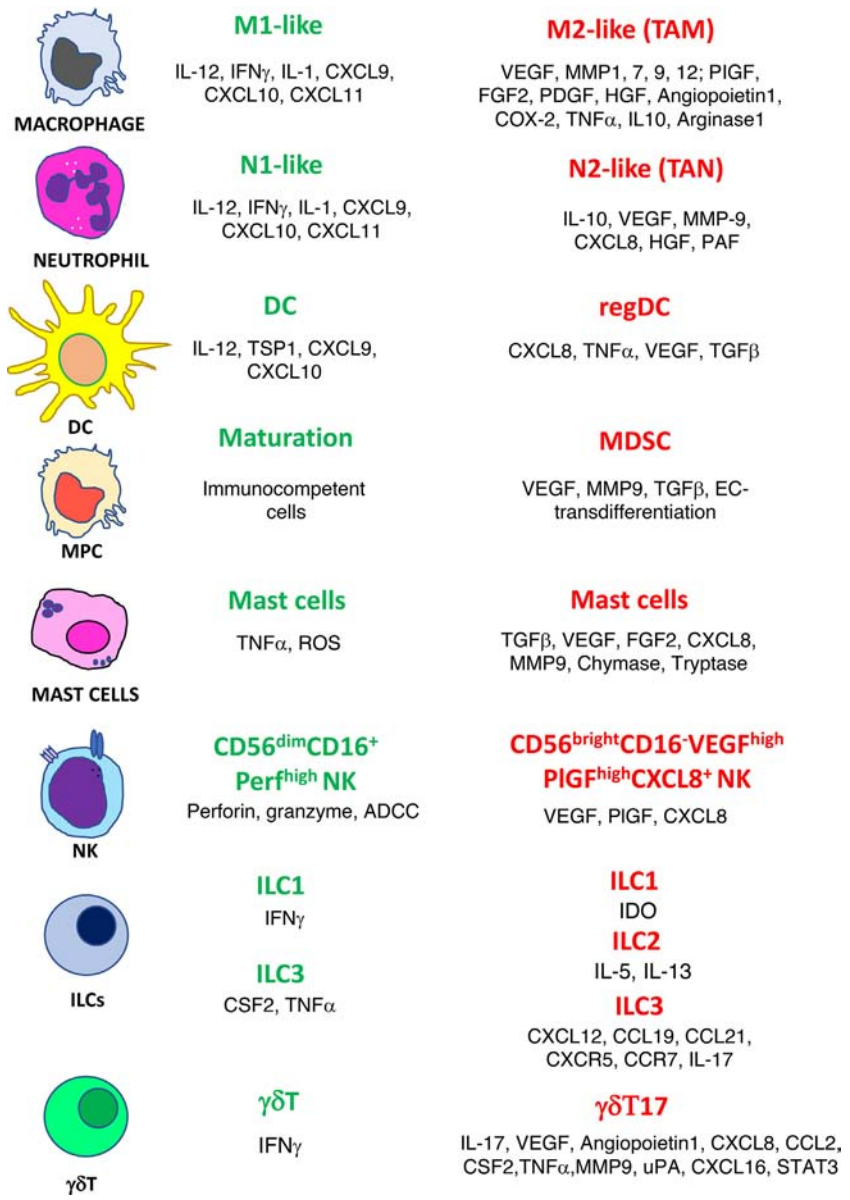

FIGURE 1 | Phenotype switch of innate immune cells in cancer. Antitumor/antiangiogenic (green text) and protumor/proangiogenic (red text) features are listed for macrophages, neutrophils, dendritic cells (DC), myeloid precursor cells (MPC), mast cells, natural killer cells (NK), innate lymphoid cells (ILCs), and γδT cells.

trigger an inflammatory response, and collaborate with other immune cells to activate adaptive T lymphocyte responses through antigen processing and presentation. These activities are related to a classical activation state, which is type 1 T helper (TH1) cell associated and INFγ and/or LPS-dependent, and is referred to as M1. This condition is favorable to immune response. Macrophages can be alternatively activated by IL-4 and/or IL-13 signals from TH2 cells, eosinophils, and/or basophils in the surrounding microenvironment. This polarization is involved in parasite control and wound healing and is termed M2 (11). M2 macrophages are associated with chemical and physical tissue damage in which they mediate tissue homeostasis and repair *via* remodeling and angiogenesis, in a spectrum of differentiation states. *In vivo*, the plasticity and diversity of macrophages are responsible of a spectrum of different activation states strictly depending on an array of concordant but also discordant stimuli, such as hypoxia, chemokines, colony-stimulating factor 1 (CSF1), TGFβ, adenosine, and prostaglandin E2 (PGE2), that do not fit with the M1/M2 classification (12). For these reasons, M1-like is the preferred term used in this review and indicate a polarization state of macrophages that are able to orchestrate cytotoxic antipathogen and antitumor responses, whereas M2-like are cells that have the common functional feature of favoring tumor cell fitness, new blood vessel formation, as well as suppressive activities toward adaptive immune cells (13, 14). Tumor-associated macrophages (TAMs), which share many features with M2-like macrophages (**Figure 2**), represent the major cell population of tumor-infiltrating leukocytes (15). TAMs also show consistent differences between diverse types of cancers (16, 17). Elevated TAM infiltration has been correlated with poor clinical outcome in many types of cancers, such as ovarian, breast, prostate, cervical, and thyroid cancers, Hodgkin's lymphoma, cutaneous melanoma, lung, and hepatocellular carcinomas (14, 18–22). Conversely, other reports on colorectal, prostatic, and lung cancers have detected a positive role of infiltrating macrophages favoring increased patient survival (23–25). During cancer development, macrophages are recruited in the tumor stroma by several inflammatory mediators, such as chemokines: CCL2 (also known as MCP-1), CCL5, CXCL12 (also known as SDF-1), cytokines: vascular endothelial growth factor (VEGF), CSF1, and activated complement elements. Blood monocytes, blood monocytic MDSCs, cells, or tissue-resident macrophages (26–28) are subverted in their phenotype and functions to differentiate into TAMs (14). However, TAMs are not fixed in an irreversible

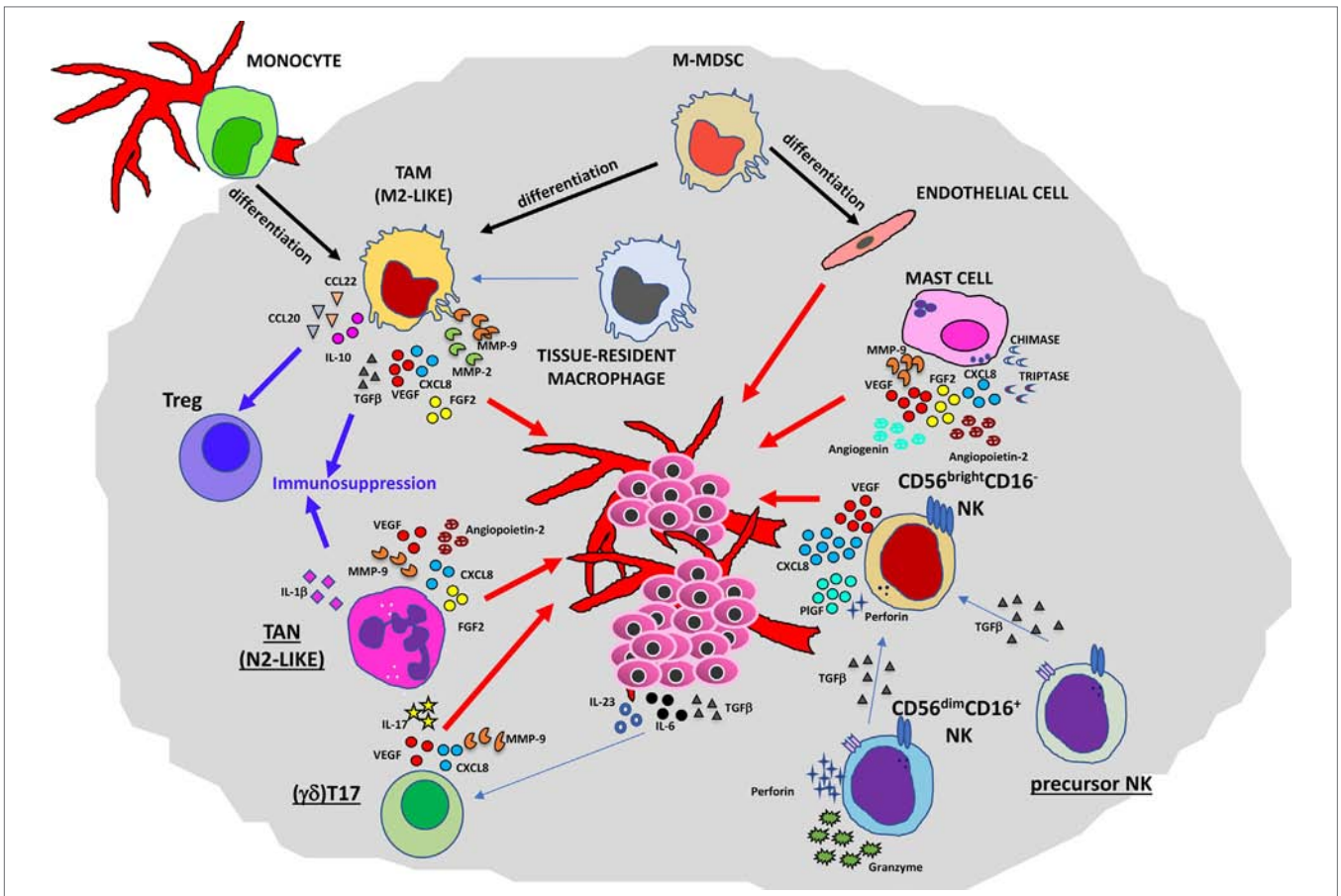

FIGURE 2 | Contribution of innate immunity to tumor angiogenesis. Soluble mediators (chemokines, cytokines, and enzymes) within the tumor microenvironment act directly or indirectly as proangiogenic factors produced by macrophages [M2-like tumor-associated macrophages (TAMs)], neutrophils [tumor-associated neutrophil (TAN), N2-like], myeloid-derived-suppressor cells (MDSCs), mast cells, and natural killer (NK) cells.

phenotype, they maintain their plasticity and eventually could be targeted by specific therapeutic approaches to re-educate them to M1-like antitumor functions (29). Accumulating evidence have shown that TAMs can act as key cellular mediators, interconnecting chronic inflammation with cancer development and progression (3, 30).

Several lines of research have pointed out the role of TAMs in the regulation of tumor cell invasion, angiogenesis, lymphangiogenesis, and metastasis (11, 19). In primary tumors (**Figure 2**), they can promote angiogenesis (the "angiogenic switch") triggering the activation and the recruitment of endothelial cells (ECs), essentially by producing multiple proangiogenic factors, including VEGFA, epidermal growth factor (EGF), basic fibroblast growth factor 2 (FGF2), chemokines CXCL8 (also known as IL-8), CXCL12, TNFα, semaphorin 4D, adrenomedullin, and thymidine phosphorylase (31–34). These factors produced by TAMs are responsible for the proliferation of ECs and the induction of sprouting, tube formation, and maturation of new vessels. Macrophages have been shown to play a critical role in tumor lymphangiogenesis by producing VEGFC and VEGFD (35–37). TAM activities can also have an impact on degradation and remodeling of the extracellular matrix (ECM), through the production of different classes of enzymes and proteases, such as matrix metalloproteinases (MMPs in particular MMP2 and MMP9), plasmin, urokinase plasminogen activator (uPA), and cathepsins, thereby influencing tumor invasion and the metastatic process (38–40).

Tumor-associated macrophages are also associated with resistance to different chemotherapeutic agents, involving the activation of distinct molecular pathways. In breast cancers, TAMs are able to inhibit apoptosis of cancer cells upon paclitaxel treatment *via* induction of IL-10/signal transducer and activator of transcription (STAT)3/Bcl-2 signaling (41). In patients with non-small cell lung cancer, TAMs or M2-like TAMs dampen the responsiveness to targeted therapy with EGF receptor–tyrosine kinase inhibitors (42, 43).

A highly proangiogenic M2-like TAM subset is represented by angiopoietin responsive Tie2$^+$ perivascular macrophages (35–37), which are able to induce chemotherapeutic drug resistance, favoring decreasing cancer cell responsiveness to radiotherapy (44). Specific inhibition of the angiopoietin/Tie2 axis can act in synergy with antiangiogenic treatments (45). Apart from their proangiogenic features, TAMs also play a crucial role in promoting an immunosuppressive milieu helping different tumors to escape immunosurveillance (46). Their contribution to tumor progression act also through crosstalk with other leukocytes and inflammatory and stromal cells (7, 47) within the TME. In the establishment of the immunosuppressive milieu, TAMs can directly recruit T regulatory (Treg) cells, by producing CCL20 (48) and CCL22 chemokines (49) and can activate them by secreting IL-10 and TGFβ (26). TAMs also represent an important factor for the establishment of the premetastatic niche (50, 51).

Different TAM-targeted therapeutic strategies have been developed with the aim to inhibit macrophage recruitment, to induce cell death, and to re-educate killer functions. These innovative therapeutic approaches could behave as a complement strategy in combination with antiangiogenic, cytoreductive, and/or immune checkpoint inhibitor treatments, and preclinical and clinical trial results are promising (14, 30, 52). CCL2-specific inhibition by antibodies has proven efficacious in mouse models of prostate, breast, lung, and melanoma, and this approach was synergistic with chemotherapy (53, 54). Different antibodies targeting CCL2 have entered phase I and II clinical trials (55). A CCR5 antagonist has been approved for the treatment of patients with liver metastases from advanced colorectal cancers and experimental data indicate that CCL5/CCR5 axis targeting could be suitable for clinical responses (56). Diverse compounds and antibody inhibitors that have been developed to inhibit the CSF1–CSF1R axis, could target TAM, and were evaluated in mouse models and in patients with different types of cancer (57). In diffuse-type tenosynovial giant-cell tumor showing overexpression of CSF1R, after treatment with CSF1R-blocking agents, patients experienced relevant clinical regressions (57, 58). In preclinical glioblastoma multiforme model, CSF1R blockade did not affect the TAM numbers but the M2-like TAM polarization markers were lowered, thus was associated with improvement of survival (59). Bisphosphonates, that are used to treat osteoporosis and to prevent bone metastases-related complications, can also be used to target macrophages inside the tumor (60). Moreover, bisphosphonates in combination with chemotherapy or hormonal therapy have been shown clinical synergistic effects, in different types of cancer patients, in particular for patients with breast cancer (61). In a murine model of pancreatic ductal adenocarcinoma (PDAC), the anti-CD40- and gemcitabine-treated mice induced re-education of M2-like TAM toward an M1-like macrophage and elicit effective antitumor responses (62). This lead to a phase I clinical trial in PDAC patients, the combination was well tolerated and provided some antitumor efficacy (63). A recently identified potent compound that targets TAMs is trabectedin, a synthetic form of a molecule isolated from the marine tunicate *Ecteinascidia turbinata*, which has found application in the treatment of soft tissue sarcomas and ovarian cancer patients. Trabectedin induces selective TRAIL-dependent apoptosis of monocytes, macrophages, and the monocytic component of MDSCs in blood, spleen, and tumors with the reduction of TAM numbers and angiogenesis (64, 65).

NEUTROPHILS

Neutrophils are the most abundant innate immune cells in the peripheral blood, they act as a first line of defense against invading pathogens and are crucial effectors in the acute phase of inflammation. Neutrophils are recruited in the damaged area by chemokines, in particular CXCL8, and the cognate receptors CXCR1 and CXCR2 (66). These leukocytes exert important functions such as phagocytosis, production and release of antimicrobial ROS, peptides, enzymes, and neutrophil extracellular traps (NET). Neutrophils can release a substantial quantity of different reactive soluble factors, including cytokines and chemokines (67), and are able to recruit and activate other immune cells, playing an important role in the regulation of chronic inflammation, tumor angiogenesis, and progression. Inflammatory CD66b$^+$ neutrophils can be found in high numbers in either blood or TME of different cancers and correlated with poor clinical outcome (68–74).

Neutrophils produce either proangiogenic or antiangiogenic factors (75–79), and in some cases, such as in the early phases of lung cancers, they can exert important T cell stimulatory, antitumor functions (80). Although they are characterized by a terminally differentiated phenotype and a short half-life, these cells are endowed with a certain kind of plasticity and in murine tumor models they are able to differentiate in two distinct subsets: neutrophils type 1 (N1) with antimicrobial functions, and tumor-associated neutrophils (TANs or N2) endowed with protumor and proangiogenic features (**Figure 2**) in response to TGFβ (81, 82). In response to IFNβ, TAN/N2 neutrophils can be converted to N1 type in both mouse lung cancers and human melanomas (83, 84).

Accumulating evidence has indicated TANs as key players involved in tumor angiogenesis and metastatic process in both mice and humans (**Figure 2**). The complex role of TANs in tumor angiogenesis and metastasis resides mainly in the capacity of these cells to secrete an array of diverse immunosuppressive or proangiogeneic molecules such as IL-1β, VEGF, FGF2, TGFα, hepatocyte growth factor (HGF), and angiopoietin 1 (ANG1) different chemokines such as CXCL1, CXCL8, CXCL9, CXCL10, CCL3, and CCL4 (6) and enzymes involved in ECM remodeling (MMP9). Production and expansion of neutrophils is dependent on CSF3 (G-CSF) and its receptor CSF3R. A crucial signaling pathway for cancer inflammation is STAT3 (85), which is downstream of activated CSF3R. In response to CSF3, neutrophils upregulate the expression of BV8 (also known as prokineticin-2) that induce myeloid cell mobilization and myeloid-dependent tumor angiogenesis (86). This production of BV8 depends on the activation of STAT3 (87). The tumor angiogenesis stimulation in mice by TANs and other myeloid cells is regulated by STAT3 signaling and involves VEGFA, FGF2, and MMP9 (88). MMP9-secreting neutrophils can directly contribute in the acceleration of tumorigenesis acting on skin premalignant epithelial cells in a mouse model (89). During the early stages of carcinogenesis, TANs can mediate the initial angiogenic switch in RIP1–Tag2 transgenic mice model of pancreatic neuroendocrine tumor. The MMP9-positive neutrophils were mainly found inside angiogenic islet dysplasia as well as in tumors (90). The neutrophil depletion by GR1 or Ly6G antibodies in both transgenic and tumor transplanted mice resulted in lower levels of VEGF/VEGF receptor (VEGFR) signaling and a delay of the angiogenic switch (90). TANs lack expression of tissue inhibitors of metalloproteinases (TIMP1), rendering neutrophil-derived MMP9 more potent as angiogenesis driver in the TME than cells which produce MMP9/TIMP1 complexes (91). Neutrophils with antiangiogenic features have been reported to be able to release the endogenous angiogenesis inhibitor thrombospondin-1 in peroxisome proliferator-activated receptor (PPAR)α-deficient mice, thus preventing angiogenesis and tumor growth (92). These reports suggest that PPARα is a central transcriptional suppressor of inflammation and tumor development and could be a valuable target. Group V secreted phospholipase A2 enzymes are released by human neutrophils and enhance the proangiogenic molecules VEGFA, ANG1, and CXCL8 in an autocrine mechanism (93), but also stimulate production of the antiangiogenic isoform of VEGFA, $VEGFA_{165b}$ (94). The functional outcome probably depends on the balance between proangiogenic and antiangiogenic factors and is still matter of investigation.

The ability of neutrophils to release several proangiogenic factors, MMPs, and other proteases (95) and to trap cancer cells *via* NET secretion (96) could promote cancer metastasis. TANs are required for the development of the premetastatic niche and metastases in murine models (97–99).

Recently, new data have brought clarity on the role of TANs and TAMs in the resistance to antiangiogenic therapy. Tumors activate PI3K signaling in all $CD11b^+$ cells (both neutrophils and monocytes) (100). Inhibition of one of these cell types induces a compensatory phenomenon by the other cell types, which overcomes the angiogenic blockade. Hindering PI3K in all $CD11b^+$ myeloid cells generate a long-lasting angiostatic effect (100).

IMMATURE MYELOID CELLS (MDSC AND DC)

Immature myeloid cells are innate immunity cells that infiltrate the TME, having a critical role in the proangiogenic activities and in tumor immune evasion (**Figure 1**). The immature myeloid cells include MDSCs and DCs, also indicated as regulatory (reg) DCs (101, 102). The immature phenotype is due to constitutive activation of STAT3 that perturbs the differentiation process of these cells. MDSCs comprise in mice and humans two distinct immature myeloid cell types: the polymorphonuclear MDSC (PMN-MDSC) characterized by neutrophil features, and the monocytic MDSC (M-MDSC) having markers of monocytes. Recently, several articles have described exhaustively both MDSC and DC phenotypic characteristics and they will not be discussed here (103–105). Several tumor-derived factors, among which CSF3, IL-1β, and IL-6, have been implicated in recruitment, activation, and expansion of MDSCs. These molecules contribute to the STAT3 activation of immature MDSCs, rendering them potent proangiogenic and immunosuppressive cells (106).

Monocytic MDSCs have been intensively studied and recognized as immunosuppressive cells as well as proangiogenic effectors in cancer (107). Murine data suggested that MDSCs are also able to differentiate into ECs (108, 109). Recent data have suggested that MDSCs in human peripheral lymphoid organs are mainly represented by PMN-MDSCs, with immunoregulatory role and are involved in the tumor-specific T cell tolerance. In the TME, there is accumulation of the M-MDSC counterpart, which is more suppressive and can rapidly differentiate to TAMs. These events might imply that targeting only one myeloid cell subset (macrophages vs. granulocytes or *vice versa*) may not be sufficient for obtaining a long-lasting immunotherapeutic effect. An investigation performed in two transplantable and two transgenic tumor murine models has shown that the tumor-induced hypoxia triggers the upregulation of CD45 tyrosine phosphatase activity in TME residing MDSCs, resulting in downregulation of STAT3 and differentiation of MDSCs into TAMs (106). There is no hypoxia in the spleens, thus CD45 downregulation of STAT3 does not occur in this organ. Use of STAT3 inhibitors in tumor-bearing mice resulted in depletion of MDSCs in the spleen but not in tumors.

Myeloid-derived suppressor cells and TAMs are regulated by metabolic constraints within the TME, and this represents a crucial factor of the signaling network regulating the expression of specific transcriptional programs with distinct protumor functions (110). Several amino acids in the TME are converted to immunomodulatory molecules such as nitric oxide, polyamines, and kinurenines. Amino acids consumption by myeloid cells decrease the availability of essential nutrients for T cells (111). The energetic metabolism of tumor-infiltrating MDCSs showed peculiar features in both mouse and human samples, such as a preferential augmented fatty acid uptake and their oxidation rather than glycolysis (112, 113). Targeting fatty acid oxidation inhibited tumor growth and combination with low dose chemotherapy blocked the MDSC immunosuppression (113). Myeloid cells in the TME produce increased fatty acid synthase in response to CSF1, which causes PPARβ/δ-dependent expression of genes, like VEGF, IL-10, and arginase 1 (Arg1), involved in the proangiogenic and immunosuppressive responses (114). A promising therapeutic approach is based on the reprogramming and the re-education of the metabolism of MDSCs in the TME, with appropriate drugs in combination with immune checkpoint inhibitors (115).

Myeloid-derived suppressor cells are also characterized by the ability to express high amounts of NADPH oxidase, which is responsible for the production of ROS in the form of superoxide anion, hydrogen peroxide, and peroxynitrite. MDSCs present also an increased expression of Arg1 and of inducible forms of nitric oxide synthase 2 genes, and they release diverse inhibitory cytokines, contributing to the immunosuppressive effects in the TME (116).

Myeloid DCs, also known as conventional (c)DCs, consists of multiple cell subsets with potent antigen-presenting cell capacity, therefore playing a fundamental role in the activation of T-cell adaptive responses against pathogens and tumor cells. However, tumor-associated cDCs or regulatory DC (regDCs) in the TME display altered functions with impaired cross-presentation capacity, express low levels of co-stimulatory molecules, and have high-proangiogenic abilities. These changes depend on diverse conditions that are established during tumor progression, for example, hypoxia, production of PGE2, IL-10, adenosine, and increased levels of lactate (117–119).

One of the major mechanisms contributing to DC dysfunction in tumor-bearing animals and in patients with different cancers is the abnormal accumulation of lipids (120). Growing evidence shows that cDCs can drive either immunosurveillance or accelerated tumor progression depending on the environment. In both mouse and human ovarian cancers, $CCR6^+$ cDCs are recruited massively in the TME through the tumor-derived β-defensins and are induced to become proangiogenic cells, favoring tumor vascularization, and growth in response of tumor VEGF (121).

Depleting DC numbers in the tumor-bearing host at early stages of the disease correlates with faster tumor development in a murine model of ovarian cancer. DC inhibition at advanced stages induces on the contrary significant delays in the malignant progression (122).

During tumor progression, the hypoxia-induced regDCs remain in an immature state and acquire tolerogenic immunosuppressive properties and proangiogenic activities, for instance, by secretion of galectin-1 (123, 124). Galectin 1 is able to bind VEGFR2 and neuropilin-1, mirroring the effect of VEGF on ECs, thereby promoting angiogenesis (123–125). Moreover, regDCs are involved in the expansion and activation of Treg cells through TGFβ release, reinforcing the induction of the immunosuppressive functions of the TME (126–128). Induction of adenosine receptor A2b is triggered by the hypoxia-induced factor (HIF)-regulated elements during tumor hypoxia and is involved in skewing DCs to TH2 triggering phenotype, sustaining M2-like macrophage induction, and reinforcing tumor angiogenesis (129). Although regDCs and MDSCs have cell-type specific functional properties, their capability of regulating tumor angiogenesis in the TME appears similar to the one of M2-like TAMs and N2 neutrophils, leading to production of several soluble factors such as VEGF, FGF2, BV8, and MMP9 (130).

MAST CELLS

Mast cells (MCs) are bone marrow-derived multifunctional immune cells first identified in human tumors by Paul Ehrlich in the 1870s (69, 131). MCs and their mediators exert a host protective immune response against noxious agents, viral and microbial pathogens (132–135), but are also associated with a detrimental role in allergic diseases (69). Increased number of MCs have been observed in tumor and peritumor tissues of cancer patients (136); their role in cancer insurgence and progression is tumor dependent (69, 131). Contrasting roles of MCs in supporting or inhibiting tumor progression have been reported (131). In solid neoplasms including thyroid, gastric, pancreatic, bladder cancers, prostate adenocarcinomas, and hematological malignancies, MCs have been associated with protumorigenic activity (69, 131, 137). In breast cancer (131) and in murine model of prostatic neuroendocrine tumors (137), MCs have antitumor activities. These data clearly suggest that the role of MCs in cancer is tumor-type dependent and is tuned by the local microenvironment (**Figures 1** and **2**). Antitumor activities by MCs are related to their ability to induce target cell cytotoxicity by releasing TNFα or by induction of ROS. Protumorigenic activities of MCs include contribution to the induction of an acidic and immunosuppressive TME, through adenosine production in the extracellular milieu. Prometastatic functions of MCs are mediated by the release of TGFβ, which induce tumor cells to undergo epithelial to mesenchymal transition. MC releases proangiogenic factors including FGF2, VEGFA, TNFs, CXCL8 (69, 131), diverse proteases, such MMPs (MMP9 mostly), as well as chymase and tryptase that modify pro-MMPs to their active forms (5, 138). MC deficient tumor-bearing mice show a reduced angiogenesis and metastatic capacity (138, 139). In renal cell carcinoma, infiltrating MCs have been found to support angiogenesis by modulating PI3K/AKT/GSK3β/AM signaling (140). Following activation of c-KitR/SCF, MCs can release tryptase that, acting on PAR2 in tumor cells, induce endothelial and tumor cell proliferation in a paracrine manner, leading to tumor cell invasion and metastasis (141). Tryptase released by MCs sustain angiogenesis in pancreatic cancers by activating the angiopoietin-1 pathway. Tryptase producing MCs correlate with angiogenesis in locally advanced colorectal cancer patients (142).

Immunohistochemical analysis showed that tryptase-positive MCs in multiple myeloma were associated with higher levels of MMP9, ANG2, and angiogenin (143) and could contribute to vasculogenic mimicry (144). Tryptase appears the key mediator for protumor activity of MCs, since it is involved in cell growth, tumor-induced angiogenesis, and invasion (145, 146), thus it appears to be a promising target for MC-related angiogenesis. Tryptase inhibitors originally designed as anti-allergic drugs could exert promising antitumor and antiangiogenic activity and could be proposed as repurposed drugs also in combination with immune therapy.

γδT17 CELLS

Gammadelta T cells are lymphoid cells characterized by unique features resembling innate cells in their capacity to recognize conserved non-peptide antigens expressed by stressed cells. They also resemble adaptive cells because of their ability to undergo clonal expansion and to develop antigen-specific memory (147). These cells are involved in the early phase of immune responses and produce pro-inflammatory factors such as IFNγ and TNFα and IL-17, activating other effector immune cells against virus, bacteria, and tumor cells but also stimulating inflammation and exacerbation of autoimmune diseases. They comprise different functional subsets.

Although there are some conflicting data on the role of γδT cells inside the TME, it is believed that the subset γδT17 cells, specialized in the IL-17 release, can actively participate in the angiogenic process (147, 148) (**Figures 1** and **2**). It has been shown that γδT17 cells release IL-17, CXCL8, CFS2 (also known as GM-CSF), and TNFα, and are able to support survival of MDSCs (149). Tumor cells over-expressing IL-17 showed significant tumor growth and new vessel formation (150). Since IL-17 has no direct effect on the proliferation of ECs, the proangiogenic effect is likely to be exerted through the enhancement of VEGF and/or CXCL8 by tumor cells (151). On the contrary, mice lacking IL-17 showed limited tumor growth and the vascular density in tumor tissues was decreased (152). There is evidence that IL-17 responsiveness can be an independent prognostic factor for overall survival in colorectal patients (153), high expression of IL-17 was shown to be associated with high microvessel density and was associated with VEGF production from tumor cells. More recently, it has been shown that IL-17 activates STAT3 in non-small cell lung carcinomas (NSCLC) cells and that treatment of HUVECs with IL-17 *in vitro* promoted the formation of vessel-like tubes in a dose-dependent manner (154). The GIV protein (Gα-interacting vesicle-associated protein, also known as Girdin) modulates the crucial signaling pathways in processes including macrophage chemotaxis, wound healing, and cancer metastasis and can be a target of STAT3 activation in NSCLC cell lines. IL-17-dependent STAT3/GIV signaling pathway is responsible for VEGF release from cancer cells and promotion of tumor angiogenesis, and GIV expression positively correlates with IL-17$^+$ cell presence and increased microvessel densities and predicts poor survival of NSCLC patients (154).

IL-17 in the TME in the CMS-G4 fibrosarcoma tumor model was largely derived from tumor-infiltrating γδT cells, and anti-cytokine mAb treatment revealed that the γδT cells require the presence of IL-6, IL-23, and TGFβ signaling (152). In gallbladder cancer (GBC) patients, γδT17 cells are increased in peripheral blood and in the population of tumor-infiltrating lymphocytes (155). GBC patients with high γδT17, TH17, and Treg cells showed poor overall survival (155). A GBC (OCUG-1) cell line that is responsive to IL-17, treated with cell-free supernatant from γδT17 cells, upregulates VEGF production, and this effect is IL-17 dependent (155). The proangiogenic action of γδT17 cells on GBC was confirmed by protein angiogenesis array performed on cell-free supernatants derived from these cells. The assay showed IL-17-dependent upregulation of several important angiogenesis factors in OCUG-1 cells, such as VEGF, angiogenin, uPA, MMP9, CCL2, CXCL16, CSF2, and coagulation factor III, but also stimulation of production of antiangiogenic factors, including thrombospondin-1, TIMP1, serpine-1, and platelet factor 4. A recent report has shown that IL-17-secreting γδT cells are dependent on CCR6 for homing to inflamed skin (156). Drugs targeting CCR6 or factors involved in γδT17 cell proangiogenic polarization should be studied for potential use in addition with immunotherapy.

INNATE LYMPHOID CELLS

Innate lymphoid cells represent a recently identified heterogeneous family of mononuclear hematopoietic cells, found mostly in solid tissues (157–160). Based on their lymphoid morphology, surface antigens, transcription factor expression, and cytokine productions (TH1, TH2, and TH17-like), ILCs have been classified into three major groups, termed as ILC1, ILC2, and ILC3 (161). ILC1s are characterized by IFNγ release and are Tbet dependent; ILC2 produce type 2-cytokines, such as IL-5 and IL-13, and require GATA3 expression; ILC3s produce IL-17 and/or IL-22 and are dependent on RORγt (162). ILCs are endowed with potent pleiotropic effects in early responses against infections and are involved in several pathologies including cancer. Aberrant activation, proliferation, and functions of ILCs support severe inflammation and damages in diverse organs, including the gut, lung, liver, and skin (163–168). Whether ILCs can be defined as friends or foes in cancer insurgence and progression is still a matter of debate (157, 158, 160). ILCs are characterized by high-cell plasticity and can be easily interconverted into their different subsets upon TME stimuli [especially ILC1–ILC3 interconversion (169)].

IFNγ$^+$ ILC1s have been associated with both antitumor and protumor effects (**Figure 1**), the latter induced by triggering of MDSCs and inducing indoleamine 2,3-dioxygenase activity (157). A protective role exerted by a novel type of ILC1-like cells has been shown in a murine model of mammary carcinogenesis (170). NK cells, that will be discussed, later have also been included in the ILC1 subclass.

ILC2s can release type 2 cytokines, such as IL-5 and IL-13, and CSF2 in response to IL-25 and IL-33. IL-13/IL-13R interaction in breast cancer and cholangiocarcinoma cells in association with recruitment and induction of TGFβ-producing MDSCs and Treg has been reported to induce tumor cell growth and migration (171), and tumor immune escape (172). Release

of IL-13 by ILC2s promotes M2-like TAM polarization and amplification (172).

Among the ILC subgroups, ILC3s are the more investigated for their contribution to carcinogenesis. They comprise several subsets: lymphoid tissue inducer (LTi) cells, first discovered for their function in the formation of lymphoid tissue during organogenesis, NCR (NKp46, NKp44)$^+$ ILC3 and NCR$^-$ ILC3. Overall, the pro-tumor activities of ILC3s are mainly linked to the induction of chronic inflammation by secretion of IL-17 and IL-22, in particular in the gut, through their response to IL-23 (173).

ILC3s preserve epithelial integrity and maintain tissue homeostasis by secretion of IL-22. Production of IL-17 by ILC3s can have a role in promoting tumorigenesis, tumor growth, and angiogenesis (174–176). Growing evidence from mouse tumor models marks ILC3s as cells involved in the recruitment of MDSCs, Treg cells, and in the promotion of M2-like macrophages in the TME. At the moment, the real contribution in human cancers remains to be fully elucidated (177, 178). ILC3s have also been shown to play a role in carcinogenesis in models of bacteria-induced colorectal cancer, through the release of IL-22 (179). The involvement of LTi-like ILC3s has been shown in the induction of tumor migration *via* lymphatics in patients with triple-negative breast cancers (180). In the 4T1.2 syngeneic mouse breast model, ILC3s are recruited in the primary tumor through CCL21, and then they trigger tumor stromal cells to release CXCL13, which leads to the induction of lymphotoxin and receptor activator of nuclear factor κ-B ligand, that in turn promotes lymphangiogenesis and stimulate tumor cell motility (180). A correlation exists between invasive aggressive behavior in breast cancer patients and gene expressed by ILC3s such as CXCL13, CCL19, CCL21, and CXCR5 and CCR7 (181). ILC3s have been shown to promote the formation of tertiary lymphoid structures (TLS), involved in tumor progression and lymph nodal metastasis (182). The protumor or antitumor roles of TLS are still debated (183, 184). NKp46$^+$ NKp44$^+$ LTi-like ILC3s are present in the TME near intra-tumor TLS and may interact directly with tumor cells by sensing and recognizing transformed cells through the NKp44 receptor. Tumor-infiltrating NKp46$^+$ NKp44$^+$ LTi-like ILC3s are endowed with ability to release several types of pro-inflammatory cytokines and chemokines, and their increased numbers correlated with intra-tumor TLS and predict favorable clinical outcome (185). Accumulation of neuropilin (NRP)1$^+$ LTi-like ILC3s has been found in inflamed tissues of patients with chronic obstructive pulmonary disease and in smokers, in association with VEGF production (186). Immunohistochemistry analysis of inflamed tissues revealed that the majority of RORγτ$^+$NRP1$^+$ cells were co-localized with blood vessels and in the alveolar parenchyma, suggesting their contribution to angiogenesis and induction of lung TLS. Apart from IL-22 and IL17, the pro-inflammatory LTi-like NRP1$^+$ ILC3 subset was also found to release CSF2, TNFα, B-cell-activating factor, and CXCL8, possibly contributing to angiogenesis.

Due to the recent discovery of the non-NK ILCs and the incomplete knowledge of the role in tumor and angiogenesis, targeting strategies have not been yet developed.

NK CELLS

Natural killer cells are bone marrow-derived large granular effector lymphocytes of the innate immune system that can potentially control tumor growth by their cytotoxic activity (187), which are now classified as a subset of ILC1 (161). Based on surface density expression of CD56, an isoform of the human neural cell adhesion molecule, and of CD16, the low-affinity Fcγ receptor, two main subpopulations of peripheral blood NK cells have been identified in humans: the CD56^{dim}CD16$^+$ and the CD56^{bright}CD16$^{-/low}$ NK cell subset, representing about 90–95% of peripheral blood NK cells and about 5–10% of peripheral blood NK cells, respectively. CD56^{dim}CD16$^+$ NKs can release high quantity of perforin and granzymes and are cytotoxic when encountering cells with high-activating ligands and low inhibitory (mostly class I MHC) ligands or when mediating antibody-dependent cell cytotoxicity (187). Although weak long-term cytokine producers, these cells have the ability to quickly (2–4 h) secrete high amounts of cytokines (188, 189). CD56^{bright}CD16$^{-/low}$ NKs, are poorly cytotoxic, but can release several cytokines, including IFNγ, TNFα, and GM-CSF. However, there is an increasing awareness of the complexity of NK cell subsets and the role of the TME (190–193). Mature NK cells express the PD-1 receptor, and engagement with the programmed death-ligand 1 (PD-L1) ligand results in impaired antitumor NK cell activity (194, 195). Disruption of this PD-1/PD-L1 by blocking antibodies partially restores their antitumor activity (194, 195). Another recently identified NK checkpoint is the IL-1R8 (also known as SIGIRR, or TIR8), which is expressed on human and murine NK cells (196). Mice lacking IL-1R8 are protected against chemically-induced tumors and metastatic dissemination (196). Mice lacking the cytokine-induced SH2-containing protein CIS also had protection toward chemically induced tumors and metastatic disease (197).

A third NK cell subset has been identified in the decidua during pregnancy, termed decidual or uterine NK cells (dNK). dNK cells acquire the CD56^{superbright}CD16$^-$KIR$^+$ phenotype (198), are poorly cytotoxic, and secrete proangiogenic cytokines, including VEGF, placental growth factor (PlGF), CXCL8, and IL-10 (198–200) and are critical for decidual vascularization and spiral artery formation (199, 201). Early on in pregnancy, dNK increase up to 70% of the local lymphocytes and 30–40% of all decidual cells (202). While it has been exhaustively demonstrated that NK cells have important proangiogenic roles in the uterine vasculature, their contribution to tumor angiogenesis still represent a poorly explored topic (**Figure 1**). The TME has been extensively reported to be crucial in shaping NK cell functions (203). We were the first to report a proangiogenic NK cell polarization in peripheral blood (TANKs) and tumor-infiltrating NK cells (TINKs) (204) in NSCLC patients. We showed that the CD56^{bright}CD16$^-$ NK cells, the predominant subset infiltrating NSCLC tissues and a minor subset in adjacent lung and peripheral blood, are associated with VEGF, PlGF, and IL-8 production (**Figure 2**). Functional assays indicated that supernatants derived from NSCLC CD56^{bright}CD16$^-$ NK cells induce EC chemotaxis and formation of capillary-like structures *in vitro*, and that these effects were even stronger in TANKs isolated from subset of squamous carcinoma patients than in adenocarcinoma.

TGFβ is associated with dNK polarization (205, 206) and is present in the TME. A combination of TGFβ, hypoxia, and a demethylating agent induces a dNK-like phenotype in healthy donor NK cells (207). A recent report indicated that TGFβ converted NK cells into other ILC1 subpopulations that were unable to control local tumor growth and metastasis (208). We observed that TGFβ1 upregulates VEGF and PlGF in healthy donor NK cells (204).

Tumor-infiltrating NK cells operate within a hypoxic TME. Hypoxia has been extensively reported to modulate immune cell response as well as driving angiogenesis (209). Murine NK cells genetically depleted of HIF1α continued to have impaired cell cytotoxicity, yet tumors grew more slowly in these mice (210). Tumors in these mice had numerous immature vessels with hemorrhages that resulted in severe hypoxia, which favored metastasis. Genetic inactivation of STAT5, which is necessary for NK cell-mediated cancer immunosurveillance, increases VEGFA in NK cells and stimulates angiogenesis in mouse lymphoma models and on healthy donor-derived NK cells (211). The aminobiphosphonate zoledronic acid, largely employed as an immunomodulatory agent and able to decrease VEGF levels, has been surprisingly found to synergize with IL-2 in inducing proangiogenic features in TINKs, acting on VEGF/VEGFR1 axis (212). Thus, therapeutic intervention could act as a double edge sword in NK cell response to tumors.

PHARMACOLOGICAL AND IMMUNOTHERAPEUTIC COMBINATION TARGETING THE TME

Extensive studies on TME led to a shift from a tumor-centered view of cancer onset to the role of a more complex tumor ecosystem in which cellular and molecular components are as influential as cancer cells themselves for cancer development and metastatic behavior. This knowledge led to the rapid development of therapeutic approaches aimed at restoring altered/aberrant host immune cell response, by accelerating/pushing efficient tumor eradication, stimulating immune cells of the host (213). The use of immune checkpoint blockers (ICBs) induces reactivation of key immune cell players and has been demonstrated to have great clinical benefits in several tumors (214). Available ICBs target cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1) receptor, and its ligand PD-L1. Known ICBs are: Ipilimumab, a mAb-blocking CTLA4, approved in patients with unresectable or metastatic melanoma. Pembrolizumab, a mAb-blocking PD-1, initially licensed for use in patients with unresectable or metastatic melanoma experiencing disease progression on ipilimumab. Pembrolizumab has been recently made available for other types of cancer (metastatic Non-Small Cell Lung Cancer, Head and Neck Cancer, Hodgkin's Lymphoma, Urothelial Carcinoma and Gastric Cancer). Nivolumab is another mAb directed to PD-1 approved

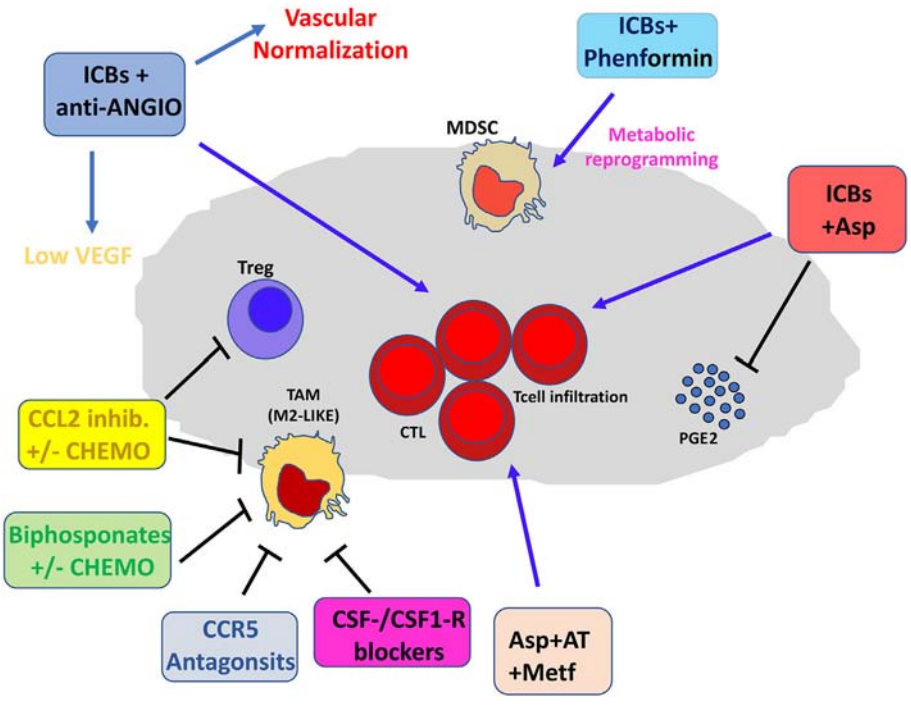

FIGURE 3 | Pharmacological and immunotherapeutic combination targeting the tumor microenvironment (TME). Immune checkpoint blockers (ICBs) can be employed in diverse pharmacological combinations resulting in clinical benefit for patients. ICBs + antiangiogenic agents result both in inhibition of aberrant angiogenesis and vascular normalization with subsequent efficient T cell infiltration. CCL2 inhibitors combined with chemotherapy dampen induction of M2-like tumor-associated macrophages (TAMs) in the TME and T regulatory (Treg) proliferation. Bisphosphonates + chemotherapy target M2-like TAMs. Anti-Angio, VEGF inhibition and eventually angiopoietin-2 blockade; Asp, aspirin; AT, atenolol; Metf, metformin; Chemo, standard chemotherapeutic drugs.

for use in individuals with unresectable or metastatic melanoma non-responding to other treatments, as well as in patients with metastatic NSCLC, or after platinum-based chemotherapy. Atezolizumab is a PD-L1-blocking antibody for the treatment of locally advanced or metastatic urothelial carcinoma. Despite the strong clinical success of cancer immunotherapy with checkpoint inhibitors and other immune modulating agents, most patients still do not experience a durable response (215) and many do not respond at all. To overcome this issue, several strategies combining immune to targeted therapy have been developed.

The gut microbiome, which has a significant influence on the local and systemic immune system, can influence the outcome of ICB therapy in preclinical mouse models and humans (216–219). A recent study on the gut and oral microbiome of a cohort of melanoma patients undergoing an anti-PD-1 therapy revealed crucial differences in the diversity and composition of the patients' gut microbiome of responders vs. non-responders (216). Analysis of patient fecal microbiome in responding melanoma patients indicated significantly higher relative abundance of bacteria of the *Ruminococcaceae* family that also correlated with presence of $CD8^+$ T cells in the TME. Fecal microbiota transplantation in germ-free recipients showed that mice which had been transplanted with stool from responders to anti-PD-1 therapy had significantly reduced tumor size and higher density of $CD8^+$ T cells in comparison to mice receiving stool from non-responders to PD-1 blockade (216). Another recent study on different epithelial tumors in mice and patients indicated correlations between clinical responses to ICBs and the relative abundance of *Akkermansia muciniphila* (217). Hence, the gut microbiome can strongly influence the outcome of cancer patients receiving PD-1 blockade therapy. However, the mechanisms related to these immunomodulatory effects of *A. muciniphila* remain elusive. It is conceivable that an integral intestinal barrier is associated with a minor systemic inflammation, and specific bacterial families such as *Ruminococcaceae* and/or *A. muciniphila* may induce beneficial bacterial metabolites that prevent leaky colon and systemic immunosuppression, paving the way to the possibility to manipulate the gut ecosystem to implement ICB therapy (218).

All recent preclinical and clinical data suggest that the localization, quality, and quantity of non-cancerous cells, including lymphoid and myeloid cells, within the TME play a major role in shaping response to immune checkpoint blockade (**Figures 3** and **4**). Other TME cells, such as fibroblast and ECs, could

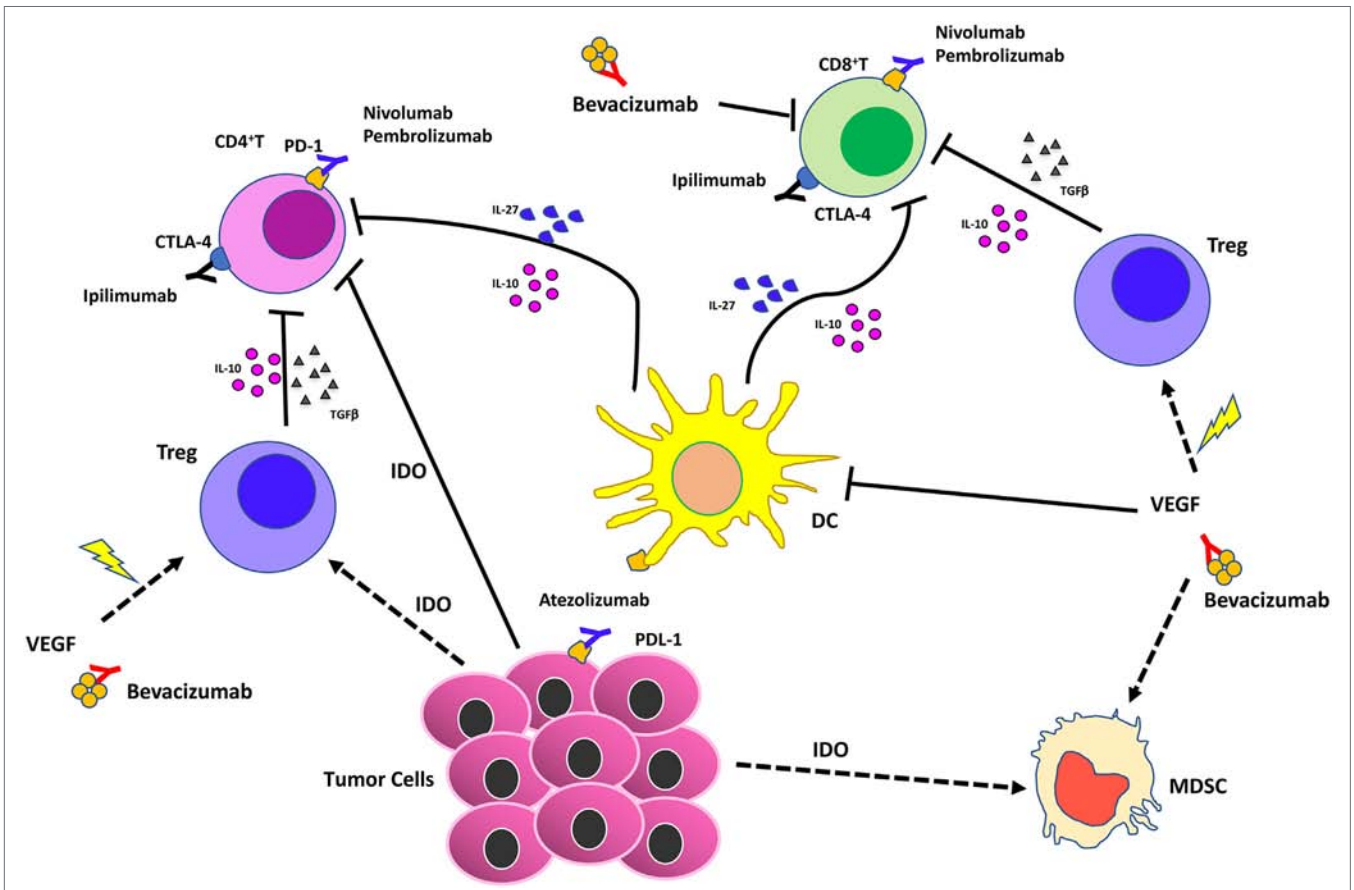

FIGURE 4 | Effects of vascular endothelial growth factor (VEGF) inhibition combined with immunotherapy in the tumor microenvironment. Immune checkpoint blockers (ICBs) combined with antiangiogenic drugs act synergistically on different cell of innate immunity by (i) reducing VEGF in the tumors that supports angiogenesis; (ii) supporting vascular normalization to stabilize blood vessels and enhance therapeutic agent delivery, T cell infiltration, and activation; (iii) blocking dendritic cells, myeloid-derived suppressor cells, T regulatory (Treg)-mediated immunosuppression.

contribute to shaping the immune contest. An emerging role is demonstrated for the angiogenic factor VEGF.

Vascular endothelial growth factor blocks T cell infiltration into the tumor by inhibition of adhesion molecules on ECs (220). VEGF has also been reported to inhibit antigen presentation by DCs, to enhance the Treg expansion, and to mediate PD-1 upregulation on tumor-infiltrated T cells (221, 222). Antiangiogenic treatments such as anti-VEGF antibody bevacizumab and the diverse multi-tyrosine kinase receptor inhibitors targeting the VEGFR family have been largely employed in the clinic, combined with chemotherapy, in particular in colorectal and renal cancer. They have shown significant but moderate benefits in patients' overall survival (223). Excessive pruning of vessels following anti-VEGF treatment has been reported to associate with increased hypoxia that, through upregulation of CXCL12/CXCR4 axis and HIF1α, supports M2-like TAM, MDSC, and Treg recruitment, thus supporting tumor progression (223). Tumors show considerable variation in their responses to antiangiogenic therapy, however, given the immunosuppressive action of VEGF (47, 222, 224), VEGF inhibitors could combine with the ICBs to enhance therapeutic effects.

Therefore, combination with antiangiogenic agents, and/or anti-inflammatory drugs has a strong rationale (47, 225, 226) but it is still in its infancy. Preclinical and clinical studies in renal cancer showed that the combination of anti-CTLA-4 with sunitinib (227) resulted in decreased Treg and increased $CD8^+$ T cell infiltration (**Figure 4**). Conversely, increased PD-L1 expression has been observed following treatments with sorafenib, sunitinib, or bevacizumab in a HIF1α-dependent and -independent manner (228). Growing evidence supports the notion that the targeting of VEGF signaling could result in the induction of tumor vasculature normalization, enhancement of immune cells extravasation, and synergy with immunotherapy (229–231). The combination of bevacizumab and ipilimumab has been reported to be associated with clinical benefits in patients with melanoma (232), and has been found to target Galectin-1 (233–235). Blocking of VEGFA and angiopoietin-2 using a bispecific antibody in murine models resulted in activation of cytotoxic T lymphocytes, which upregulated PD-L1, and inhibition of PD-1 axis further improved the efficacy of this therapy (236). Another rationale for the combination of ICBs and antiangiogenic agents is that antiangiogenic agents "normalize" the tumor vasculature, inducing intra-tumor high endothelial venules, thus favoring enhanced T-cell infiltration, antitumor CTL activity, and tumor cell destruction (236, 237). ICBs in combination with antiangiogenic agents may act as a promising strategy also to dampen the proangiogenic features of immune-infiltrating cells, such as TAMs, MDSCs, and NK cells, acting as re-polarizing agents (226, 238, 239).

Chronic inflammation, another relevant hallmark of cancer (3), directly stimulates angiogenesis to support tumor progression (5, 7) and immune suppression (16, 17, 107, 225, 226). The immunosuppressive inflammatory TME is a key obstacle to cancer immunotherapy (**Figures 3** and **4**). Thus, targeting chronic inflammation could be one strategy to combat the immunosuppressive TME and enhance the activities of ICBs. One example is targeting the PI3Kγ, which has a strong effect on myeloid cells, preventing immune suppression and enhancing the effects of ICBs *in vivo* (240, 241) (**Figures 3** and **4**). Another example of therapy that could synergize with ICBs is targeting the CXCR2 axis, which recruits neutrophils into the premetastatic niche (98).

The combination of anti-inflammatory agents with ICBs can be exploited to support immunotherapy. Regular use of aspirin, the most commonly employed nonsteroidal anti-inflammatory drug, has been widely reported to reduce incidence and mortality of colorectal cancer (242) and many other adenocarcinomas (243). A recent U.S. population-based study reported a stronger survival association of post-diagnosis aspirin use in CRC patients with lower-level PD-L1 expression when compared with those with higher-level of PD-L1 expression (244). Experimental data supported a synergistic effect between aspirin and anti-PD1 antibody in mutant Braf(V600E) melanoma cells (245). The synergistic effects resulted also in increased T cell-mediated immune responses and decreased PGE2 production (245). In experimental models, we showed that aspirin or the beta-blocker agent atenolol can augment the activity of metformin, a biguanide largely employed in type 2 diabetes management and that have been associated to reduced risk of developing diverse cancers,

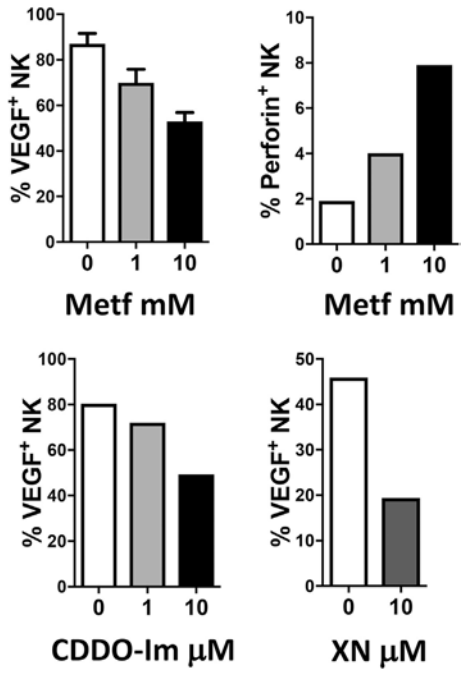

FIGURE 5 | Examples of effects of repurposed drugs and phytochemicals on natural killer (NK) cell repolarization. The biguanide metformin (Metf), the synthetic triterpenoid 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole (CDDO-Im) and the hop flavonoid xanthohumol (XN) can decrease vascular endothelial growth factor (VEGF) production in non-small cell lung cancer associated NK cells and at the same time can upregulate perforin production. Graphs show data obtained from multicolor flow cytometry analysis of total NK cells ($CD45^+CD14^-CD3^-CD56^+$ cells) from peripheral blood samples of patients with non-small cell lung carcinomas (as in 204, protocol number 0024138/2013), exposed for 24 h to the compounds at indicated concentrations. These examples sustain the action of anti-inflammatory chemopreventive drugs in innate immune cells repolarization supporting the rationale for future combinations with immunotherapy.

including breast cancers (**Figure 4**), targeting both neoplastic cells and the TME (246, 247). Metformin and phenformin affect the angiogenesis pathway (248–250) and modulate the immune response and the microbiome (251, 252). Phenformin enhances PD-1 immunotherapy (115). CDDO-Im (a synthetic triterpenoid: 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole) has an extensive documentation as an immunomodulation agent (253–255), and xanthohumol (XN) (a prenylated chalcone flavonoid) is an antileukemia agent (256–259) and is a polarizing agent in murine models of breast cancer (260). Phytocompounds and their synthetic derivatives are able to polarize macrophages inducing anti-tumorigenic phenotype/functions (253, 260–262). For example, we show that NSCLC patient TANKs treated with metformin, CDDO-Im, and XN decreases VEGF production (**Figure 5**) and increases perforin content. Thus, we would like to indicate the use of non-toxic or low-toxic re-polarization agents endowed with anti-inflammatory chemopreventive properties to be combined with ICBs.

CONCLUSION

The immune checkpoint inhibitors have posed a distinct milestone in cancer therapy. However, several patients do not respond to the ICBs, or have a relapse, with eventual long-term toxicity (i.e., autoimmune diseases). The polarized TME is crucial in the outcome of the patient response to an ICB, thus treating an inflamed or vascularized TME, could theoretically enhance the efficacy of these drugs. We suggest to combine ICBs with drugs that inhibit VEGF (232) or to employ drugs that eliminate the protumor inflammatory cells (for example, trabectedin to eliminate TAMs) or to treat with anti-inflammatory agents that will "re-polarize" the immune cells, for example, the repurposed drugs (metformin) and phytochemicals and their synthetic derivatives (CDDO-Im and XN) or both. Since phytochemicals and their synthetic derivatives often protect the cardiovascular system from chemotherapy induced damage (248, 263, 264), we propose, as a first-line therapy for difficult and metastatic tumors, to pretreat with phytochemicals or synthetic derivatives, then continue treatment and add sequentially a VEGF blocker, ICBs, and chemotherapy (to trigger the immunogenic cell death). This will set the stage for the ICBs to become highly effective in additional patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Targeting Autophagy in the Tumor Microenvironment: New Challenges and Opportunities for Regulating Tumor Immunity

Bassam Janji[1]*, *Guy Berchem*[1,2] and *Salem Chouaib*[3,4]

[1] Laboratory of Experimental Cancer Research, Department of Oncology, Luxembourg Institute of Health, Luxembourg City, Luxembourg, [2] Centre Hospitalier du Luxembourg, Department of Hemato-Oncology, Luxembourg City, Luxembourg, [3] INSERM U1186, Gustave Roussy Cancer Center, Villejuif, France, [4] Thumbay Research Institute for Precision Medicine – Gulf Medical University, Ajman, United Arab Emirates

Cancer cells evolve in the tumor microenvironment, which is now well established as an integral part of the tumor and a determinant player in cancer cell adaptation and resistance to anti-cancer therapies. Despite the remarkable and fairly rapid progress over the past two decades regarding our understanding of the role of the tumor microenvironment in cancer development, its precise contribution to cancer resistance is still fragmented. This is mainly related to the complexity of the "tumor ecosystem" and the diversity of the stromal cell types that constitute the tumor microenvironment. Emerging data indicate that several factors, such as hypoxic stress, activate a plethora of resistance mechanisms, including autophagy, in tumor cells. Hypoxia-induced autophagy in the tumor microenvironment also activates several tumor escape mechanisms, which effectively counteract anti-tumor immune responses mediated by natural killer and cytotoxic T lymphocytes. Therefore, strategies aiming at targeting autophagy in cancer cells in combination with other therapeutic strategies have inspired significant interest to overcome immunological tolerance and promote tumor regression. However, a number of obstacles still hamper the application of autophagy inhibitors in clinics. First, the lack of selectivity of the current pharmacological inhibitors of autophagy makes difficult to draw a clear statement about its effective contribution in cancer. Second, autophagy has been also described as an important mechanism in tumor cells involved in presentation of antigens to T cells. Third, there is a circumstantial evidence that autophagy activation in some innate immune cells may support the maturation of these cells, and it is required for their anti-tumor activity. In this review, we will address these aspects and discuss our current knowledge on the benefits and the drawbacks of targeting autophagy in the context of anti-tumor immunity. We believe that it is important to resolve these issues to predict the use of autophagy inhibitors in combination with immunotherapies in clinical settings.

Keywords: autophagy, hypoxia, tumor microenvironment, immune response, tumor immunity

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***Correspondence:**
Bassam Janji
bassam.janji@lih.lu

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INTRODUCTION

While initially considered as a disease of cells with deregulated gene expression, cancer progression is now considered to be largely influenced by the tumor microenvironment. It is now well established that factors in the tumor microenvironment play a key role in cancer progression, metastasis, and resistance to the therapies (1). In addition to malignant cells, the tumor microenvironment

contains different subsets of immune cells, fibroblasts and cancer-associated fibroblasts, tumor vasculature and lymphatics, as well as pericytes and sometimes adipocytes (2). Effector immune cells infiltrating tumors, notably T lymphocytes and natural killer (NK) cells mediating adaptive and innate immunity, respectively, are basically the major immune cells able to kill cancer cells in the tumor microenvironment (3). Although these immune effectors are recruited to the tumor site, they are exhausted and their anti-tumor functions are often downregulated in response to micro-environmental factor such as hypoxia.

It is now widely accepted that the oxygen consumption of solid tumors is increased due to the tumor volume and elevation of the respiratory activity of different cell populations within a tumor. The increase in the oxygen consumption leads to the establishment of hypoxic tumor microenvironment. The hypoxic tumor microenvironment is a characteristic feature of locally advanced solid tumors and a major hallmark that contributes to tumor resistance to several therapies including chemotherapy, radiotherapy, and immunotherapy (4). While mounting experimental evidences highlight the role of hypoxia at primary tumors, the role of hypoxia in the metastatic dissemination and at the metastatic niches is only being unraveled. Indeed, hypoxia signaling pathway is involved in multiple steps of the metastatic cascade, including local invasion and migration, intravasation and extravasation, establishment of the pre-metastatic niche, and survival and growth at the distant site. The role of hypoxia in metastasis control is reviewed in many excellent reviews (5–7).

Hypoxia within the tumor is characterized by a condition where the pressure of oxygen is lower than 5–10 mm Hg. Such condition results from an insufficient and/or inadequate oxygen supply to the tumor bed. In normal tissues, the oxygen pressure is basically higher than that in the corresponding tumors. The oxygen pressure within the tumor likely depends on the initial oxygenation of the tissue as well as the heterogeneity and the size of the tumor. **Table 1** shows the percentage of oxygen (reported as a median) in some healthy organs or tissues and their corresponding tumors. Adapted from Ref. (8).

Hypoxia is not only resulted from decrease in O_2 partial pressure in arterial blood, but also from pathological conditions, such as anemia (anemic hypoxia), which restrict the ability of blood vessel to carry O_2. It can also be generated from dramatic decrease in tissue perfusion or defect of cells to use O_2. The level of O_2 in tissue is finely tuned by blood flow regulatory mechanism, which is adapted according the consumption level of O_2 in the tissue. Therefore, hypoxia can be generated in a particular tissue or organ if the system regulating blood flow fails to meet the level of O_2 demand, thus impacting the function this tissue or organ. It should be noted that the term hypoxia has been used in several publications in a somewhat careless manner. Indeed, the *in vitro* experimental conditions described in many papers were routinely conducted under atmospheric O_2 levels ranging from 18 to 21% O_2. However, physiological normoxia comprises between 1 and 13% O_2. Therefore, interpreting results when performing research under varying O_2 conditions require a comprehensive understanding of physiological parameters that define the appropriate *in vitro* model.

TABLE 1 | The median percentage of O_2 in some organs and in their corresponding tumors.

Tissue/organ	Median % O_2	Corresponding cancer	Median % O_2
Brain	4.6	Brain tumor	1.7
Breast	8.5	Breast cancer	1.5
Kidney cortex	9.5	Renal cancer	1.3
Liver	4.0–7.3	Liver cancer	0.8
Lung	5.6	Non-small cell lung cancer	2.2
Pancreas	7.5	Pancreatic tumor	0.3
Rectal mucosa	3.9	Rectal carcinoma	1.8

Hypoxia induces disorganized tumor microvasculature and such abnormal tumor vascular network often fails to rectify the oxygen deficit. While normal tissue is composed of mature and well-organized blood vessels, abnormal tumor vasculature is largely composed of immature vessels characterized by increased permeability, vessel diameter, vessel length, vessel density, tortuosity, and interstitial fluid pressure. Such characteristics of tumor vasculature compromise the delivery of chemotherapeutic drugs and nutrients (9). While the role of hypoxia in tumor resistance to chemotherapy and radiotherapy is currently well described (10), emerging evidence points to its involvement in tumor resistance to immunotherapy. Indeed, experimental and clinical evidence suggests that the hypoxic tumor microenvironment is responsible for the establishment of large number of mechanisms suppressing the anti-tumor immune functions [reviewed in Ref. (11)]. We have shown that the anti-tumor immune response is dramatically impaired under hypoxic stress (12–17). It has been reported that the tumor-killing function of immune cells present in the hypoxic tumor microenvironment is largely attenuated and the immune cells at the hypoxic area of tumors displayed an anergic phenotype induced by malignant cell-derived factors (18). In addition, immune cells in the tumor microenvironment not only fail to perform their anti-tumor effector functions, but also they are co-opted to promote tumor growth (19). Thus, a hypoxic tumor microenvironment not only contributes to chemotherapy and radiotherapy resistance, but also induces the evasion of tumor cells from immunosurveillance. The compelling evidence for the involvement of hypoxia in tumor resistance to anti-cancer therapies makes it a high priority target for cancer therapy. Several preclinical and clinical trials have been initiated using hypoxia-activated prodrugs that target hypoxic tumor compartments or hypoxic bone marrow niches. However, despite compelling evidence highlighting the role of hypoxia in therapy resistance, several hypoxia-activated prodrugs failed to show efficacy in clinical trials (20). Such failure could be attributed to the lack of predictive biomarkers for hypoxia-activated prodrugs and to some technical challenges of assaying such drugs in appropriate clinical settings (20).

HYPOXIA INDUCIBLE FACTOR-1α (HIF-1α) IS THE MAJOR HYPOXIA SENSOR

Hypoxic is sensed to a large extent by the HIF-1α. Briefly, the structure of HIF-1α composed of two oxygen-dependent degradation domains (ODDD) at the N-terminal (N-ODDD) and the

C-terminal (C-ODDD) parts. In addition, HIF-1α displayed two transactivation domains (TADs), one N-terminal, which overlaps with the C-ODDD, and another C-terminal (21). HIF-1α is constantly synthesized in an O_2-independent manner under normoxia, however, it is rapidly degraded by the ubiquitin proteasome system (UPS) in O_2-dependent mechanism (22). Thus, under hypoxic stress, the decrease in the O_2 pressure prevents the degradation of HIF-1α leading to its accumulation in the cytoplasm. It should be noted that, under normoxic conditions, the half-life of HIF-1α is very short, which is less than 5 min (23). The degradation of HIF-1α under normoxic conditions is related to its ability to be hydroxylated on proline residue 402 and/or 564 in the ODDD by prolyl hydroxylase domain protein 2 (PHD2) and its subsequent binding to the von Hippel–Lindau tumor suppressor protein (pVHL). pVHL is a component of an E3 ubiquitin-protein ligase complex that targets HIF-1α for proteolysis by the ubiquitin proteasome pathway (24).

Three prolyl hydroxylase domain (PHD) enzymes (PHD-1, PHD-2, and PHD-3) regulating HIF-1α proteasomal degradation have been identified (25, 26). Under hypoxia, the low O_2 level inhibits the activity of PHD2, and HIF-1α is no longer hydroxylated and its proteasomal degradation event is blocked (26). Therefore, HIF-1α is accumulated in the cytoplasm and then translocation to the nucleus. In the nucleus, HIF-1α dimerizes with HIF-1β and the HIF-1α/HIF-1β heteromer binds to the hypoxia responsive element in target genes before recruiting coactivators and inducing the transcription of several downstream target genes (27). More than 800 genes involved in several pathways and biological processes are reported to be transcriptionally activated by HIF-1α (21) since they contain in their promoter the core sequence 5′-[A/G]CGT-3′, which in most cases is ACGTG (28). Two other isoforms of HIFs family HIF-2α and HIF-3α have been identified; but only HIF-2α is stabilized by oxygen-dependent hydroxylation similar to HIF-1α (29). HIF-1α and HIF-2α share similar structure of their DNA binding and dimerization domains but differ in their TADs (30). HIF-3α functions as an inhibitor of HIF-1α and HIF-2α.

AUTOPHAGY ACTIVATION BY HYPOXIC STRESS IN THE TUMOR MICROENVIRONMENT

Macroautophagy (hereafter referred as autophagy) is an evolutionarily conserved cellular catabolic process responsible for the degradation of damaged proteins and organelles to produce alternative energy source necessary for maintaining cell homeostasis and viability. Although autophagy is executed at basal level in all cells, it is frequently increased in established tumors (31).

Basically, autophagy process contains three major steps: (i) the induction and phagophore formation; (ii) phagophore elongation and autophagosome formation; and (iii) fusion, degradation, and recycling. Briefly, the first step is initiated by a nucleation step or the formation of phagophore that involves two protein complexes: the class-III PI3K/Vps34, Atg6/Beclin1, and Atg14 and Vps15/p150 complex and the serine/threonine kinase Atg1/ULK1, which is a positive regulator of autophagosome formation. The maturation of the phagophore requires several autophagy-related proteins (ATG). During this step, portions of the cytoplasm are engulfed and the microtubule-associated protein 1 light chain 3 (LC3)-I is lipidated to LC3-II. During the maturation, the phagophore is closed by the action of LC3-II and BECN1 proteins, and this step is required for the formation of autophagosome. Materials intended to be degraded are finally sequestered in the autophagic vacuole that will be fused with lysosomes and subjected to degradation by lysosomal hydrolases (32).

Several studies reported that advanced tumors could be addicted to autophagy to maintain their energy balance (33, 34). Indeed, in cancer patients' high autophagic index is correlated with less responsive to cancer therapy and worse survival compared with those with a low autophagic index (35). Therefore, autophagy has been recently considered as a major process in regulating the progression of hypoxic tumors.

Under hypoxia, autophagy is basically activated by three major pathways (36): low O_2 pressure; unfolded protein response; and energy depletion. In this review, we will describe how autophagy is activated by low O_2 level in tumors and summarize recent data describing how autophagy activation under low O_2 pressure operating in tumor cells as a major resistance mechanism to anti-tumor immune response.

Hypoxia is a major characteristic of almost 50–60% of tumors (37), and that increased autophagy induces tumor cell survival (38). The stabilization of HIF-1α under hypoxia leads to its translocation to the nucleus. In the nucleus, HIF-1α induces the expression of downstream target genes, the BH3-only protein Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and the related protein, BNIP3L (39). The upregulated expression of BNIP3 and BNIP3L dissociates Beclin1 from Bcl-2 and activates autophagy.

HYPOXIC TUMOR CELLS ACTIVATE AUTOPHAGY TO ESCAPE CYTOTOXIC T-LYMPHOCYTES (CTL)-MEDIATED KILLING

Several mechanisms have been described to induce hypoxic tumor cell escape from CTL-mediated killing. Bellow, we will briefly describe those involving autophagy activations.

Hypoxia-Induced Autophagy Regulates Phospho-Signal Transducer and Activator of Transcription 3 (STAT3) Degradation

Signal transducer and activator of transcription 3 is a transcription factor that can be activated through phosphorylation by cytokine and growth factor signaling pathways including interleukin (IL)-6 (40), epidermal growth factor, and vascular endothelial growth factor (41). Following phosphorylation, STAT3 promotes tumor cell survival, proliferation, angiogenesis/metastasis, and immune escape (42–44). It has been reported that the immune escape properties of phospho-STAT3 relies on its ability to induce several genes responsible for immunosuppression (45–48). We have previously reported for the first time that hypoxic lung

carcinoma cells can evade CTL-mediated killing by activating autophagy and that targeting autophagy by silencing ATG5, and Beclin1 was sufficient to restore their CTL-mediated killing (16, 49). We provided evidence that targeting autophagy in hypoxic cancer cells led to the accumulation of the adaptor protein sequestosome1 (SQSTM1/p62). Accumulated SQSTM1/p62 bound selectively to pSTAT3 and induced its selective degradation by the UPS. These data highlight targeting autophagy as a valuable strategy to improve CTL-mediated killing of hypoxic cancer cells. This statement was further supported by *in vivo* data using hydroxychloroquine (HCQ) as autophagy inhibitor in B16-F10 tumor-bearing mice (16). Thus, the effect of HCQ on the tumor growth of B16-F10 melanoma was assessed alone or in combination with a tyrosinase-related protein-2 (TRP2) peptide-based vaccination strategy. A synergistic effect on the inhibition of tumor growth was observed by combining HCQ with TRP2 vaccination, indicating that targeting autophagy represents an innovative strategy to improve the anti-tumor effect of TRP2-based vaccine.

Hypoxia-Induced NANOG Expression Activates Autophagy by Regulating BNIP3L

In addition to the mechanism described above, other studies showed that hypoxia impaired CTL-mediated lysis by transcriptionally upregulating the stem cell self-renewal transcription factor NANOG (50, 51). It has been reported that targeting NANOG in hypoxic cells restored CTL-mediated tumor cell killing. In this regards, a link between NANOG expression and the phosphorylation of STAT3 has been proposed, since NANOG depletion results in the inhibition of STAT3 phosphorylation and its nuclear translocation. More recently, a direct regulation of autophagy inducer gene BNIP3L by NANOG has been reported by chromatin immunoprecipitation and luciferase reporter assays showing that NANOG binds directly to the enhancer sequence of BNIP3L and activates its transcription. These data strongly argue that the pluripotency factor NANOG and autophagy cooperate to induce resistance to CTL under hypoxia (52).

HYPOXIA-INDUCED AUTOPHAGY LEADS TO TUMOR CELLS ESCAPE FROM NK-MEDIATED KILLING

Similar to CTL, NK cells of the innate immune system able to recognize and kill tumor cells (53). The recognition and the killing of tumor cells by NK depend on the balance between the expression of activating and inhibitory receptors on the surface of NK cells and their corresponding ligands on the surface of tumor cells (54). Similar to CTL, NK cells kill their target following the establishment of immunological synapse (55) and the secretion of cytotoxic granules containing perforin and granzymes. In tumor cells, the secreted granules induce cell death by apoptosis (56). NK cells are also able to kill their target by tumor necrosis factor superfamily dependent mechanism (57). Below, we will briefly describe the major autophagy-related mechanisms responsible from tumor escape form NK-mediated killing.

Hypoxia-Induced Autophagy in Tumor Cells Degrades NK-Derived Granzyme B

We have reported that autophagy activation in tumor cells impaired NK-mediated killing by selective degradation of NK-derived granzyme B in the lysosome compartment. Using GFP granzyme B-expressing NK cells, we provided evidence that the level of granzyme B is significantly lower in hypoxic tumor cells compared with normoxic tumor cells. Targeting autophagy by knocking down Beclin1 in hypoxic tumor cells was sufficient to rescue the granzyme B level in hypoxic cells and restore NK-mediated lysis (12, 58, 59). These data clearly suggest that during its intracellular trafficking in hypoxic tumor cells, granzyme B is exposed to a high risk of being targeted to autophagosomes and subsequently to the lysosome compartment to be degraded (**Figure 1**). While autophagy has long been considered as a process of non-selective bulk degradation, new evidence suggested that it can be a selective degradation process under stress conditions. The selectivity of autophagy to degrade specific proteins depends on several cargo protein including SQSTM1/p62. In keeping with this, no data are available so far describing whether granzyme B is selectively degraded by autophagy or it is

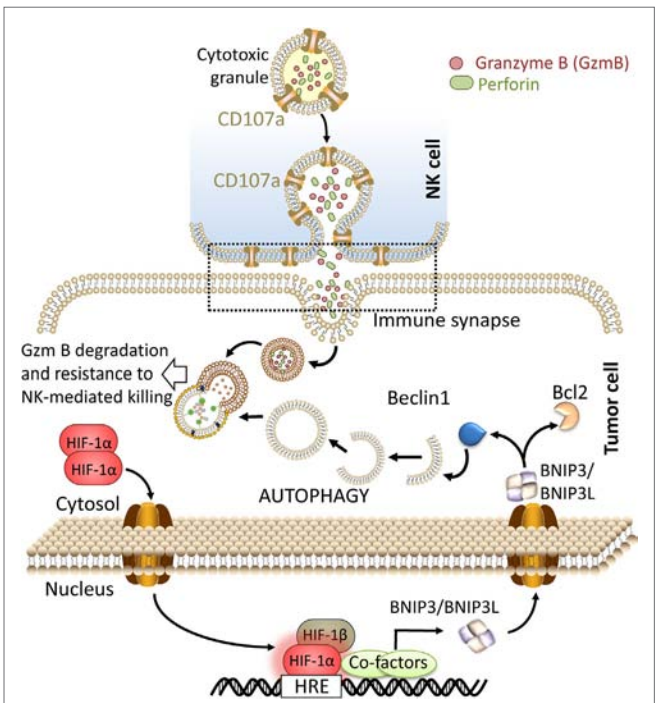

FIGURE 1 | Targeting autophagy in hypoxic tumor cells restores natural killer (NK)-mediated tumor cell killing by preventing the degradation of granzyme B. The recognition of tumor cells by NK leads to the release of cytolytic granules containing perforin and granzyme B from NK cells. These cytotoxic granules enter to the tumor cells through endocytosis and traffic to enlarged endosomes called "gigantosomes." Following the formation of pores in the "gigantosome" membrane, granzyme B is released in the cytoplasm and initiates cell death. Under hypoxia, excessive autophagy leads to the fusion of "gigantosomes" with autophagosomes and the subsequent degradation of granzyme B. Degraded granzyme B is no longer able to induce tumor cell death, therefore, targeting autophagy prevents the degradation of granzyme B and restores NK-mediated lysis.

just an "innocent victim" subjected to non-specific degradation under hypoxia in tumor cells.

Targeting Autophagy Induces a Massive Infiltration of NK Cells into the Tumor Bed

Based on our data showing that targeting autophagy restores tumor cell susceptibility to NK-mediated lysis *in vitro*, we investigated whether blocking autophagy reduces tumor growth in an NK-dependent manner. We used BALB/c and C57BL/6 mice transplanted with syngeneic murine 4T1 breast adenocarcinoma and B16-F10 melanoma tumor cells, respectively. We first showed that the tumor growth of these two syngeneic mouse models is primarily controlled by NK cells as the depletion of host NK cells significantly increases tumor growth. We next assessed the impact of targeting Beclin1 on the tumor growth. Our data showed that targeting Beclin1 resulted in a significant decrease in the tumor growth presumably as a consequence of potentiation of tumor cell killing by NK cells. The decrease in the tumor growth was no longer observed when NK cells were depleted. In keeping with this, we showed a massive infiltration of NK cells into Beclin1-defective compared with control B16-F10 tumors. Mechanistically, we showed that the infiltration of NK cells is related to the ability of Beclin1-defective tumor cells to overexpress CCL5 cytokine responsible for the trafficking of NK cells to the tumor (**Figure 2**). The infiltration of NK cells was completely abrogated when CCL5 was silenced in Beclin1-defective tumor. Furthermore, we showed that the overexpression of CCL5 involved the activation of the transcription factor c-Jun by JNK (60).

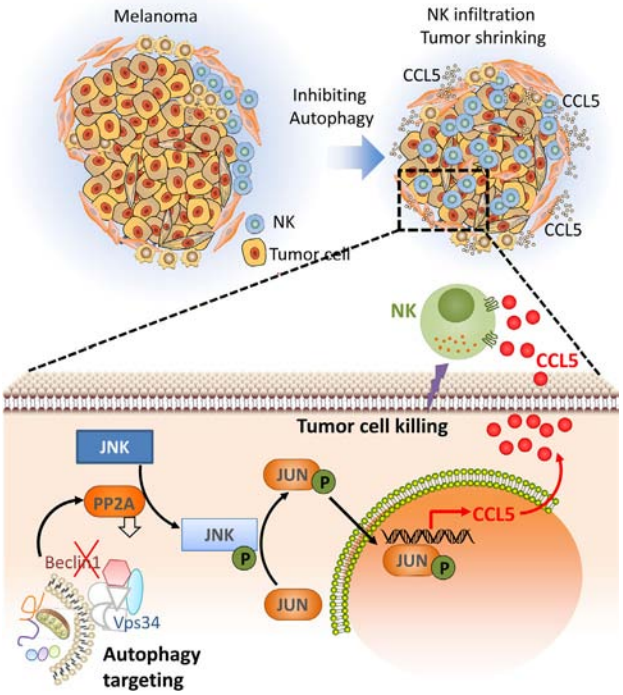

FIGURE 2 | Targeting autophagy leads to tumor regression by inducing the infiltration of natural killer (NK) cells into the tumor bed. Targeting autophagy in tumor cells induces the expression of CCL5 cytokine. Through paracrine mechanism, CCL5 binds its receptors expressed on the surface of NK cells and induces the recruitment of functional NK cells to the tumor bed. Functional NK cells recruited to the tumor kill cancer cells leading to tumor regression. The lower part describes the molecular mechanism underlying the expression of the chemotactic cytokine CCL5. Briefly, targeting Beclin1 leads to a decrease in the activity of the protein phosphatase 2A by a mechanism not yet understood. Such a decrease enhances the phosphorylation of JNK that subsequently phosphorylates c-JUN. Phosphorylated c-JUN binds to the promoter of Ccl5 and induces its transcription. CCL5 released by Beclin1-defective tumor cells binds to CCL5 receptor on the surface of NK cells, and induces their infiltration. Functional NK cells recruited to the tumor site kill cancer cells and thereby reduce the tumor volume.

TARGETING AUTOPHAGY IN THE CONTEXT OF CANCER THERAPY: FRIEND OR FOE?

Several lines of evidence supports the concept that autophagy activation is associated with cancer cell resistance to chemotherapy (61, 62), radiotherapy (63, 64) and immunotherapy (12, 16, 58) either by supporting cell metabolism directly (65) or through the impairment of cell death pathway (66). Therefore, several preclinical and clinical studies have been undertaken to develop drugs able to inhibit autophagy (67). Basically, pharmacological inhibitors of autophagy pathway can be classified into three classes: (i) inhibitors of the initiation step of autophagy; (ii) inhibitors of the nucleation of phagophore; and (iii) inhibitors of the fusion of autophagosomes with lysosomes [reviewed in Ref. (67, 68)]. In this review, we will not describe all drugs inhibiting each step of autophagy but briefly describe the action of those displaying potent anti-tumor activities.

Chloroquine (CQ) has been approved for decades in the treatment of malaria and arthritis, and currently used as autophagy inhibitors. CQ blocks the last step of autophagy process before the fusion of autophagosomes with lysosomes (69). Therefore, several clinical trials are currently evaluating CQ or its derivative HCQ alone or in combination with chemotherapy or radiotherapy in patients with several types of cancers (70). Briefly, a significantly prolonged median survival of glioblastoma (GBM) patients (33 months compared with 11 months) was observed using CQ combination with temozolomide and radiotherapy (40). The combination of CQ with radiotherapy also reported in a pilot and phase II clinical trials to improve the survival of non-small cell lung carcinoma, squamous cell lung carcinoma, and breast and ovarian cancer patient with brain metastasis (71). Another phase I/II clinical trial using CQ in combination with radiotherapy in GBM showed no significant improvement in the survival (72) due to an inconsistent inhibition of autophagy between patients and dose-limiting toxicities that prevented the use of high CQ doses. In some trials, CQ was also used as monotherapy, notably in patients with metastatic pancreatic cancer, but no clinical benefit was observed. This failure to provide clinical benefit could be related to inconsistent autophagy inhibition was reported (72) and the limited potential for CQ as single agent to improve end-stage disease outcomes. However, in PDX preclinical model, the single treatment with HCQ was effective (73). The combination of HCQ and gemcitabine in

preoperating setting of patients with pancreatic adenocarcinoma induced a decrease in the serum tumor marker cancer antigen 19-9 in 61% (74). In the context of cancer immunotherapy, the effect of CQ has been evaluated in combination with high-dose interleukin-2 (HDIL-2) in preclinical murine hepatic metastasis model. Combining CQ with HDIL-2 enhanced IL-2 immunotherapeutic efficacy and limit toxicity by increasing long-term survival, decreased toxicity associated with vascular leakage, and enhanced immune cell proliferation and infiltration in the liver and spleen (75).

Based on studies described above, it appears that the clinical response to autophagy inhibitors varied widely. The major difficulties were the identification of appropriate pharmaco-dynamic biomarkers to evaluate the change in autophagy (70). Therefore, none of them formally confirmed that inhibiting autophagy in cancer cells provides therapeutic benefits to cancer patients (76). It remains to be defined whether the lack of therapeutic benefits is related to the lack of the specificity of CQ to inhibit autophagy. Indeed, it should be highlighted that CQ and HCQ are non-selective autophagy inhibitors since they lead to the reduction of nutrient scavenging (77, 78). They could also alter tumor pH, thus affecting other drugs bioavailability when combined with conventional cytotoxic chemotherapies (79). Currently, there is a major interest in developing selective new drugs inhibiting autophagy as an important survival mechanism of tumors.

Lys05 is dimeric form of CQ displaying more potent autophagy inhibitor than CQ, which displays more potent accumulation properties in the lysosome. Lys05 is, therefore, a new lysosomal autophagy inhibitor with a strong potential to be developed into a drug for cancer. It has been reported that Lys05 is a potent anti-tumor drug *in vitro* and in several preclinical mouse model. The potent autophagy inhibition property of Lys05 relied to the bivalent aminoquinoline rings, C7-Chlorine, and a short triamine linker. Since Lys05 is a potent inhibitor of autophagy it can be used at low doses, which are well tolerated and associated with strong anti-tumor activity (80).

Another druggable autophagy target proteins have been recently proposed, which include Beclin-1 and Vps34 (or PI3K class-III) (81). Both of them are involved in the early step of autophagy initiation (82, 83). SAR405 is a kinase inhibitor of Vps18 and Vps34. The inhibition of Vps34 leads to an impairment in the lysosomal function, thus affecting vesicle trafficking between late endosome and the lysosome. The Vps34i (SAR405) has been developed following chemical optimization with highly potent and selective inhibitor of vesicle trafficking from late endosomes to lysosomes. SAR405 inhibits also starvation- and mTOR-dependent induction of autophagy (84, 85).

Another autophagy druggable protein is the serine/threonine kinase ULK1/Atg1 involved in the core autophagy pathway. Cell-based screen allowed identification of a potent ULK1 small molecule inhibitor SBI-0206965. This drug is highly selective ULK1 kinase inhibitor *in vitro* and suppressed ULK1-mediated phosphorylation events in cells. The anti-tumor activity of SBI-0206965 has been proved *in vivo*, thus providing a strong rationale for it use in the clinic (86). NSC185058 has been identified as an effective inhibitor of ATG4B activity. NSC185058 showed a negative impact on the development of Saos-2 osteosarcoma tumors *in vivo* (87). Inhibition of ATG4B using NSC185058 was reported to reduce autophagy and tumorigenicity of GBM cells and to improve the impact of radiotherapy on GBM growth in mice (88). These results suggest that ATG4B is another suitable anti-autophagy target and a promising therapeutic target to treat osteosarcoma.

Beside its role in supporting tumor growth and resistance to therapies, preclinical results suggest that intact autophagic responses in cancer cells are dispensable for the initiation of an appropriate danger signaling and thus for the initiation appropriate anti-cancer immune responses in syngeneic tumor models treated with immunogenic chemotherapy or radiotherapy (89, 90). Indeed, by contrast to autophagy-defective tumors, autophagy-competent tumors attracted dendritic cells and T lymphocytes into the tumor bed. Inhibiting autophagy impaired the immunogenic release of adenosine triphosphate (ATP) from dying tumor cells and subsequently blocked the ATP-dependent recruitment of immune cells (89).

In addition its impact on tumor cells, it has been observed that autophagy actively participates in the intracellular antigen processing for major histocompatibility complex (MHC) class-II and I presentation as well as in extracellular antigen processing for MHC class-II presentation. It has been also reported that autophagy is involved in the cross-presentation of antigens for MHC class-I presentation and in MHC class-I internalization [reviewed in Ref. (91)]. In keeping with this, it appears that the autophagic machinery plays an important role in many aspects of the antigen presentation and therefore raises the question about the net outcome of inhibiting autophagy on the adaptive immunity.

In addition to the role of autophagy in antigens processing, autophagy plays a functional role in different immune cell type. Briefly, in macrophages autophagy plays a crucial role in macrophage homeostasis by different mechanisms [reviewed in Ref. (59)]. The autophagic activity is increased in DCs compared with other cell types. Such autophagic activity is related to intensive processing of extra- and intra-cellular antigens for the MHC class-I and -II presentation (92).

The role of autophagy in T cells was also addressed. In the context of naive T cells, it has been reported that tumor-derived metabolite lactate selectively inhibits FAK family–interacting protein of 200 kDa (FIP200; also known as RB1CC1) in naive T cell leading to autophagy deficiency, apoptosis and poor anti-tumor immunity in ovarian cancer patients, and tumor-bearing mice (93).

In tumor cells, suppression of FIP200 suppresses the initiation and progression of mammary tumor breast cancer driven by the PyMT oncogene. In addition, FIP200 conditional knockout mice display elevated expression level of interferon (IFN)-responsive genes associated with increased infiltration of effector T cells in the tumor microenvironment triggered by the production of CXCL10 chemokine (94). In regulatory T (Treg) cells, autophagy plays a major role in their lineage stability and survival fitness. Specific ablation of autophagy-related genes Atg7 or Atg5 in Treg induces apoptosis and loss of Foxp3 transcription factor (95). In $KRas^{G12D}$-driven lung cancer mouse model, it has been reported that ablation of Atg5 favors adenosinergic signaling *via*

a HIF-1α pathway, as well as the infiltration of tumors by Tregs, thus influencing inflammatory and immunosurveillance mechanisms that can stimulate and control carcinogenesis, respectively (96). Pharmacological blocking of autophagy by CQ enhances IL-2 immunotherapeutic efficacy and limit toxicity. Combining CQ with IL-2 increases long-term survival, decreases toxicity associated with vascular leakage, and enhances immune cell proliferation and infiltration in the liver and spleen (75). These results support the use of autophagy inhibitors as a novel clinical strategy to enhance the efficacy of IL-2-based immunotherapy for cancer patients. Similarly, the ablation of autophagy-related gene GABARAP, inhibits the tumor formation incidence in mice and by enhancing the immune response through increased secretion of IL-1β, IL-6, IL-2, and IFN-γ from stimulated macrophages and lymphocytes (97).

Furthermore, autophagy seems to be an important mechanism for the development, maintenance, and survival of T lymphocytes (98–100). Moreover, the interaction of B cells with CD4+ T cells requires autophagy that promotes the presentation of antigens by MHC class-II molecules through a mechanism reminiscent to that described for DCs (101, 102).

CONCLUDING REMARKS

Given the impressive impact of targeting autophagy on tumor immunity is the ultimate question that arises whether targeting autophagy would improve or impair the efficacy of cancer immunotherapy. Based on our current knowledge available so far, it is difficult to draw a clear statement about this question. In this review, we provided some clues to argue that blocking autophagy for therapeutic purposes requires careful consideration. Although targeting autophagy appears to improve the anti-tumor immune response, it should be highlighted that such strategies must consider the potential negative or positive impact on immune cells. Therefore, it is important to evaluate the net outcome of targeting autophagy in the context of the TME rather than analyzing the impact of targeting autophagy at the cellular level. Moreover, considering this complex role of autophagy in the tumor microenvironment it is still difficult to draw a clear statement whether, when, and how autophagy has to blocked or enhanced for the benefit of cancer patients.

AUTHOR CONTRIBUTIONS

BJ, GB, and SC wrote the manuscript. BJ designed figures.

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Pancreatic Ductal Adenocarcinoma: A Strong Imbalance of Good and Bad Immunological Cops in the Tumor Microenvironment

Etienne D. Foucher[1†], Clément Ghigo[2†], Salem Chouaib[3,4], Jérôme Galon[5], Juan Iovanna[2] and Daniel Olive[1]*

[1] Team Immunity and Cancer, CRCM, Aix Marseille Univ, CNRS, INSERM, Institut Paoli-Calmettes, Marseille, France, [2] Team Cellular Stress, CRCM, Aix Marseille Univ, CNRS, INSERM, Institut Paoli-Calmettes, Marseille, France, [3] INSERM UMR1186, Integrative Tumor Immunology and Genetic Oncology, Gustave Roussy, Equipe Labellisée par La Ligue Contre Le Cancer, EPHE, Faculté de Médecine, Université Paris-Sud, Université Paris-Saclay, Villejuif, France, [4] Thumbay Research Institute for Precision Medicine, Gulf Medical University, Ajman, United Arab Emirates, [5] Laboratory of Integrative Cancer Immunology, INSERM, UMRS1138, Paris, France

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Edited by:
Jason Roszik,
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Reviewed by:
Evangelos P. Misiakos,
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Ying Ma,
University of Texas MD Anderson Cancer Center, United States

***Correspondence:**
Daniel Olive
daniel.olive@inserm.fr

[†]These authors have contributed equally to the work.

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal cancers with very few available treatments. For many decades, gemcitabine was the only treatment for patients with PDAC. A recent attempt to improve patient survival by combining this chemotherapy with FOLFIRINOX and nab-paclitaxel failed and instead resulted in increased toxicity. Novel therapies are urgently required to improve PDAC patient survival. New treatments in other cancers such as melanoma, non-small-cell lung cancer, and renal cancer have emerged, based on immunotherapy targeting the immune checkpoints cytotoxic T-lymphocyte-associated antigen 4 or programmed death 1 ligand. However, the first clinical trials using such immune checkpoint inhibitors in PDAC have had limited success. Resistance to immunotherapy in PDAC remains unclear but could be due to tissue components (cancer-associated fibroblasts, desmoplasia, hypoxia) and to the imbalance between immunosuppressive and effector immune populations in the tumor microenvironment. In this review, we analyzed the presence of "good and bad immunological cops" in PDAC and discussed the significance of changes in their balance.

Keywords: pancreatic ductal adenocarcinoma, immune infiltrate, tumor microenvironment, immunosuppression, hypoxia, immune checkpoint

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth-leading cause of cancer-related death in the world, with a 5-year survival rate of less than 5%. Each year more than 350,000 people worldwide are diagnosed and more than 340,000 die of the disease. The incidence is rising, and some reports project an over twofold increase in the number of new PDAC cases and PDAC deaths by 2030 (1).

The only curative treatment is complete surgical resection. Unfortunately, fewer than 20% of patients are candidates for surgery since their cancer has usually already spread before diagnosis. For this small subgroup of patients undergoing surgery, adjuvant treatment with the chemotherapy

drug gemcitabine, Erlotinib, or more recently FOLFIRINOX has been shown to slightly improve survival (2, 3).

It appears that tumors develop multiple immunosuppressive mechanisms to down-regulate the innate and effector arms of the immune system, thus compromising most of the immunotherapeutic strategies that have been proposed during the last decade. In PDAC, the tumor microenvironment (TME) seems to play a pivotal role in tumor escape. A large number of cells or mechanisms participate together to improve the proliferation of tumor cells (4, 5). One of these is immune cells themselves, in particular immunosuppressive leukocytes that we will discuss in this review. Other components contribute toward PDAC cancerogenesis such as cancer-associated fibroblasts (CAFs) and extracellular matrix proteins. Together, these components interact with tumor cells to develop a pro-tumor environment and support proliferation. Another important mechanism called hypoxia exerts a strong impact on the structure of the tumor tissue (angiogenesis) and also on cells in the TME where hypoxia induces the development of immunosuppressive cell populations. Together these components participate toward inducing the desmoplastic reaction in the TME, which increases the "sealing off" (high level of intra-tumor blood vessel pressure) from effector immune cells (failing upon immune cell recruitment) and from drug delivery (chemoresistance) (6).

In this review, we focus on the organization and the role of infiltrating anti- or pro-tumor immune cell populations (referred to as "good and bad immunological cops," respectively) during the course of PDAC and discuss the state-of-the-art of immunotherapy in PDAC.

INFLAMMATION AND IMMUNE CELL INFILTRATE IN THE TME

The link between tumor growth and inflammation has been greatly illustrated in the literature. The three Es (Elimination/Equilibrium/Escape) of cancer immunoediting perfectly reflect the development of pancreatic cancer and the immune population evolution in the TME (7). While inflammation is classically associated with an anti-tumor Th1 immune response (cancer immunosurveillance/elimination phase), tumor-associated inflammation is chronic, smoldering, and detrimental and participates toward tumor cell development and the accumulation of immunosuppressive leukocytes (equilibrium and escape phases) (**Figure 1**). In some cancers such as PDAC, Kras or myc

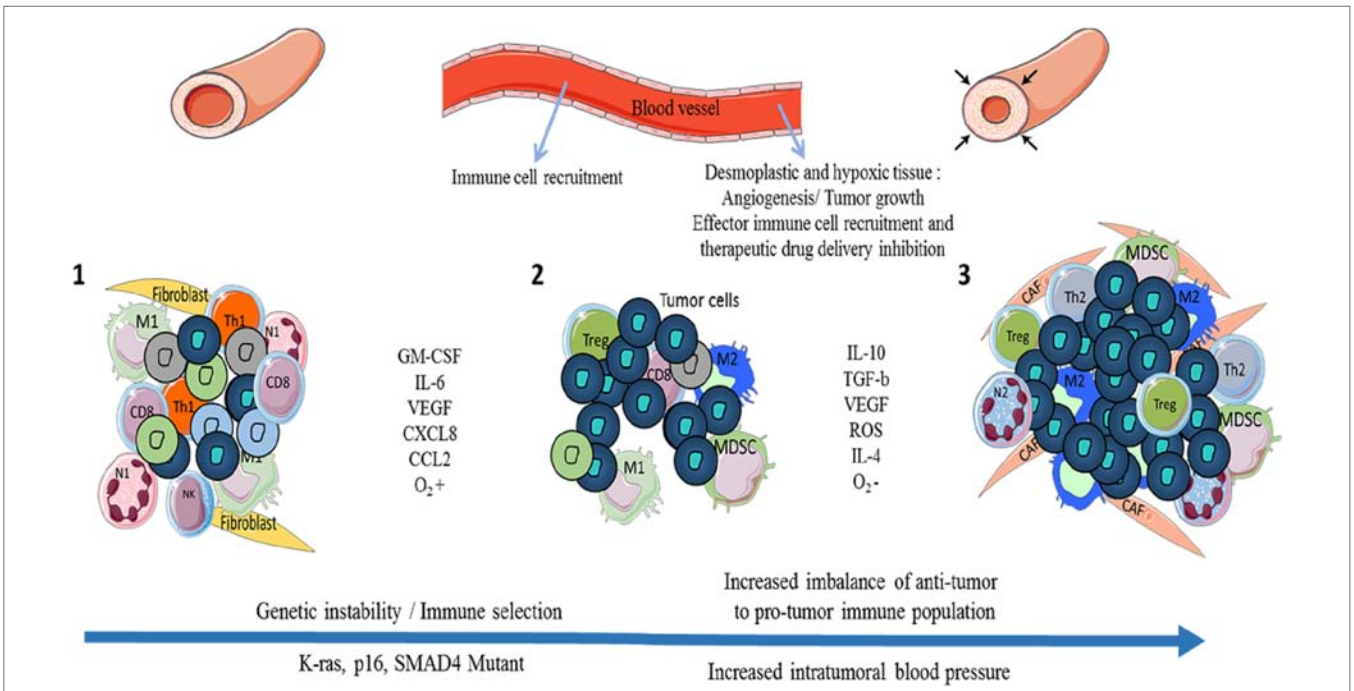

FIGURE 1 | Evolution of the immune cell population and pancreatic ductal adenocarcinoma (PDAC) development through the three Es of cancer immunoediting. During cancer immunosurveillance (1), immune effector cells M1 macrophages and N1 neutrophils are recruited to the tissue in order to eliminate heterogenic mutant/tumor cells. While these immune cells kill most tumor cells, specific resistant tumor clones (in dark blue) survive (2). An equilibrium between anti- and pro-tumor immune cells is maintained until tumor cells and immunosuppressive immune cells develop tumor escape mechanisms via the secretion of pro-tumor factors (IL-10, TGF-β, etc.) and inhibitory co-signaling molecules (3). Tumor escape induces the growth of tumor cells, angiogenesis, metastasis, the establishment of an immunosuppressive microenvironment with the presence of Tregs, tumor-associated macrophages (TAMs) such as M2, CAFs, myeloid-derived suppressive cells (MDSCs), tumor-associated neutrophils (TANs) such as N2 and with hypoxia and desmoplasia, which increase the pro-tumor impact and create a barrier (high blood pressure) against therapeutic drug delivery and recruitment of effector immune cells. M1: anti-tumor macrophages, M2: pro-tumor macrophages, N1: anti-tumor neutrophils, N2: pro-tumor neutrophils, CD8: $CD8^+$ T cells, Th1/Th2: $CD4^+$ Th1 (anti-tumor) or Th2 (pro-tumor) T cells, Treg: regulatory T cells, CAFs: cancer-associated fibroblasts.

oncogenes are responsible for such chronic and smoldering inflammation in the TME (8, 9). Regardless of origin, this inflammation allows cancer cells to establish the tumor escape and development processes (10, 11). In PDAC, despite the hypoxia and hyaluronan-induced development of desmoplastic stroma, the TME is composed of several immune cell populations (12). At early stages, effector cells such as natural killer (NK) cells, $CD8^+$ T cells, and $CD4^+$ T cells can be present and activated. Nevertheless, during the selection of resistant tumor cells (during the elimination process) and the development of the escape mechanism, the TME induces the recruitment of monocytes and neutrophils, which then have acquired an anti-inflammatory phenotype (M2 and N2 respectively), the recruitment of myeloid-derived suppressive cells (MDSCs), the recruitment and/or the polarization of regulatory T cells (Tregs) or Th17, and the recruitment of Th1 to Th2 cell shift (13, 14). Furthermore, $CD8^+$ T cells, NK cells, and dendritic cells are deactivated or exhausted in order to inhibit anti-tumor function. Of course, the transformation of pro-inflammatory to anti-inflammatory in the TME increases the tumor growth and angiogenesis and correlates with poor survival (**Figures 1** and **2**) (13).

GOOD COPS

Effector Immune Cells

$CD8^+$ T Cells

Tumor infiltrated $CD8^+$ T cells (also called cytotoxic T lymphocytes; CTLs) are immune effector cells that can kill cancer cells using perforin and granzyme molecules. Analysis in peripheral blood has revealed significantly decreased circulating CTLs and lower perforin expression levels in pancreatic cancer patients compared with healthy controls (15). Immunohistochemistry on pancreatic cancer samples showed a higher cellular infiltration compared to normal pancreas and survival studies have shown that higher levels of tumor infiltrating $CD4^+$ and $CD8^+$ T cells are associated with longer survival (16).

Shortly after T-cell activation, cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) is translocated to the plasma membrane. This co-receptor molecule binds to B7 ligand with a higher affinity than does the co-receptor CD28, leading to inhibition of the T-cell activation. Furthermore, PDAC cells express PD-L1, which binds to PD1 expressed on activated T cells (17). Interaction between these molecules leads to T-cell anergy or death and consequently promotes tumor progression (18).

The restoration of exhausted $CD8^+$ T cells and recovery of their effector role represent one of the main therapeutic objectives toward the destruction of cancer cells.

$CD4^+$ T Cells

$CD4^+$ T cells (T helper cells) play an important role in the immune response by secreting several cytokines that modulate the function of B and $CD8^+$ T cells. Their peripheral blood levels are reduced in patients with pancreatic cancer compared to healthy controls (19). Naive $CD4^+$ T cells can differentiate into the following two main subsets: Th1 cells, which support cell-mediated immune responses by secreting IL-2 and IFN-γ (activate macrophages and $CD8^+$ T-cell proliferation), and Th2 cells, which induce humoral immune responses by secreting IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (stimulate B-cell proliferation and induce B-cell antibody class switching) (20). In PDAC cancer, the shift from Th1 to Th2 cells is correlated to reduced survival.

NK Cells

Natural killer cells are cytotoxic lymphocytes of the innate immune system. Activation of these cells is determined by the balance between activating and inhibitory receptor stimulation. Analysis of peripheral blood mononuclear cells has revealed reduced levels of NK cells in patients with PDAC compared to healthy controls (19). Patients were also found to have significantly lower levels of two activating receptors (CD226 and CD96) on their circulating NK cells compared to healthy controls (21). The decrease in the level of activating receptors on NK cells could indicate dysfunction of these cells and may represent a factor promoting PDAC progression. These data suggest that reactivation of NK cells via these activator receptors could be a new target for cancer immunotherapy.

BAD COPS

Anti-Inflammatory Myeloid Cells

Tumor-Associated Macrophages (TAMs)

Monocytes recruited to the tumor site can differentiate into TAMs. In the majority of solid tumors such as in PDAC, TAMs represent the most abundant immune population in the TME. Tumor cells express many factors including CCL2 (under hypoxic conditions), M-CSF or GM-CSF, IL-10, TGF-β, and IL-6, all of which favor the recruitment and generation of TAMs (22). At early cancer stages, TAMs can be polarized into an anti- (M1) or pro- (M2) tumor phenotype (23, 24), whereas at advanced stages, they are mainly present as the M2 subtype ($CD14^+$ $CD163^+$) (23) and their presence is associated with bad prognosis in PDAC (25, 26). As reported by Cui et al, TAMs play large roles in the promotion of tumor growth and development of an immunosuppressive microenvironment. They do this by secreting angiogenic factors (IL-6, VEGF, and MMP), as well as immunosuppressive factors (IL-10 TGF-β), that promote the generation of an immunosuppressive cell population and inhibit effector T cells, and also other factors such as chemokines and cytokines that promote metastasis and epithelial–mesenchymal transition (27). Therefore, TAMs represent an important therapeutic target for inhibition at the level of their activation, recruitment, and survival or for the reprogramming of polarization (27, 28). Shibuya et al. showed that multimodal neoadjuvant chemotherapy could decrease the number of immunosuppressive infiltration cells such as myeloid cells (29).

Tumor-Associated Neutrophils (TANs)

Analogous to the M1 and M2 dichotomy for TAMs, TANs exhibit a pro-tumor N2 profile with pro-tumor function through the influence of TGF-β (30). Furthermore, pancreatic cancer cells attract neutrophils through the secretion of chemokines, such

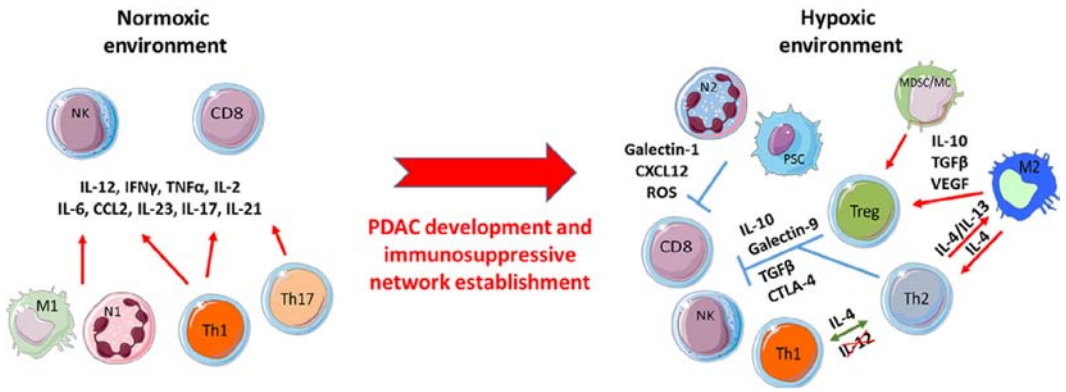

FIGURE 2 | Pancreatic ductal adenocarcinoma (PDAC) development induces the shift of pro-inflammatory to immunosuppressive immune populations. At early PDAC stages, a primarily anti-tumor immune population favors the effector T-cell functions of such as $CD8^+$ and natural killer (NK) cells toward the prevention of tumor cell growth. Despite this initial anti-tumor response, over time chronic activation of these effector immune cells brings about a smoldering inflammation process that selects resistant tumor clones resulting in the promotion and development of immunosuppressive immune populations under hypoxic stress.

as CXCL8 and CXCL16 (31). Few studies have evaluated the function of TANs; however, those with the N2 profile have been shown to produce matrix metallopeptidases including MMP-8, MMP-9, neutrophil elastase, reactive oxygen species (ROS), and VEGF and some inflammatory cytokines including TNFα and GM-CSF, which promote tumor and immune cell proliferation (metastatic potential) and favor chronic inflammation (31, 32).

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells are a population of cells defined by their immature state, myeloid origin and capacity to suppress the immune response. Through factors in the TME, they can acquire phenotypic and functional characteristics of TAMs and TANs and are thus called mononuclear (Mo-) or granulocytic (G-) MDSCs (14, 31). They are strongly immunosuppressive by their ability to inhibit T-cell proliferation, IFNγ production, and effector T-cell function and to favor Treg generation through the secretion of ROS, Arg1, and iNOS (33, 34). They also promote tumor growth by VEGF and MMP9 secretions. High concentration of MDSCs in the peripheral blood is associated with poor prognosis in PDAC (35).

Mast Cells (MCs)

Mast cells can release cytotoxic granules and have the capacity to recruit other immune cell populations by chemokine secretion. They have been found in significantly higher numbers in PDAC compared to those in normal pancreatic tissue (36), where they support tumor growth and angiogenesis and inhibit anti-tumor immunity. The MCs accumulate within the TME, along with macrophages, through the action of tumor-derived chemoattractants such as MCP-1 and RANTE and by tumor-secreted VEGF and FGF (37). MC accumulation correlates with higher tumor grade, diminished survival, and lymph node metastasis.

In vitro, MCs induce PDAC cell proliferation and migration (angiogenesis and metastasis) by secreting factors including secretin, VEGF, and IL-8 and tumor growth factors including PDGF and proteases (38, 39).

Anti-Inflammatory Lymphoid Cells

Tregs

In PDAC and solid tumors, $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs are strongly associated with poor prognosis and inversely correlated to the presence of $CD8^+$ T cells, with more advanced disease presentation, a lower chance of surgical resection and a poorer survival after resection (36, 40). Patients with PDAC have increased numbers of Tregs. They produce IL-10 and TGF-β and express CTLA-4; thus, they inhibit effector T cells and induce M2 profile TAMs and N2 profile TANs (41).

Th17 Lymphocytes

The role of Th17 cells in cancer is highly controversial. Their function seems to depend on the type of cancer, the tumor stage, and the localization (42). In PDAC, while some evidence favors a higher level of Th17 cells in advanced stage tumors, other data in a murine model of pancreatic cancer support Th17 induction increasing survival (43, 44). This inconsistency can be explained by the plasticity of Th17 cells and their ability to promote smoldering inflammation at early stages (45–47). Indeed, Th17 cells are polarized on the one hand by IL-6, IL-23, and Il-1β with pro-inflammatory functions (impact on smoldering inflammation and recruitment of inflammatory immune population) and on the other hand by TGF-β, which induces anti-inflammatory functions (impact on tumor growth, immunosuppressive microenvironment, and angiogenesis) (46, 48). Furthermore, the shift of Th17 to Treg, explained by the plasticity of these cells, is important. In PDAC, patients were shown to exhibit Th17/Treg disorders with higher Treg and lower Th17 cells (49).

Th2 Lymphocytes

Th2 cells ($GATA\text{-}3^+$ $IL\text{-}13^+$ $IL\text{-}4^+$), in contrast to Th1 cells, are anti-inflammatory T cells. In PDAC, the TME and CAFs were shown to induce the polarization of Th2 cells by IL-13-mediated

dendritic cell secretion *in vitro* (50). The Th2 cells produce IL-13 and IL-4 and thereby induce M2 macrophages or TAMs, which further increases the anti-inflammatory TME. Furthermore, via an amplification loop and T-cell plasticity, Th2 cells inhibit Th1-cell polarization and induce themselves. In tumor tissue, Th2 T-cell infiltrates are a predictive marker of poor prognosis, confirmed by the shift of Th1 to Th2 cells within the TME (13).

γδ T Cells

γδ T cells are "unconventional" T cells. Unlike αβ T cells, these lymphocytes do not require antigen processing and major histocompatibility complex presentation of peptide epitopes. In contrast to current dogma, one study using a mouse model and human samples showed that γδ T cells have no anti-cancer properties in pancreatic cancer (51). *In vivo* deletion of γδ T cells using a neutralizing antibody resulted in a robust protection against oncogenic progression. The analysis also revealed that infiltrating γδ T cells express high levels of T-cell exhaustion ligands (PD-L1 and Galectin-9) and may block the immune response by immune checkpoint inhibition. Altogether these data suggest that, in PDAC, γδ T cells promote pancreatic oncogenesis and that their deletion or reactivation could be a novel therapeutic strategy. Surprisingly, the key regulator of Vγ9Vδ2 function BTN3A1 was found to act as a critical marker of PDAC prognosis and is detectable either by IHC or by its soluble receptor sBTN3A1 (52).

Other Main Anti-Inflammatory Mechanisms

Hypoxia

Pancreatic cancer stroma is composed of several main components: CAFs, immune cells and associated cytokines, adipocytes, and endothelial cells. These stromal components are involved in the production of highly toxic conditions including low pH and low oxygen environment (hypoxia). To define the hypoxic status of pancreatic cancer, one study measured tissue oxygenation of the tumor and normal adjacent pancreas during pancreaticoduodenectomy surgery (53). Results of this study showed that PDAC are highly hypoxic compared to normal pancreas.

Cancer cells under hypoxic conditions are more resistant to radiation and chemotherapy (54, 55). This ability to survive is mainly conferred by the hypoxia-inducible pathway involving transcription factors able to induce the expression of several genes controlling cell survival, glycolysis, and other cellular metabolism events. Recent evidence supports the hypothesis of hypoxia being one cause of radioresistance. Indeed, Hajj et al. showed that radiation therapy in combination with TH-302 (a hypoxia-activated pro-drug) allowed tumor growth delay in an orthotopic model of PDAC by comparison with the outcome following these two treatments given separately (56). This TH-302 compound is currently being tested in a pancreatic cancer Phase I clinical trial in combination with Nab-paclitaxel and gemcitabine.

Despite the high levels of hypoxia found in pancreatic cancer, which would be expected to promote angiogenesis, PDAC remains poorly vascularized. This poor vascularization limits blood flow to the tumor and is associated with prominent desmoplasia, which prevents drug delivery and could impede the immune response (57). This hypoxia seems to impact on several escape mechanisms and could therefore be a relevant target for next generation therapeutic options.

Pancreatic Stellate Cells (PSCs)

In non-inflamed pancreas, PSCs are resident cells involved in maintaining tissue homeostasis by regulating extracellular matrix turnover (58). During pancreatic injury, quiescent PSCs are activated and transform into myofibroblast-like cells. These activated PSCs secrete extracellular matrix proteins, which generate fibrosis and limit drug delivery to cancer cells (59). Inordinate secretion of extracellular matrix proteins is also linked to hypoxia (see paragraph above) and promotes cancer cell proliferation.

Pancreatic stellate cells can also modulate immune cells via their secretion of cytokines. Indeed, secretion of CXCL12 by activated PSCs reduces the migration of $CD8^+$ and $CD4^+$ T cells, NK cells, and Tregs to the juxtatumoral compartment within close proximity to the tumor (60). Another study showed that PSCs secreted Galectin-1, which mediated immunosuppression of $CD8^+$ T cells and promoted T-cell apoptosis (61). All these data suggest that PSCs could be a good target to enhance immunotherapy for PDAC.

IMMUNOTHERAPY IN PDAC: STATE-OF-THE-ART

Pancreatic ductal adenocarcinoma is currently recognized as one of the deadliest human malignancies. Compared to other cancers, PDAC shows marked resistance to conventional forms of chemotherapy and often develops without early symptoms making its detection and early diagnosis very difficult, greatly limiting treatment capability. No current treatment option has demonstrated long-term benefit in patients with advanced disease who are not eligible for surgery, which represents the majority (80%) of PDAC cases. Although some risk factors have been identified (such as tobacco use, family history of PDAC, and a personal history of pancreatitis, diabetes, or obesity), few patients diagnosed with PDAC have identifiable risk factors (1, 62). For many years, gemcitabine monotherapy was the only treatment available for this cancer (2). More recently, studies found that using gemcitabine in combination with FOLFIRINOX and nab-paclitaxel was more effective than gemcitabine monotherapy (3). Unfortunately, this combination therapy prolonged survival by only a few months and actually increased toxicity.

New therapies are thus urgently needed to combat this highly lethal cancer and further extend the lives of affected patients. Immune-based strategies to treat various cancers during the early stages of development, as well as new immunological approaches to treat advanced disease, are showing significant promise where other approaches have failed (63, 64). In PDAC, potential immunology-based therapies have provided new hope and can be divided into three main subtypes: (i) therapeutic vaccines aimed, as those protecting against infection, to stimulate the immune system to produce tumor-specific T cells and B cells (65); (ii) adoptive therapy in which *ex vivo* expanded cytotoxic cells are injected into the tumor to kill cancer cells (66); and (iii)

immune checkpoint inhibitors. After their activation, T cells express "blocker" molecules called immune checkpoints, which allow them to return to normal. Cancer cells divert this blocking mechanism by expressing ligands of immune checkpoint resulting in T-cell anergy. New treatments based on monoclonal therapy have been established to counteract T-cell inhibition by immune checkpoint. Antibodies targeting CTLA4, PD1, and programmed death 1 ligand (PDL1) have demonstrated significant efficacy in non-small-cell lung cancer, renal cancer, and melanoma (67).

Unfortunately, immune checkpoint inhibitor monotherapy targeting these three molecules appears to be ineffective in PDAC (68). One explanation for this resistance could be found in the composition of the immune cell infiltrate. As discussed earlier, several cell subtypes found in the PDAC TME have potent immunosuppressive functions. MDSCs promote pro-tumor macrophages, decrease cytotoxic T cells, and recruit Treg lymphocytes. TAMs inhibit T-cell function and secrete immunosuppressive factors (69). Treg lymphocytes secrete immunosuppressive cytokines (IL-10 and TGF-β) and limit $CD8^+$ T-cell activation by the consumption of IL2 available by IL2Rα (70). Together, these cells generate an immunosuppressive environment, which likely interferes with immune checkpoint inhibitors.

Another reason that could explain the immune-based therapy inefficiency is the desmoplastic feature of PDAC stroma caused by hypoxia and TME components, as discussed earlier. Novel therapies targeting these two last obstacles are urgently needed which, when combined with immune checkpoint inhibitors, are expected to provide substantial benefits to patients with PDAC. Furthermore, CTLA4, PD1, and PDL1 may not be the major immune checkpoint molecules involved in immune system inhibition in PDAC. A complete analysis of the immune checkpoint molecules expressed by cancer cells in PDAC could help decipher how immune system inhibition is set up and thus reveal new targets.

Finally, the biology and genetics in PDAC also appear to be very important (11, 71). Indeed, several genetic and transcriptomic studies have demonstrated the classification of PDAC into two or more subtypes including basal versus classic or immunogenic versus non-immunogenic (72). Chen and Mellman recently described cancer-immune phenotyping into the following three different subtypes: the immune-desert, the immune-excluded, and the inflamed tumor (73).

Future immunotherapies should now consider such phenotyping in order to adapt therapeutic strategies to specific groups of patients with the aim of increasing patient survival.

CONCLUSION

In PDAC and most solid tumors, the TME and, in particular, the immune network play a pivotal role in their development. From the elimination phase where effector immune cells eliminate and select specific resistant tumor cells to the equilibrium and escape phases, tumor cells induce an immunosuppressive TME. These may be found to target myeloid cells and Tregs, as the most abundant cells in the TME of PDAC. PDAC is a devastating disease that is mostly diagnosed at advanced stages at which strong immunosuppressive immune populations and desmoplastic environment have already developed, likely explaining the inefficiency of current immunotherapies in this cancer. The relation between the PDAC's biology, genetic, and immune network seems to be very closed and important to adapt therapy for each patient. That is why, further studies are needed to better understand the escape mechanisms relating to immunosuppression in order to reveal the best immune checkpoint therapeutic strategies.

AUTHORS NOTE

DO team was labeled "Equipe FRM DEQ 201 40329534." DO is the senior scholar of the Institut Universitaire de France.

AUTHOR CONTRIBUTIONS

EF and CG prepared the manuscript collaboratively with input from SC, JG, JI, and DO.

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