



# The Cancer Cell Dissemination Machinery as an Immunosuppressive Niche: A New Obstacle Towards the Era of Cancer Immunotherapy

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Although cancer immunotherapy has resulted in unprecedented survival benefits to subsets of oncology patients, accumulating evidence from preclinical animal models suggests that the immunosuppressive tumor microenvironment remains a detrimental factor limiting benefit for many patient subgroups. Recent efforts on lymphocyte-mediated immunotherapies are primarily focused on eliminating cancer foci at primary and metastatic sites, but few studies have investigated the impact of these therapies on the highly complex process of cancer cell dissemination. The metastatic cascade involves the directional streaming of invasive/migratory tumor cells toward specialized blood vessel intravasation gateways, called TMEM doorways, to the peripheral circulation. Importantly, this process occurs under the auspices of a specialized tumor microenvironment, herewith referred to as “Dissemination Trajectory”, which is supported by an ample array of tumor-associated macrophages (TAMs), skewed towards an M2-like polarization spectrum, and which is also vital for providing microenvironmental cues for cancer cell invasion, migration and stemness. Based on pre-existing evidence from preclinical animal models, this article outlines the hypothesis that dissemination trajectories do not only support the metastatic cascade, but also embody immunosuppressive niches, capable of providing transient and localized immunosubversion cues to the migratory/invasive cancer cell subpopulation while in the act of departing from a primary tumor. So long as these dissemination trajectories function as “immune deserts”, the migratory tumor cell subpopulation remains efficient in evading immunological destruction and seeding metastatic sites, despite administration of cancer immunotherapy and/or other cytotoxic treatments. A deeper understanding of the molecular and cellular composition, as well as the signaling circuitries governing the function of these

dissemination trajectories will further our overall understanding on TAM-mediated immunosuppression and will be paramount for the development of new therapeutic strategies for the advancement of optimal cancer chemotherapies, immunotherapies, and targeted therapies.

**Keywords:** cancer immunotherapy, tumor microenvironment, endothelial energy, lymphocyte exclusion, lymphocyte exhaustion, metastasis, macrophages, T cells

## INTRODUCTION

Molecular investigations of the intricate and reciprocal interactions between tumor and immune cells have been at the frontier of cancer research in the past decade, a trend that will likely continue given the recent development of highly effective cancer immunotherapies (1–7). In general, antitumor immunity is strongly reliant on the trafficking of CD8<sup>+</sup> T cells in both primary and metastatic tumor microenvironments (TMEs) and can be characterized as a highly dynamic and tightly regulated process (8–10). There is abundant preclinical and clinical evidence that the presence of tumor-infiltrating lymphocytes (TILs) correlates with favorable clinical outcomes (11–17), but contradictory results have also been reported (18, 19). Moreover, recent studies have demonstrated that the spatial distribution patterns of TILs within the tumor microenvironment may play an even more drastic role in determining the prognostic outcome, than the density of TILs alone (20–22). In yet other studies, the co-assessment of immune cell signatures related to specific functional status (or subtypes) of TILs may be critical for a more accurate assessment of prognostic outcomes (23–30). These observations collectively suggest that T cell trafficking into the TME is one of the critical aspects of antitumor immunity. The overall immune landscape in the TME is therefore a key determinant for the efficiency of CD8<sup>+</sup> T cell-mediated antitumor immunity in either natural, induced or engineered immune responses.

The intricate relationship between immune and cancer cells in the context of tumor development and progression has long been recognized (31). Since the initial proposal of the cancer immunosurveillance theory (32, 33), numerous immunotherapies have been developed including monoclonal antibodies (34, 35), chimeric antigen receptor (CAR) T cells (36–38), and tumor vaccines (39, 40). Despite the success, which is primarily seen in hematological malignancies, such as in leukemia and lymphoma (41, 42), the efficacy of these treatment modalities has been less dramatic in solid tumors, such as in breast, colorectal, and prostate cancers (31, 43). The lack of promising outcomes in these solid tumor types is likely a multifactorial and cumulative result arising not only from intrinsic defects of antitumor immunity, but also from the intricate relationships among tumor cells, immune cells, and their surrounding microenvironment, which can obfuscate these antitumoral immune responses (44–46). Although the majority of these mechanisms will not be detailed as they are beyond the scope of the current perspective, here we focus on the emerging

roles of the tumor-infiltrating myeloid cell population in limiting antitumor CD8<sup>+</sup> T cell responses.

A plethora of terminally differentiated myeloid cells and/or their immature counterparts, including monocytes, macrophages, neutrophils, and myeloid derived suppressor cells (MDSCs) among others, have been identified in the tumor stroma, whereby they conspire with tumor cells to promote the acquisition of metastatic hallmarks (47–52). In this heterogeneous landscape, a flurry of proangiogenic and proinflammatory cytokines (VEGF, IL6, etc.) rising from hypoxic and acidic microenvironments instigate myeloid cell infiltration and activation (53–60). There is now ample evidence that this myeloid cell-dominated milieu constitutes a rather inhospitable and antagonistic microenvironment for T cell trafficking and further promotes T cell exhaustion and deactivation (61–68). As such, the latest advances in immunotherapy have been directed at overcoming the immunosuppressive mechanisms within the tumor microenvironment, with a special focus on counteracting the function of protumoral myeloid cell populations (31).

It is undeniable that modern immunotherapies, including immune checkpoint blockade (anti-PDL1, anti-CTLA4, etc.) and adoptive transfer of genetically engineered T cells to express a receptor that is specific for a tumor antigen have revolutionized cancer treatment (41, 69–72). However, most such studies have primarily evaluated cancer cell growth and proliferation endpoints, such as primary and metastatic tumor burden, to document their efficiency as potential anticancer treatment modalities. The degree to which natural or engineered antitumor immunity can successfully target the highly invasive and migratory tumor cell subpopulation is poorly understood. As seen by the high recurrence rates in many solid malignancies, invasive/migratory tumor cells can evade the cytotoxic effects of chemotherapy, radiotherapy, and other treatments, as well as escape immunological detection and destruction (47, 73, 74). Cancer cell dissemination is regulated by a specialized network and subsets of myeloid cells, which form dedicated niches for the nurturing of migratory/invasive cancer cells (47, 50, 75–77). In this perspective, we propose that certain myeloid cell subsets, particularly perivascular M2-like macrophages, are contextually associated with cancer cell dissemination trajectories, offering a localized immunosuppressive niche to the metastasizing tumor cell population, while in the act of active dissemination. We conclude that thorough understanding of these immunosuppressive mechanisms in the tumor microenvironment at the molecular level will lead to more effective therapeutic targeting of cancer metastasis and will possibly improve the outcome of modern immunotherapies.

## THE CANCER CELL DISSEMINATION TRAJECTORY: TUMOR MICROENVIRONMENT THAT REGULATES THE INITIAL STEPS OF THE METASTATIC CASCADE

From earliest portrayals to more recent representations, two generic components have been distinguished as integral parts of the metastatic cascade, a cancer cell dissemination step and a cancer cell growth/proliferation step at the metastatic site, the latter also known as colonization step (78–84). Both these steps are regulated and may even be reinforced by a diverse array of biological programs in the tumor microenvironment, including epithelial-to-mesenchymal transition (EMT), invasion/migration, chemotaxis, and dormancy (85–92), among others. Recent advances in the underlying mechanisms of cancer cell dissemination have indicated that cancer cells that have undergone EMT, and thus have lost epithelial polarity and gained mesenchymal properties, participate in a reciprocal juxtacrine-paracrine signaling loop with tumor-associated macrophages (TAMs), eventually leading them to the underlying vasculature for subsequent intravasation. Cancer cell intravasation, however, does not occur along the entirety of the cancer-associated endothelium, but is rather restricted to specialized intravasation sites, known as Tumor MicroEnvironment of Metastasis (TMEM) doorways. In this section, we will briefly discuss the factors that underlie the spatial and functional relationship between the disseminating tumor cell subpopulation and the TMEM doorways, a critical ingredient that regulates the initial steps of the metastatic cascade in primary tumors.

Cancer cell intravasation doorways, also known as TMEM doorways, constitute intratumoral niches characterized by the physical juxtaposition of a tumor expressing high levels of the actin-regulatory protein Mammalian enabled (MENA), a perivascular macrophage and an endothelial cell, and represent an independent prognostic indicator of metastatic risk in breast cancer patients (93–96). Perivascular macrophages residing in TMEM doorways express the tyrosine kinase receptor TIE2, thus assuming an M2-like polarization status and tumor-promoting effects. Under the tight regulation of TIE2 signaling, TMEM macrophages secrete large amounts of vascular endothelial growth factor (VEGF), which in turn, functions in a paracrine fashion on the TMEM endothelial cell to promote the reversible breakdown of endothelial cell-to-cell adhesions, localized vasculature opening, and the subsequent intravasation of invasive/migratory tumor cells from the immediate area surrounding the TMEM doorway. Despite that the precise role of the TMEM tumor cell in the TMEM triad has not yet been clearly elucidated, high-resolution microscopy has suggested the presence of invadopodia stemming from TMEM tumor cells and extending in between the underlying vasculature (73). Thus, the current understanding is that TMEM tumor cells pinpoint the breaching point of the endothelial wall following TIE2-dependent TMEM doorway activation.

Formation of active TMEM doorways has not only been observed in primary tumors, but also in their respective loco-

regional and distant metastatic sites, such as in the lymph nodes and lungs, respectively (75, 93, 97–99). Indeed, prior work has documented that established lymph node metastases attract TIE2<sup>+</sup> macrophages in the perivascular niche, which in turn assemble TMEM doorways *de novo* (99). More importantly however, photoconversion experiments that can specifically label tumor cells in metastatic lymph nodes and observe their behavior in real time have indicated that cancer cells within metastatic foci are capable of utilizing TMEM doorways to re-disseminate to tertiary metastatic sites, such as to the lungs (99). Overall, these studies support TMEM doorway-mediated cancer cell dissemination as a universal mechanism of cancer cell dissemination at all stages of cancer progression.

Within the constantly evolving landscape of tumor cell heterogeneity, it is crucial to appreciate that not all tumor cells are equally capable of cancer cell dissemination *via* TMEM doorways. Rather, only a small subset of tumor cells in primary tumors is co-opted to utilize TMEM doorways for intravasation in the peripheral circulation. Expression profiling studies have specifically identified this subset as overexpressing an alternatively spliced isoform of the actin-regulatory protein Mammalian enabled (MENA), called MENA<sup>INV</sup>, and having concurrently lost expression of the antimetastatic and cell cohesion-promoting alternatively spliced isoform MENA11a (100–104). MENA is one of the key members of the Ena/VASP family of proteins, involved in regulation of cell movement, shape and adhesion (105), mainly through regulating actin filament polymerization and rate of filament elongation during the formation of cellular protrusions (106, 107). Cancer cells that overexpress MENA<sup>INV</sup> are characterized by formation of extracellular matrix-degrading cellular protrusions, called invadopodia, by increased sensitivity to chemotactic factors in the tumor microenvironment such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF), which both facilitate cancer cell invasion and migration (75, 98, 107–114). It is therefore not surprising that MENA<sup>INV</sup>-expressing tumor cells are preferentially co-opted for TMEM-mediated cancer cell intravasation. It should be noted that MENA<sup>INV</sup>-expressing cancer cells also share markers and phenotypic characteristics that indicate they have undergone epithelial-to-mesenchymal transition (EMT), which is a crucial landmark of metastatic dissemination (85, 115–118). However, in the current perspective we will primarily refer to the migratory/invasive tumor cell compartment as the MENA<sup>INV+</sup> cancer cell subpopulation, given that prior studies have suggested that MENA isoform switching is crucial for the establishment of metastatic disease (103, 104, 106).

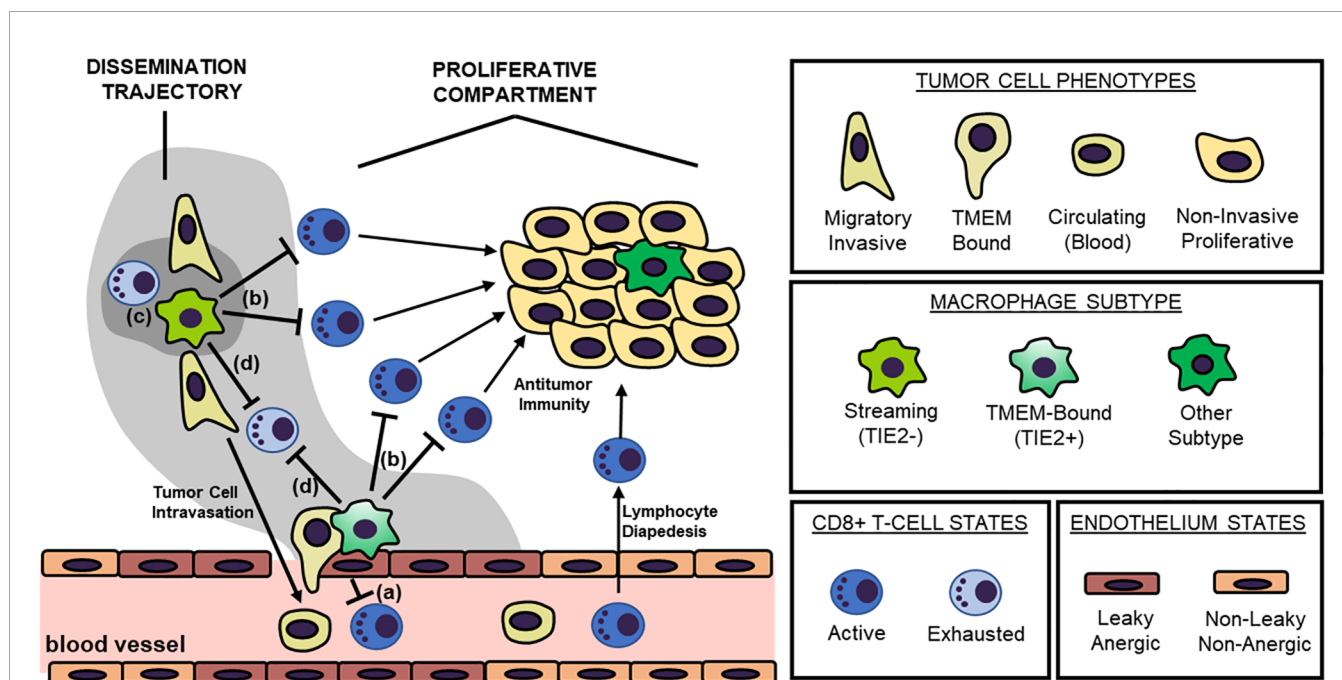
Multiphoton intravital imaging studies in live mice have suggested that MENA<sup>INV</sup>-expressing tumor cells migrate along collagen fibers with partnering TAMs in the tumor microenvironment. A well-described, reciprocal paracrine loop between the two cell types, involving colony stimulating factor-1 (CSF1) secretion from the tumor cell and epidermal growth factor (EGF) secretion from the macrophage, leads to the chemotactic attraction of one cell towards the other, coupling them in sequence in a unique migratory pattern called “cancer cell streaming” (104, 119, 120). The specific targeting of either of

these factors, either pharmacologically or *via* genetic engineering, is sufficient by itself to disrupt cancer cell streaming and suppress cancer cell dissemination (104, 121–123). Eventually,  $MENA^{INV+}$  tumor cells reach down to the perivascular niche, whereby they utilize pre-existing TMEM doorways to intravasate into the blood vessel (104, 114, 124, 125). Chemotactic factors, such as hepatocyte growth factor (HGF) and stromal derived factor-1 (SDF1), either secreted by the cancer endothelium itself or by cells associated with the endothelium (e.g. TMEM macrophages), are responsible for the directed migration of the entire cancer cell “streams” towards the TMEM doorway (126, 127).

A few studies have previously investigated the mechanisms *via* which  $MENA^{INV}$  expression is induced in the migratory/invasive cancer cell subset. Although the exact mechanism has not been deciphered at the molecular level, there is strong indication that TAMs streaming with tumor cells are crucial for  $MENA^{INV}$  induction in the latter. Specifically, *in vitro* co-culture experiments have indicated an up to 50-fold increase in  $MENA^{INV}$  expression when tumor cells were co-cultured with macrophages, and this

phenotype was demonstrated to be contact-dependent, suggesting that juxtacrine signaling loop may also be elicited during the cancer cell streaming behavior (124). In support to these observations, the *in vivo* depletion or suppression of macrophage differentiation has shown a significant reduction of cancer cell dissemination (128).

In this perspective, the described  $MENA^{INV+}$  migratory/invasive cancer cell population partnered with intratumoral TAMs during streaming, along with corresponding TMEM doorways used during the intravasation process, will be collectively referred to as the “Dissemination Trajectory” (Figure 1). Indeed, it is expected that different signaling pathways, cytokine/chemokine profiles, and metabolic patterns will characterize the dissemination trajectories versus the more proliferative compartments of solid tumors. Here, we explore dissemination trajectories as immunosuppressive landscapes, in an effort to explain current translational and clinical observations on why natural or engineered antitumor immunity is not efficient in preventing the metastatic cascade, albeit demonstrating promising results in eliminating tumor growth potential.



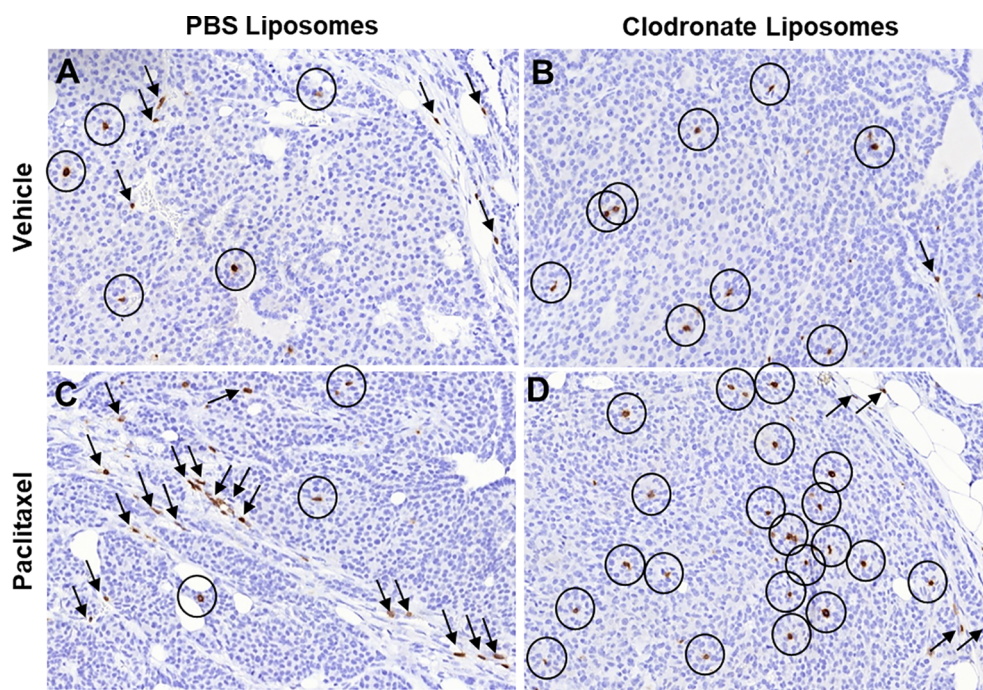
**FIGURE 1** | The “Dissemination Trajectory” Working Model of Metastatic Dissemination. Two major cellular prerequisites are necessary for cancer cell dissemination: a TMEM doorway and a highly invasive, highly migratory cancer cell subsets streaming toward TMEM doorways. TMEM doorways are composed of three cell types, a  $TIE2^+$  macrophage, an endothelial cell and a tumor cell forming an invadopod in the vasculature, and signaling conversation among these three cells results in localized vascular opening to facilitate transendothelial migration of the highly invasive, highly migratory cancer cell subset. The highly invasive and migratory cancer cell subsets participate in a reciprocal paracrine and juxtacrine signaling loop with intratumoral macrophages that do not express  $TIE2$ , resulting in the increased induction of the actin-regulatory protein  $MENA^{INV}$ . Eventually, these interactions result in the so called “streaming migration”, which is directed toward TMEM doorways, and  $MENA^{INV}$ -facilitated transendothelial migration and metastatic dissemination. TMEM doorways and their streaming  $MENA^{INV+}$  cancer cell subsets are herewith referred to as “dissemination trajectories”. These specialized microenvironments are distinguishable from other tumor compartments with rapidly dividing tumor cells that do not share similar molecular pathways, here described as “proliferative compartments”. Four layers of immunosuppressive mechanisms dominate within the dissemination trajectories, that result in the development of immune deserts further facilitating the process of metastatic dissemination. These mechanisms postulate that: (a) the TMEM endothelium is anergic, thus not allowing for T cell diapedesis; (b) dissemination trajectories do not support cytokine/chemokine matching for allowing T cell chemotaxis; (c) dissemination trajectories have a unique metabolic landscape that is refractory for T cell chemotaxis and/or function; and finally (d) dissemination trajectories are characterized by the induction of immune checkpoint signaling, that promoted exhaustion of T cells. Overall, tumor-associated macrophages (TAMs) within these dissemination trajectories play the pivotal role in regulating all four layers of immunosuppression, although secondary mechanisms have also been identified.



## THE CANCER CELL DISSEMINATION TRAJECTORY AS AN IMMUNOSUPPRESSIVE NICHE

A substantial amount of preclinical and clinical studies has indicated that tumor-associated myeloid cells, predominantly tumor-associated macrophages (TAMs), neutrophils, and myeloid-derived suppressor cells (MDSCs), sustain an immunosuppressive tumor microenvironment, which is particularly refractory to both T cell trafficking and antitumor T cell functions (4, 129–134). Extensive research in this field has additionally concluded that the specific targeting and/or elimination of this myeloid-driven immunosuppressive program can render the natural, induced, and engineered immunological responses against tumors more concrete and effective (135–137). In line with the above, here we first provide proof-of-principle evidence of this notion using the Mouse Mammary Tumor Virus Polyoma Middle-T antigen (MMTV-PyMT) mouse model of breast carcinoma, which successfully recapitulates human breast cancer progression (138). During the natural progression of MMTV-PyMT carcinomas, T cells are spatially restricted to the peritumoral stromal sheaths and

are visually excluded from multicellular tumor cell cohorts (**Figure 2A**), insinuating structural and/or functional impediments of intratumoral T cell trafficking. However, upon the pharmacological depletion of TAMs *via* the administration of clodronate liposomes, intratumoral T cell trafficking is clearly improved (**Figure 2B**), pinpointing TAMs as the responsible structural and functional impediments to T cell trafficking. It has been previously shown that immune cells can excessively infiltrate primary tumors as a result of a cytokine surge, induced by cytotoxic factors, such as chemotherapy treatment (47, 73, 74, 139). Indeed, administration of paclitaxel, a taxane-based chemotherapy known to inflict prometastatic modifications as a consequence of a cytokine surge (139–144), results in a dramatic increase of TILs, which are otherwise restricted to the peritumoral stromal sheaths (**Figure 2C**). This distribution pattern appears to be the consequence of immunosuppressive TAMs, because clodronate-mediated depletion of TAMs in the chemotherapy setting facilitates the intratumoral trafficking of T cells that have responded to the chemotherapy-driven cytokine surge (**Figure 2D**). Of note, similar observations by other groups have corroborated our findings using a diverse array of macrophage suppression or



**FIGURE 2** | Immunohistochemical indication of how different pharmacologic modifications of the immunosuppressive tumor microenvironment may affect T cell trafficking into tumors. **(A–D)** Immunohistochemistry for T cell specific marker CD3 in tumor sections from mouse mammary tumor virus – polyoma middle T antigen (MMTV-PyMT) mice, developing spontaneous breast carcinomas. The images are high power fields (x40), representative from a total of three mice in each experimental condition. Circles, CD3<sup>+</sup> T cells infiltrating the tumor nests; Arrows, CD3<sup>+</sup> T cells infiltrating the tumor stroma. Notice the significant changes in intratumoral versus stromal T cell infiltration upon different treatments that modify the immunosuppressive microenvironment.) In breast carcinoma, T cells are found in both tumor cell nests and the tumor stroma **(A)**. Upon macrophage depletion with clodronate liposomes, most T cells can leave the stroma and penetrate the tumor cell nests **(B)**. However, treatment with cytotoxic chemotherapy is known to induce lymphocyte infiltration and significantly larger number of T cells is found compared to the vehicle **(C)**. Notably however, most of these T cells are restricted in the tumor stroma, as chemotherapy attracts immunosuppressive myeloid cells at the same time, resulting in lymphocyte exclusion **(C)**. If such immunosuppressive myeloid cells are depleted through clodronate liposomes in chemotherapy-treated tumors, the increased influx of T cells is now relocated in the tumor nests **(D)**. Immunohistochemistry was performed in archival tissue from experiments originally conducted in the manuscript by Karagiannis et al. (139), in which ethical approval for the use of the experimental mice was also obtained (139).

re-polarization strategies (77, 145, 146). In conclusion, these experimental data along with accompanied literature collectively demarcate the detrimental impact of TAMs in T cell trafficking and distribution in primary tumors.

As described above, specialized tumor microenvironments within primary carcinomas comprising of TMEM doorways and their associated prometastatic MENA<sup>INV+</sup> cancer cell compartments, herewith defined as *dissemination trajectories*, are both structurally and functionally supported by distinct TAM subsets (47). Given the experimental and literature evidence on the immunosuppressive properties of TAMs described above, here, we surmise that dissemination trajectories signify immunosuppressive niches, reminiscent of *immune deserts*. The term “immune desert” is used here as an interchangeable term for collectively describing tumor microenvironments with immune excluded and immune desert phenotypes, as defined in multiple prior studies (147–150). It is hereby suggested that at least four distinct mechanisms may contribute to the function of dissemination trajectories as immune deserts in the primary tumor microenvironment: First, dissemination trajectories are sites of endothelial anergy; Second, they represent sites of lymphocyte exclusion; Third, they represent sites of metabolic reprogramming, refractory to anti-tumor lymphocyte functions; Fourth, they constitute sites of lymphocyte exhaustion. Collectively, the aforementioned immunosuppressive mechanisms (**Figure 1**) significantly undermine the capacity of the tumor-infiltrating lymphocytes for targeting the disseminating cancer cell population, thus allowing for a narrow, but solid window of opportunity for the successful execution of the initial steps of the metastatic cascade.

### (a) Dissemination Trajectories as Beacons of Endothelial Anergy

Lymphocyte migration needs to be precisely coordinated to contribute to effective T cell trafficking in both physiological and neoplastic contexts. This process can be summarized into selectin-dependent leukocyte rolling, chemokine-driven integrin activation, integrin-dependent leukocyte tethering in the vascular wall, and leukocyte diapedesis (8, 9, 151–153). It should be mentioned that this process is primarily mediated by lymphocyte-endothelial cell interactions, and as a consequence, the integrity and functionality of the endothelium in either a physiological or neoplastic context, could have a dramatic effect on T cell trafficking. Under the control of growth factors and abnormal contextual signals, the tumor (neo)vasculature often displays a high angiogenic potential coupled to irregular distribution, enlarged vessels, excessive branching morphology, microhemorrhaging, and disturbed blood flow, when compared to traditional blood vessel architecture and physiology (154–158). Another decisive factor contributing to tumor endothelium instability, and consequently to defective T cell trafficking into the tumor tissue, is the failure to support endothelial integrity and functions *via* adequate mural cell (e.g. pericyte) coverage (159–162). One could intuitively, but erroneously, assume that high endothelium instability/permeability should render immune cell trafficking much easier. However, circulating lymphocytes require specialized molecular signatures (e.g. selectin, integrin

and chemokine profiles) in tissue endothelial barriers to help with their homing into tissues (163–167). These molecular signatures, which are magnanimously present in High Endothelial Venules (HEVs) of various lymphoid organs for example (168–171), are characteristically disrupted or absent in tumor endothelia, rendering them “leakier” and insensitive to pro-inflammatory signals (172). This phenotype, known as “endothelial anergy”, is characterized by impaired adherence of effector T cells to the endothelial cells and their subsequent extravasation to the tumor microenvironment (9, 173).

Although macrophages provide essential trophic factors to facilitate generation and retention of pericytes in certain developmental contexts (174, 175), at least one study has previously indicated that TMEM doorways are devoid of NG1<sup>+</sup> pericyte coverage (128), signifying one potential signature of endothelial anergy at TMEM doorways. In yet other studies, it has been shown that M2-polarized TAMs may in contrast support macrophage-pericyte interactions in the tumor microenvironment, but such interactions lead to enhanced neovascularization and tumor progression (176–179), again flagging the immediate surroundings of M2-like TAMs as potential niches of endothelial anergy.

For a long time, it has been theorized that the tumor vasculature is under constant and simultaneous control of proangiogenic and antiangiogenic factors, with vascular endothelial growth factor-A (VEGFA) representing a well-known paradigm of angiogenesis inducers (155, 180–186). However, prior evidence also suggests that different blood vessel subtypes in the tumor microenvironment do not all respond homogeneously to anti-VEGF treatment (187), inferring the presence of contextual factors promoting heterogeneity in VEGFA expression and activity. Indeed, the increase of VEGF around TMEM doorways may be the cause of the overall heterogeneity of VEGF expression around blood vessels in tumors. Under the transcriptional control of the Ang2-Tie2 signaling axis, TIE2<sup>+</sup> TMEM macrophages can locally release large quantities of proangiogenic factors, most prominently VEGFA, which is critical for both eliciting an angiogenesis program and sustaining TMEM function and TMEM-mediated cancer cell dissemination (128, 188–190). Importantly, VEGFA regulates blood vessel wall permeability *via* a variety of mechanisms, for instance *via* increasing endothelial cell fenestration at lower concentrations, or *via* breaking down and dissolving the endothelial cell adherens and tight junctions at higher concentrations (191–197). The latter is especially critical in the process of metastasis because it provides an effective paracellular passageway for the disseminating cancer cell subpopulation into the blood circulation (128). Indeed, the conditional ablation of the VEGFA gene *via* targeted expression of Cre recombinase under the control of the macrophage-specific promoter that regulates transcription of the colony stimulated factor-1 receptor (CSF1R) in a mouse model of breast carcinoma results in successful assembly of TMEM-doorways, which are otherwise entirely incapable of breaking down endothelial junctions and facilitating cancer cell transendothelial migration and intravasation (128). Overall, these data suggest that TMEM

doorways within the dissemination trajectories reflect to TMEs with high VEGFA expression and activity, suggesting that they function as candidate beacons of endothelial energy within the tumor microenvironment.

TMEM doorways are functionally regulated by M2-like TAMs, which represent a prominent source of angiogenic molecules in the perivascular niche (47, 51, 75, 189, 190, 198–201). Besides the well documented VEGFA, TAMs release a plethora of other proangiogenic factors, such as tumor necrosis factor- $\alpha$ , basic fibroblast growth factor, thymidine phosphorylase, urokinase-type plasminogen activator, adrenomedullin, and semaphorin-4D (47, 189, 190, 201–205). These proangiogenic factors are known to downregulate the expression of adhesion molecules (ICAMs, VCAMs, and selectins), which are actively involved in lymphocyte trafficking, thus resulting in endothelial energy and lymphocyte tolerance (9, 172).

In summary (Figure 3A), TMEM doorways likely serve as proponent components of endothelial energy in the tumor microenvironment, subduing intratumoral recruitment of CD8<sup>+</sup> T cells. Possible contributors of endothelial energy at TMEM doorways are the reported defects in pericyte coverage, as well as the localized, high concentration of VEGFA and other proangiogenic molecules secreted by the TMEM macrophage. These mechanisms may together prompt a highly permeable vasculature at TMEM doorways, which is otherwise unable to support T cell trafficking due to the lack of characteristic molecular signatures for lymphocyte diapedesis.

## (b) Dissemination Trajectories as Crossroads for Lymphocyte Exclusion

Among the critical mechanisms leading to inadequate T cell trafficking into solid tumors are those culminating in mismatching between bioavailable chemokines in the tumor microenvironment and chemokine receptors expressed on the surface of cytotoxic T cells (206). The disruption of the immunosuppressive chemokine/cytokine network either pharmacologically or *via* genetic manipulations in animal models can therefore reliably covert the tumor microenvironment into a receptive niche for T cell trafficking and further sensitize tumors to immunotherapy (207). The dissemination trajectories are functionally and contextually associated with distinct macrophage subtypes, which represent a prominent source of immunosuppressive cytokines and chemokines in the tumor microenvironment (47, 73–75, 206). As mentioned, perivascular TMEM doorway macrophages, express high levels of the tyrosine kinase receptor TIE2 (also known as CD202b), and the mannose receptor MRC1 (also known as CD206), suggesting that they are skewed towards an M2 (or M2-like) phenotype according to the traditional macrophage polarization spectrum (47, 119, 208, 209). In this perspective, we support the working model that M2-like macrophages within dissemination trajectories represent the major orchestrators of chemokine/chemokine receptor mismatching that leads to inadequate CD8<sup>+</sup> T cell trafficking (206).

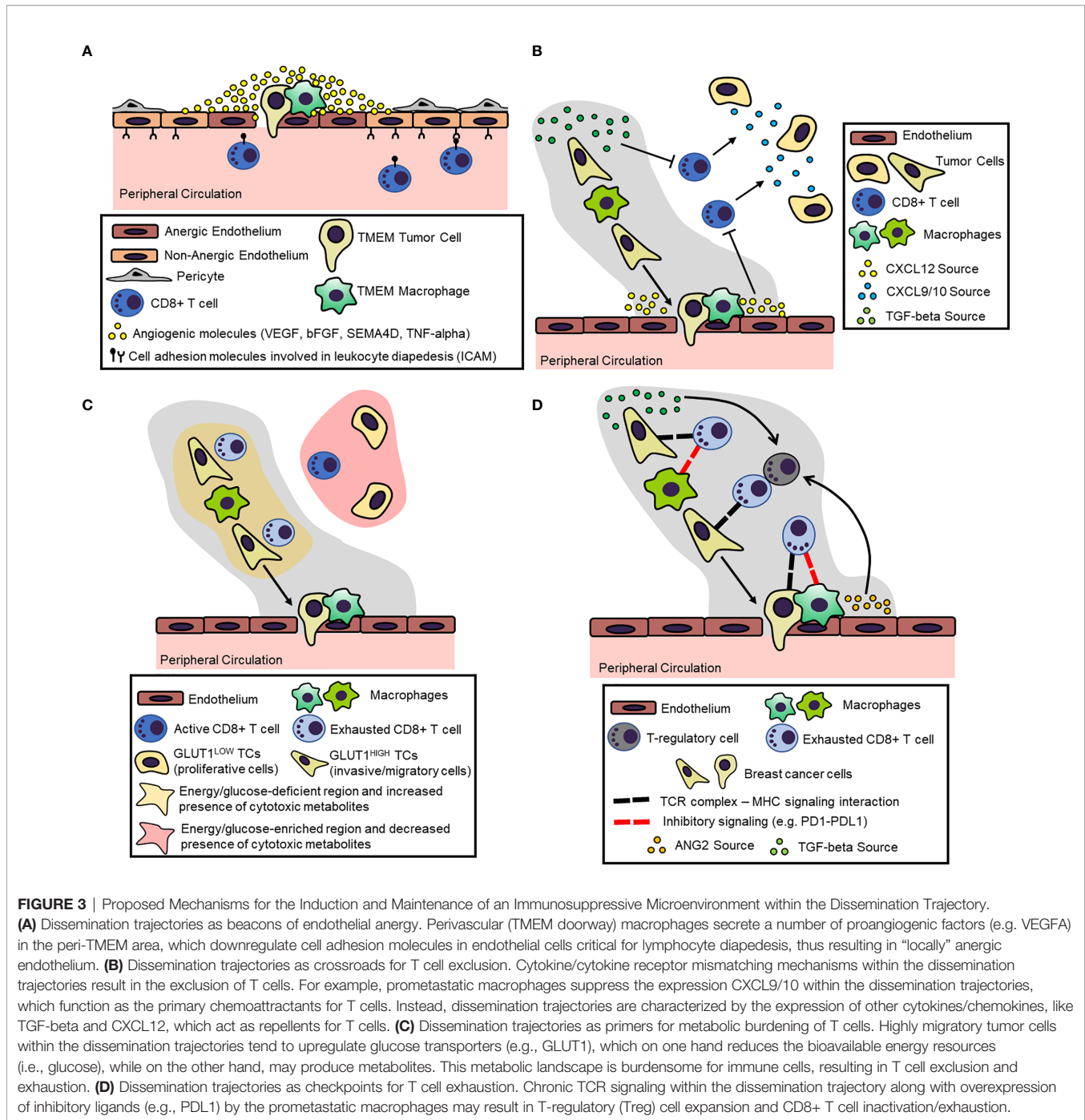
Peripheral monocytes are usually recruited within tumors *via* the CCL2/CCR2 chemokine pathway and transdifferentiate into M2-like macrophages under the regulation of the CSF1/CSF1R

pathway (210). It is now strongly documented that CSF1-dependent macrophage polarization into M2-like phenotype leads to the acquisition of an immunosuppressive macrophage subtype, characterized by T cell exclusion (145, 206, 211–213). Indeed, the depletion of tumor-associated macrophages *via* inhibiting either CSF1/CSF1R or CCL2/CCR2, are both capable of overcoming T cell exclusion within tumors (145, 214). There is sufficient evidence that M2-like macrophage functions are antagonistic to Th1 immunological responses, which would theoretically favor antitumoral immunity. Specifically, M2-like macrophages may suppress the interferon-gamma (IFN)-mediated responses that culminate in the induction of CXCL9 and CXCL10 chemokines, which, in turn, are able to attract CXCR3<sup>+</sup>CD8<sup>+</sup> memory T cells (215). The critical association between CXCR3-binding ligands CXCL9/10 and CD8<sup>+</sup> T cell trafficking has been well documented (216–220). Although the dominance of M2-like macrophages within the dissemination trajectories can by itself account for the suppression of such favorable Th1 immunological responses, several macrophage-independent mechanisms of Th1 suppression have also been reported in this context. For example, certain tumors (e.g. ovarian carcinomas) can use epigenetic mechanisms to silence the expression of CXCL9 and CXCL10. Moreover, nitrosylation by reactive oxygen species (ROS) in the tumor microenvironment may result in altered proteolytic processing of CXCL11, another chemoattractant of CD8<sup>+</sup> T cells (221), which incapacitates its binding-induced signaling (222).

It has been demonstrated that once homed in tumors under the control of CCL2/CCR2 and CSF1/CSF1R pathways, M2-like macrophages begin to also express the chemokine receptor CXCR4, possibly under the control of the pleiotropic cytokine TGF $\beta$  (210). The *de novo* expression of CXCR4 may force prometastatic macrophages into a unidirectional migration toward the perivascular niche where CXCL12, the chemokine ligand of CXCR4 is abundantly expressed, and where they eventually assemble TMEM doorways (210). Although many sources of TGF $\beta$  within the tumor microenvironment have been reported (223–225), human monocytes and macrophages can also activate TGF $\beta$  *via* the expression of integrin  $\alpha$ v $\beta$  (226). TGF $\beta$  has been previously documented as among the strongest immunosuppressive cytokines, capable of excluding T cells from human and murine tumors (227, 228). These observations collectively suggest that TGF $\beta$  expression within dissemination trajectories represent a critical mechanism of lymphocyte exclusion as a result of cytokine/cytokine receptor mismatching.

As mentioned, CXCR4<sup>+</sup> macrophages within dissemination trajectories can chemotactically respond to the presence of the CXCL12 ligand at the perivascular niche (210). Prior evidence suggests that mesenchymal stromal cells, such as cancer-associated fibroblasts (CAFs) and possibly mural cells coating the blood vasculature, serve as the primary source of CXCL12 production and secretion (229–234). In a recent model of cancer cell dissemination, the chemotactic migration of CXCR4<sup>+</sup> macrophages with their partnering MENA<sup>INV+</sup> tumor cells, has been rendered as the possible driving force for the observed streaming migratory behavior within dissemination





trajectories (210). Moreover, a concrete body of evidence supports that intratumoral distribution of CXCL12 inversely correlates with the presence of T cells (235), although it is not yet clear whether the CXCL12/CXCR4 pathway can directly suppress T cell trafficking into CXCL12-enriched microenvironments (230, 236). Indeed, pharmacological inhibition of the CXCL12/CXCR4 pathway alleviates the tumor microenvironment from the lymphocyte exclusion phenotype (236). On one hand, CXCL12 appears to be a critical chemokine for cancer cell dissemination

(231, 233, 237, 238); still, it may comprise a major chemokine/chemokine receptor mismatching mechanism for the trafficking of T cells into dissemination trajectories.

In summary (**Figure 3B**), the immunosuppressive M2-like macrophages may orchestrate the expansion of a cytokine/chemokine network, which excludes T cells from the dissemination trajectories. Foremost, M2-like macrophages seem to directly suppress the expression of the CXCR3-binding ligands CXCL9 and CXCL10, which are the primary chemokine



attractants for CD8<sup>+</sup> T cells. Moreover, the reciprocal interactions among disparate cells within dissemination trajectories seem to be highly dependent on the induction and contextual expression of several cytokines and chemokines, including but not limited to CXCL12 and TGF $\beta$ , which may disrupt lymphocyte trafficking and exclude CD8<sup>+</sup> T cells from the landscape.

### (c) Dissemination Trajectories as Primers for Metabolic Burdening of Lymphocytes

In general, sugars, amino acids, and fatty acids are the major fuel sources utilized by eukaryotic cells, but rapidly proliferating tumor cells tend to exhaust them, thus subjecting both tumor and immune cells to nutrient-deficient microenvironments and imposing considerable bioenergetic constraints on their functions (76, 151, 239–242). Cancer cells tend to upregulate the expression of glucose transporters, such as GLUT1 (243, 244), amino acid transporters, such as ASCT2 and LAT1 (245–247), and fatty acid elongation enzymes, such as FAS (248–250), to facilitate their adaptation to energy-deficient microenvironments. This metabolic reprogramming does not only limit nutrient availability for cytotoxic CD8<sup>+</sup> T cells, but may also generate metabolic byproducts that may overwhelm T cell function, survival, and expansion (151). In this chapter, we briefly explore certain mechanisms, *via* which the metabolic landscape within dissemination trajectories may interfere with lymphocyte trafficking and function.

Foremost, the metabolic machinery of MENA<sup>INV+</sup> tumor cells within dissemination trajectories remains poorly understood. However, it is generally known that fatty acids are primarily required by rapidly dividing tumor cells to form new plasma membrane lipid bilayers, thus explaining why most tumors overexpress FAS and malignant transformation depends on lipogenesis (251). However, neither migratory tumor cells nor effector T cells seem to heavily depend on fatty acid oxidation, although the development of antitumor memory T cells is affected (252–254), suggesting that such pathways may not be as immunocompromising within the dissemination trajectories. On the other hand, there are certain lines of evidence suggesting that the highly migratory/invasive cells that have undergone EMT tend to express high levels of the glucose transporter GLUT1, which partially supports high energy demands for the active process of invasion and migration (255, 256). Accordingly, it has been shown that proteolytic modifications of the extracellular matrix by highly migratory cells *per se* can also promote GLUT1 expression and aerobic glycolysis (257). Concomitantly, GLUT1 overexpression has been associated with low T cell trafficking in renal cell and squamous cell carcinomas (258, 259), suggesting that dissemination trajectories could potentially limit both T cell trafficking and their functional capacity in a GLUT1-dependent manner.

The metabolic landscape within dissemination trajectories may also impair T cell functions through generation of immunosuppressive metabolites and byproducts, not only *via* the direct competition for energy resource availability. For example, indoleamine 2,3-dioxygenase (IDO), an enzyme that converts

tryptophan into kynurenines (260), is a well-established suppressor of CD8<sup>+</sup> T cell infiltration into tumors and most of the associated antitumor T cell responses (132, 261). In addition, diminished tryptophan deposits in IDO<sup>High</sup> tumor microenvironments can prevent T cell proliferation, while kynurenines can promote T cell death and interference with TCR signaling (132, 261, 262). Despite that dendritic cells have been identified as major inducers of IDO within the immune microenvironment (261), TAMs can also participate in IDO-mediated tryptophan metabolism under certain contexts (263–265), suggesting that dissemination trajectories may be characterized by the accumulation of immunosuppressive metabolites.

In summary (Figure 3C), dissemination trajectories are associated with a metabolic landscape that results in diminished T cell trafficking into tumors and associated antitumor T cell functions. On one side, highly migratory tumor cells within the dissemination trajectories may successfully outcompete TILs for the scant availability of energy resources, such as glucose, because they tend to upregulate corresponding transporters (e.g., GLUT1). On the other hand, TAMs within the dissemination trajectories may be engaged in metabolic pathways that not only deplete essential elements (e.g., tryptophan), but also produce immunosuppressive metabolites along the process (e.g., IDO-induced kynurenines).

### (d) Dissemination Trajectories as Checkpoints for Lymphocyte Exhaustion

In recent years, it has been suggested that effector T cells (CD4<sup>+</sup> and CD8<sup>+</sup>), which infiltrate tumors tend to exhibit impaired functional and proliferating capacity, characterized by progressive loss of their ability to produce their characteristic effector cytokines (i.e., TNF- $\alpha$ , IFN- $\gamma$ , IL-2) and lyse tumor cells, a state described as *lymphocyte exhaustion* (45, 151, 207, 266–269). The existence of this particular phenotype is further corroborated through experimental evidence showing that certain cancer immunotherapies, such as those that specifically target immune checkpoint pathways, may alleviate T cell exhaustion, and restore the ability to eradicate cancer cells (270). In this section, we propose that dissemination trajectories rich in M2-like immunosuppressive macrophages can yield a contextual milieu that promotes T cell exhaustion, potentially accounting for the lack of treatment response seen in many patients following checkpoint therapies.

Similar to the case of chronic viral infections, the most prominent hallmark of T cell exhaustion in the tumor microenvironment is the co-expression of a wide range of immune checkpoint receptors by the T cells (271, 272). These inhibitory receptors primarily include programmed cell death protein 1 (PD1), lymphocyte activation gene 3 protein (LAG3), T-cell immunoglobulin domain and mucin domain protein 3 (TIM3), cytotoxic T lymphocyte antigen-4 (CTLA4), band T lymphocyte attenuator (BTLA) and T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) (273). Although it is beyond the scope of the current perspective to delineate the detailed biology of these immune checkpoint pathways, it should be mentioned that intracellular

signaling *via* these receptors in T cells can generally lead to functional deficiencies characteristic of the lymphocyte exhaustion phenotype (274–279). However, in a certain context, PD1<sup>+</sup>TIM3<sup>+</sup> tumor-infiltrating T cells were functional despite the co-expression of both immune checkpoint receptors, suggesting that certain competitive intracellular pathways to unruly T cell exhaustion may also exist (280). It has been generally known that TAMs are prominent inducers of T cell exhaustion in the tumor microenvironment through interference with immune checkpoint control. For example, TAMs from renal cell carcinoma patients induce the skewing of autologous blood derived CD4<sup>+</sup> T cells towards an exhausted phenotype, with decreased production of effector cytokines and enhanced expression of PD1 and TIM3 (281). Of all immune checkpoint pathways mentioned above, the prominent expression of PDL1, a ligand for PD1, and B7-H4, a ligand for CTLA4, are perhaps the most well-known immunosuppressive mechanisms leading to macrophage-driven T cell exhaustion (282–286). Of particular interest is the fact that ligands for immune checkpoint receptors are mostly expressed by M2-like macrophages, which are also integral components of TMEM doorways, providing another attractive theory for immune evasion by the migratory/invasive cancer cell subpopulation within the dissemination trajectories.

Prior research has suggested that chronic T cell receptor (TCR) signaling in functional T cells can normally lead to elevated expression of inhibitory receptors, such as PD1, TIGIT and CTLA4 (271, 287). This observation further postulates that increased expression of these inhibitory receptors in TILs may accordingly be the result of chronic exposure to neoantigens and/or persisting tumor antigens (151, 268). However, the expression of inhibitory receptors in TILs is markedly higher compared to those in functional T cell states, suggesting that other factors, possibly microenvironmental ones, may be responsible for increased immune checkpoint control and lymphocyte exhaustion (151). In accordance with these observations, prior experimental evidence has demonstrated that certain cytokines, often expressed in the tumor microenvironment (tumor cells, cancer-associated fibroblasts, immune cells, adipocytes), such as angiopoietin-2 (ANG2), interleukin-10 (IL10), and transforming growth factor- $\beta$  (TGF $\beta$ ), are sufficient for T cell exhaustion and suppression of anticancer immunity (288–291). Although, this cytokine network leads to lymphocyte exhaustion through a variety of mechanistic pathways, both direct and indirect *via* the expansion of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> T-regulatory (Treg) cells, have been suggested (270, 272, 273, 292). Certain of these cytokines, especially TGF $\beta$ , have been discussed in prior chapters with regards to their functional relevance within dissemination trajectories. Others, like ANG2, are also critical for cancer cell dissemination, as ANG2-dependent activation of TIE2 receptor in the TMEM macrophage leads to the localized production and secretion of VEGF, which in turn, is critical for TMEM-associated vascular opening and the transendothelial migration of MENA<sup>INV+</sup> tumor cells (75, 98). Therefore, it seems that dissemination trajectories are enriched in cytokines that not only promote lymphocyte exclusion, but also lymphocyte exhaustion.

In summary (**Figure 3D**), T cells that are not excluded from and manage to eventually infiltrate dissemination trajectories have acquired an “exhausted” phenotype rendering them unable to produce effector cytokines and successfully target tumor cells. This phenotype is regulated by an abnormally high expression of immune checkpoint receptors, such as PD1, CTLA4 and TIM3, at their surface. Among other cells, M2-like immunosuppressive TAMs within dissemination trajectories express a spectrum of corresponding ligands for these inhibitory receptors, thus offering immunosuppressive “sanctuaries” around the exhausted CD8<sup>+</sup> T cells. Furthermore, the cytokine network within the dissemination trajectory, including primarily TGF $\beta$  and ANG2, among other factors, serves as a critical driver of Treg expansion and inhibitory receptor overexpression, thus maintaining and perpetuating the dysfunctional T cell states.

## CONCLUSIONS AND FUTURE PERSPECTIVES

In recent years, the molecular/cellular investigation of the immune tumor microenvironment and the comprehensive studying of the immunosuppressive mechanisms harbored therein have been at the frontier of cancer research, as an attempt to improve the already promising landscape of cancer immunotherapy (1, 86, 132, 135, 151). In this regard, we offer a fresh perspective on the topic by distinguishing disparate sets of immunosuppressive mechanisms in different tumor microenvironments. In particular, here we focused on analyzing multiple layers of immunosuppression, which involve mechanisms preventing T cell trafficking and mechanisms promoting T cell exhaustion within the specialized microenvironments dedicated to cancer cell dissemination (i.e., dissemination trajectories). This unique distinction serves a dual purpose: First, it offers an attractive explanation on why most immunotherapies do not target the migratory/invasive tumor cell subpopulation but instead are primarily restricted in promoting antitumor immunity within the more proliferative - less migratory tumor compartment. Second, it provides a rational framework on thinking the diverse immunosuppressive mechanisms as a multilayered obstacle against antitumor immunity, clearly suggesting that we should focus on targeting the immunosuppressive “network” rather than a “pathway” to be able to either restore the natural or orchestrate an engineered antitumor immunity. This perspective certainly does not aim at understating the importance of studying mechanisms of T cell exclusion and exhaustion in the proliferative and/or the cancer stem cell niches of the tumor microenvironment, given that targeting these microenvironments is also critical for establishing efficient anticancer immunity. However, this perspective aims at drawing significant attention to the frequently neglected concept of cancer cell dissemination, which may lead to a significant burden of dormant tumor cells in the distant metastatic sites, which may eventually grow into overt once they have found a way to avoid immunological detection and

acquired resistance to immunotherapy or other therapeutic modalities (293). Therefore, the rational targeting of immunosuppressive mechanisms within the dissemination trajectories would serve as a promising *antimetastatic therapy*, given that its purpose would be to improve T cell trafficking and to alleviate T cell exhaustion, thus rallying an immunological attack against the migratory/invasive cancer cell population while in the act of departure from the primary tumor.

In pursuit of understanding the escape of migratory/invasive (MENA<sup>INV+</sup>) cells from antitumor immunity, here, we propose a unified model with at least four distinct layers of immunosuppression. Foremost, we propose that endothelial anergy and cytokine/cytokine receptor mismatching mechanisms do not allow for robust T cell trafficking within dissemination trajectories, and, in case that these mechanisms are somehow breached, alternative mechanisms promoting T cell exhaustion from either metabolic burdening or immune checkpoint control may become dominant (Figure 1). It should be noted that all these individual mechanisms are strictly context-dependent and may occur simultaneously within dissemination trajectories, not in tandem. As a consequence, therapeutic targeting of these mechanisms for purposes of improving cancer chemotherapy and/or immunotherapy should consider all the aforementioned categories of immunosuppression, because counteracting a single one would likely be inadequate. Fortunately, therapeutic strategies that target each individual immunosuppressive layer in our model (Figure 1) are in development. Therefore, the greatest challenge for the next decade will fall back to eliciting the most appropriate combinations to successfully cripple the immunosuppressive niche within the tumor microenvironment, including within the dissemination trajectory. For example, prior reported antiangiogenic approaches aimed at promoting blood vessel normalization were shown to concurrently disrupt endothelial anergy, resulting in (re)sensitizing tumor blood vessels to lymphocyte diapedesis and improved T cell trafficking (294). Furthermore, immune checkpoint receptor/ligand blockade (primarily of CTLA4, PD1, and PDL1) with monoclonal antibodies has emerged as a successful therapy against intratumoral T cell exhaustion in human patients (1, 69, 291, 295, 296). Combining such antiangiogenic therapies with immune checkpoint blockade could represent the most attractive strategy to counteract immunosuppression and render cancer immunotherapy more successful (297).

Most conclusions regarding the immunosuppressive cues described in this review article have risen from literature evidence on the immunosuppressive properties of M2-like macrophages in general. The most critical aspect of the working model of spatial immunosuppression (Figure 3) is the contextual positioning of M2-like TAMs within the dissemination trajectories, either those represent “streaming” or “TMEM-doorway” macrophages. Therefore, the immunological properties of all the distinct tumor compartments are attributed to the topographical enrichment of M2-like macrophages within the dissemination trajectories rather than to unique or specific M2-like macrophage phenotypes.

As mentioned earlier, TMEM doorways are also formed in diverse metastatic sites, such as in the lungs and lymph nodes, and such *de novo* dissemination machineries may participate in the re-dissemination of cancer cells to tertiary sites, accelerating metastatic burden (97, 99). Indeed, analysis of TMEM doorways in secondary/metastatic sites suggests that their ensuing biology can mimic to great extent the biological programming of cancer cell dissemination observed in primary tumors (97, 99). It would therefore be interesting to investigate in the future if identical or similar immunosuppressive cues are recapitulated in the metastatic microenvironments that assemble “re-dissemination machineries”.

The deeper we delve into the complex circuitries involving immune cells and their associated cytokine/chemokine signatures in the tumor microenvironment, the necessity for more sophisticated technologies to study the processes they are involved with, will constantly emerge. Indeed, conclusions from many studies included in this perspective would be impossible to be drawn in the absence of high-throughput technologies for multiplex imaging and/or single cell expression profiling. In addition, high-resolution imaging (e.g., intravital fluorescence microscopy and planar bioluminescence imaging) has yielded important spatiotemporal data at single cell resolution, furthering our understanding on the immunological pathways supporting the active process of cancer cell dissemination (298–300). To complement the aforementioned efforts, such emerging technologies will additionally provide feasible tools for analyzing mutation antigen profiles, gene signatures and epigenetic modifications of both tumor and immune cells, the breadth of antibody responses, as well as the magnitude, homing capacity, cytotoxic function, and T cell receptor (TCR) repertoires of tumor-infiltrating lymphocytes. Overall, we anticipate that new technologies in this intriguing field of research will bring us a step closer to achieving personalized medicine and more promising immunotherapies.

In brief, here we describe an alternative perspective that tumor microenvironments dedicated to cancer cell dissemination may elicit strong immunosuppressive cues that prevent T cell trafficking and promote T cell exhaustion, processes that undeniably facilitate the initial steps of the metastatic cascade. Interestingly, these mechanisms are primarily orchestrated by certain well-recognized subsets of tumor-promoting TAMs (e.g., TIE2<sup>+</sup> TAMs), and their corresponding cytokine/chemokine network deployed around the cancer cell dissemination machinery. This working model of compartmentalized “immunosubversion” provides the groundwork for future studies on alleviating the immunosuppressive milieu for more optimal cancer immunotherapies.

## AUTHOR CONTRIBUTIONS

SA, GKi, PF, DE, DM, MO, and GKa: wrote the manuscript. LS and GKa: produced data and designed Figure 2. GKa: designed illustrations for Figures 1 and 3. All authors contributed to the article and approved the submitted version.



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

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