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Immune cellular components and signaling pathways in the tumor microenvironment

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Abstract

During the past decade there has been a revolution in cancer therapeutics by the emergence of antibody-based and cell-based immunotherapies that modulate immune responses against tumors. These new therapies have extended and improved the therapeutic efficacy of chemo-radiotherapy and have offered treatment options to patients who are no longer responding to these classic anti-cancer treatments. Unfortunately, tumor eradication and long-lasting responses are observed in a small fraction of patients, whereas the majority of patients respond only transiently. These outcomes indicate that the maximum potential of immunotherapy has not been reached due to incomplete knowledge of the cellular and molecular mechanisms that guide the development of successful anti-tumor immunity and its failure. In this review, we discuss recent discoveries about the immune cellular composition of the tumor microenvironment (TME) and the role of key signaling mechanisms that compromise the function of immune cells leading to cancer immune escape.

Keywords

Tumor microenvironment; Signaling pathways; Checkpoint inhibitors

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CRediT authorship contribution statement

SY, KA, QW, AC, RS wrote the main sections of the manuscript. NP generated sections of the manuscript and prepared figures. VAB generated sections of the manuscript, prepared figures, guided the co-authors and was responsible for the organization of the document.

Conflict of interest

VAB has patents on the PD-1 pathway licensed by Bristol-Myers Squibb, Roche, Merck, EMD-Serono, Boehringer Ingelheim, AstraZeneca, Novartis, and Dako. The authors declare no other competing interests.

1. Cancer immunosurveillance pathways and the tumor microenvironment

The exploitation of the immune system for the treatment of cancer has been investigated for many years. However, enthusiasm waxed and waned based on discoveries that supported or opposed the hypothesis that cancer can be subjected to immune-mediated control. It is currently understood that not only the immune system can control cancer growth but has an important role in shaping the immunogenicity of cancer cells via immunoediting [1]. During early stages of cancerous differentiation of normal cells, continuous immune surveillance results in the identification and elimination of these malignant populations through generation of adaptive anti-tumor immune responses. Tumor-associated macrophages (TAMs) have a central role in this process by mediating phagocytosis and clearance of cancer cells [2] and the presentation of cancer neoantigens to T cells [3]. Through continuous control, the immune system keeps cancer under check achieving equilibrium, even without complete elimination. The adaptive antitumor immune system can efficiently recognize neoantigens resulting from tumor-specific somatic mutations, antigens derived from oncogenic viruses, and antigens whose expression is shared with tissues at immune-privileged sites [4]. When cancer antigens yield peptides capable of binding to an individual's HLA alleles (neoepitopes), they can elicit $CD4^+$ and $CD8^+$ T cell responses [5,6] as evidenced by the presence and prognostic significance of immune infiltrates in human tumors [7–9]. However, under the continuous immune pressure cancer cells develop alterations to overcome immune attack, resulting in escape and growth of tumors that are resistant to the physiological immune mechanisms utilized to recognize and present antigens thereby engaging adaptive immune responses.

Escape mechanisms include tumor cell-intrinsic adaptations such as downregulation of tumor neoantigens and induction of protective mechanisms rendering tumors resistant to cytotoxic cells of adaptive immunity. In that regard, it has been determined that genomic alterations and changes of neoantigen load are linked to diminished immune responses [10]. Escape may also result from the development of immunosuppressive mechanisms of the TME, with the production of soluble factors such as IDO, VEGF, and TGF-β [11,12]. Alterations in the cellular populations of the TME such as recruitment of immunosuppressive myeloid-derived suppressor cells (MDSCs) that are produced during cancer-driven emergency myelopoiesis, regulatory T cells (Tregs) and tumor associated macrophages (TAMs) have important roles in shaping the properties of the TME (Fig. 1) [13]. Changes also occur in dendritic cells (DC) which lose their ability to process and present tumor antigens to T cells [14]. Expression of co-inhibitory molecules in these immune populations shapes the signaling landscape of the TME, generating primarily pro-tumorigenic cues by suppressing critical functions of myeloid cells and TAMs, such as phagocytosis and antigen presentation, and compromising the ability of T cells to mount immune responses. These signaling pathways have a key role in cancer immune escape but also represent targets for immune-based treatments that have re-shaped modern cancer therapy. Among the coinhibitory receptors, inhibitory targeting PD-1 and its ligands with blocking antibodies has been the cornerstone of cancer immunotherapy and together with CTLA-4 blocking agents have revolutionized cancer treatment. In this review, we provide a concise summary of how key coinhibitory receptors shape the function of the immune

components of the TME and discuss the role of immune populations during tumorigenesis and cancer evolution.

2. Dendritic cells are key mediators of tumor antigen presentation

Dendritic cells (DC) are professional antigen-presenting cells (APC) that play an important role in the tumorigenic and non-tumorigenic environment by activating $CD8^+$ T cells and enhancing antitumor responses. DCs make up a small minority of the tumor-infiltrating leucocytes [15]. In both human and mice, four different major subsets of DCs have been characterized: the classical DC1 (cDC1), the heterogeneous population cDC2, the monocyte-derived DC (mo-DC), and the plasma-cytoid DC (pDC) [16]. cDC1 expresses a distinct gene expression profile and specific markers [17]. It has been reported that cDC1 are critical for priming $CD8^+$ T cells whereas cDC2 are responsible for priming $CD4^+$ T cells [18] and support Th17 responses [19]. The DC2 group is a more heterogeneous population consisting of two different types of DCs, cDC2 and DC3 [20]. In agreement to that, another study revealed two distinct cDC2 populations by transcriptional analysis, cDC2A and cDC2B which express T-bet and RORγt respectively and are conserved across both human and mice [21] and might correspond to cDC2 and DC3 [22]. In humans, DC2 and DC3 derive from progenitors with differential expression of IRF8, where high expression led to the development of cDC1 and DC2, whereas low expression of IRF8 led to the development of DC3 and monocytes [23]. In contrast to cDC2 which require FLT3L for expansion, DC3 expanded and differentiated in the presence of granulocyte-macrophage stimulating factor (GM-CSF) but not FLT3L, providing further evidence that DC3s forms a distinct population within the cDC2 compartment [24]. Mo-DC can either derive directly from $CD34^+$ precursors or from monocytes [25,26]. Studies have indicated that IRF4 might be required for the differentiation of cDC2 and mo-DC, however, mo-DC do not share the same precursors as cDC2 [27,28]. Lastly, pDC are a distinct subpopulation of DC deriving from the common DC progenitors, secrete IFN-α/β and play an important role in viral infections [28].

Chemokines are mainly responsible for cDC1 recruitment and retention in the TME (Fig. 1). Tumors secrete several chemokines including CXCL9, CXCL10, and CXCL11, which can recruit Th1 and $CD8^+$ T cells, CCL20 which recruits Th17 and immature DC, and CCL2 or CCL5 which recruit monocytes [29]. NK cells can also recruit cDC1 to the TME by secretion of CCL5 and XCL1, an effect abrogated by prostaglandin E2 (PGE_2) production [30]. Inhibition of PGE2 production by ablation of COX1 or COX2 leads to increased accumulation of cDC1, tumor eradication, and increased sensitivity of tumor towards anti-PD-1 treatment [31]. Cytokines also play a role in the positioning of the DC in the TME. FLT3L secreted by NK cells and lymphocytes in mouse and human tumors has an active role in the recruitment and localization of cDC1 [32].

Generation of anti-tumor $CD8^+$ T cell responses requires cross-presentation of tumor peptides to naïve $CD8^+$ T cells on MHC class I molecules by the DC (Fig. 2A). Although priming of naïve T cells occurs in the tumor-draining lymph nodes, studies have shown that cross-presentation can also occur within the TME [33]. The antigens can travel to the lymph node either on cell debris or after their engulfment by migratory $CD103^+$ cDC1 found in

the TME, which then migrate to the tumor-draining lymph node and cross-present tumor antigens to naïve T cells, either directly or through antigen exchange to resident myeloid cells [34]. However, only the migratory $CD103^+$ cDC1, but not the lymph node resident $CD8^+$ cDC1, can prime naïve $CD8^+$ T cells [35]. CCR7 is required for cDC1 migration to the tumor-draining lymph node and ablation of CCR7 expression results in a significantly smaller migratory effect of cDC1 to tumor-draining lymph node and a diminished T cell activation profile [36]. In human tumors, CCR7 expression positively correlates with greater DC infiltration and increased patient survival [36].

Within the TME, $CD103^+$ cDC1 secrete CXCL9 and CXCL10 which recruit $CXCR3^+CD8^+$ effector T cells to the tumor [37]. In non-tumor models, CXCR3 has been previously shown to drive $CD4^+$ and $CD8^+$ T cell recruitment into the lymph nodes and in close contact to DC for T cell priming [38]. In a melanoma mouse model, CXCL9 and CXCL10 secreted by $CD103^+$ cDC1 were responsible for the recruitment of effector T cells and for controlling tumor growth [39]. $CD103^+$ cDC1 are located at distal regions of the tumor and despite their sparsity, they are good CTL activators [15]. Furthermore, cDC1 can uptake and cross-present tumor antigens to T cells more efficiently than other myeloid cells, which can also engulf tumor cells and uptake tumor antigens [15]. These $CD103^+$ cDC1 also secrete increased levels of IL-12 but not IL-10 suggesting a dominant role in facilitating increased cytotoxic function of intratumoral $CD8^+$ T cells [15].

Tumors have developed several mechanisms of immune evasion which restrict the function of cDC1 [40]. One such mechanism involves reducing the number of cDC1 in the TME. Tumors in breast and pancreatic cancers secrete the G-CSF thereby inhibiting the expression of IRF8 which is responsible for the generation of DC progenitors [41]. Similarly, reduction of FLT3L production within the tumor limits the differentiation, expansion, and survival of intratumoral cDC1 [35]. The reduced tumor infiltration by DC might also result from an impaired chemoattractant profile in the TME. PGE_2 production leads to impaired NK viability and consequently impaired CCL5 and CXCL1 chemokine production, both of which act as chemoattractants for cDC1 [30].

Impairment of cDC1 function is also possible through direct inhibition of cDC1. In a breast cancer model, IL-10 which is secreted mainly by tumor-associated macrophages in the TME, resulted in low levels of IL-12 cDC1-mediated secretion [42]. Similarly, PGE_2 reduced the production of IL-12 by cDC1 and downregulated the expression of co-stimulatory molecules [31]. In murine tumor models, lipid accumulation led to decreased ability of DC to translocate the peptide-MHC class I complex to the cell surface resulting in decreased antigen-presentation ability and T cell stimulation [43]. Tumor-derived TGF-β also inhibited the antigen presentation capacity of DC, impaired their ability to stimulate T cells, and decreased their migration capacity to the draining lymph nodes [44].

Suppression of cDC1 responses in the context of cancer is also driven by the inhibitory receptors present on the surface of DC (Fig. 2B). In patients with hepatocellular carcinoma, $PD\text{-}1^+$ DC circulate in their peripheral blood, making them potentially prone to inhibition by PD-L1, the ligand of PD-1, present in the TME [45]. PD-L1 is highly expressed on both tumor cells and immune cells present in the TME [46]. PD-L1 expression in tumor

infiltrating DC might be stabilized by phosphorylation mediated by the enzyme casein kinase 2 (CK2) [47], which is highly expressed in multiple cancers and is linked to increased cell growth, proliferation and inhibition of apoptosis [48]. Inhibition of CK2 decreased PD-L1 expression on DC and resulted in tumor suppression through release of CD80 from DC thereby allowing T cells to receive CD80-mediated co-stimulatory signals and become activated [47]. In PD-L1-deficient tumors, tumor-associated DC upregulatedPD-L1 expression [49]. The authors hypothesized that this upregulation could be an escape mechanism of the tumor by which immune cells receive inhibitory signals through the PD-L1-PD-1 axis. PD-L1 deletion on DC enhanced $CD8^+$ anti-tumor T cell responses despite the higher number of tumor-associated macrophages in the TME [50]. In addition to its canonical interaction with PD-1 in *trans,* PD-L1 can also bind to CD80 in *cis* [51,52] and this interaction disrupts the PD-1: PD-L1 axis and prevents T cell inactivation [53,54]. Blocking of PD-L1 on DC allows CD80 to re-engage with CD28 on T cells and enhance T cell priming [55]. In ovarian tumor-infiltrating DC, PD-1 inhibited the secretion of TNFα and IL-6, and downregulated the expression of CD80, CD40, and MHC class I molecules [56]. PD-1 regulated multiple NF-κB targets by recruiting the SHP-2 to the cytoplasmic domain tyrosine-based switch motif (ITSM) of PD-1 [56]. Anti-PD-1 reversed the suppressive effect of PD-1 on cytokines but had no effect on the expression of the co-stimulatory and antigen-presenting molecules [56]. Together these studies provide evidence about the critical role of DC-expressed PD-L1 in the induction of PD-1-mediated T cell immunosuppression in the context of cancer.

TIM-3, a T cell checkpoint inhibitory receptor, is highly expressed on DCs in the TME compared to normal tissues and inhibits innate immune responses through recognition of nucleic acids via the pattern recognition receptors TLR3, TLR7, and TLR9 [57]. A combination of CK2 and a TIM-3 inhibitors led to greater tumor suppression and longer survival times for the mice [47]. TIM-3 was also highly expressed on intratumoral cDC1 in a mouse model for breast cancer [58]. Antibody blockade of TIM-3 led to increased granzyme B expression by $CD8^+$ T cells and enhanced expression of CXCL9 by cDC1 [58]. Moreover, deletion of TIM-3 on DCs resulted in strong anti-tumoral $CD8^+$ T cell responses and inflammasome activation [59]. TIM-4, a receptor responsible for engulfment of dead cells, is highly expressed in normal lung cDC1, however, inhibition of this receptor results in incomplete activation of anti-tumorigenic $CD8^+$ T cells and increased tumor growth [60].

cDC2 cells have been historically considered to derive from monocytes and are recognized as cells responsible for Th2 and Th17 responses [61,62]. As mentioned earlier, they are recognized as a heterogeneous group which can be broken down into two different subsets, DC2 and DC3 [22]. In the context of cancer, these cells can travel from the tumor to the tumor-draining lymph nodes and present tumor antigens to $CD4^+$ T cells [63]. Depletion of Treg leads to increased cDC2 ability to induce strong T cell responses and increased ratio of cDC2 to Treg is correlated with better clinical outcome and responsiveness to anti-PD-1 therapy [63]. DC3 are poorly characterized in the context of cancer. They rise from granulocyte-monocyte-DC progenitors and express low levels of IRF8 [23]. They are present in human papillomavirus-associated oropharyngeal squamous cell carcinoma tumors, are potent Th1 activators, and can secrete high levels of IL-12 and IL-18 which have the potential to drive anti-tumor responses [64]. It has been reported that DC3 bear

signatures of both DC and monocyte-derived DC [22]. DC3 can stimulate memory T cells to produce IL-17 and differentiate naïve T cells into Th17 [65]. The differentiation program of these cells is regulated by GM-CSF [24]. Similarly, mo-DC can be generated in vitro from $CD34^+$ precursors by culture with GM-CSF and TNF-α or monocytes by culture with GM-CSF and IL-4 [25,26]. However, only Mo-DC generated in vitro express the DC marker *Zbtb46*, providing evidence that these in vitro generated populations might not be the true counterparts of mo-DC differentiated in vivo [66]. The role of mo-DC in the presentation of tumor antigens is currently unclear. It was reported that although mo-DC can uptake antigen they have impaired capacity to induce potent T cell responses due to increased nitric oxide (NO) production [67]. However, in a different experimental system it was found that mo-DC could induce strong anti-tumor $CD8^+$ T cell responses, which were impaired by blockade of monocyte entry to tumors [68]. Further studies are needed to fully characterize the cDC2, DC3 and mo-DC roles in the context of cancer.

pDCs are found in higher frequencies in human cancers and their presence is associated with poor prognosis and decreased survival [69]. Although pDCs normally produce IFN-α, in tumor settings including ovarian cancer, breast cancer, and melanoma, cancer-associated pDC have impaired IFN-α secretion [70]. This effect might be mediated by TNF-α and TGF-β present in the TME, which affect production of other cytokines such as IL-6, MIP-1b (CCL4) and RANTES [70]. In human ovarian cancer, pDCs are present in the tumor -but not ascites- can induce IL-10 secretion by naïve allogeneic T cells, and correlate with relapse [70]. Furthermore, when pDCs were cultured in medium from head and neck squamous cell carcinoma their ability to secrete IFN-α after TLR stimulation was significantly limited by IL-10 present in the medium, supporting the hypothesis that immunosuppressive cytokines in the TME contribute to the anti-inflammatory profile of tumor infiltrating immune cells [71]. Lastly, pDCs express the ICOS-L which can stimulate ICOS-expressing $Foxp3^+$ Treg which in turn secrete IL-10 in the TME and suppress immune responses [72]. Together, these immunological, signaling and functional features of DCs underline the pivotal role of this immune cell population in the development of anti-tumor responses and explain why DC-targeting signaling alterations in the TME might have a significant impact in anti-tumor immunity.

3. Myeloid derived suppressor cells (MDSC) inhibit anti-tumor T cell responses

The constant and prolonged secretion of danger-associated molecular patterns (DAMPs) and cytokines from the tumor cells has a direct effect on the bone marrow, through a process that is called in emergency myelopoiesis. During emergency myelopoiesis, immature myeloid cells, named MDSCs, with potent immunosuppressive properties, are produced and accumulate in the tumor microenvironment and peripheral organs. High numbers of these cells are associated with poor treatment outcome and worse clinical prognosis in various cancer types [73]. Two major subsets of MDSCs have been described in mice: Polymorphonuclear MDSCs (PMN-MDSCs) monocytic-MDSCs (M-MDSCs), which resemble morphologically and phenotypically to neutrophils and monocytes, respectively. PMN-MDSCs are identified as $CD11b^+Ly6G^+Ly6C^{lo}$ in mice and $CD11b^+CD14^-CD15^+$/

$CD66b^+$ in humans, while M-MDSCs are identified as $CD11b+Ly6G^-Ly6C^{hi}$ in mice and $CD14^+CD15^-HLA\text{-}DR^{lo/-}$ in humans [74].

Among the mechanisms proposed for MDSC-mediated suppression function and subsequent cancer immune evasion, checkpoint inhibitors might have an important role. MDSCs isolated from patients with AML express high levels of VISTA and siRNA-mediated VISTA deletion attenuated MDSC-mediated inhibition of $CD8^+$ T cells [75]. VISTA, a B7 family ligand, is predominantly expressed on the hematopoietic compartment, with highest expression observed in myeloid cells, including monocytic and granulocytic cells, and weaker expression on T cells [76]. VISTA was also identified as a homolog of PD-1, a member of the CD28 superfamily, indicating a potential function both as a ligand and a receptor [77]. VISTA transcription can be induced by HIF-1α suggesting that hypoxia is implicated in VISTA upregulation in MDSCs located in hypoxic regions of the TME [78]. VISTA deletion in MDSCs had no effects in T cell proliferation in vitro. However, VISTA deletion resulted in increased T cell proliferation under hypoxia, suggesting that regulation of MDSC function by VISTA might occur only under oxygen deprivation [78]. Furthermore, VISTA blockade in tumor-bearing mice altered the tumor infiltrating immune cell populations by decreasing MDSCs and increasing T cells with anti-tumor properties [79].

The MDSCs suppression capacity is also affected by the PD-1/PD-L1 pathway. Compared to MDSCs localized in non-cancerous tissues, tumor infiltrating MDSCs express high levels of PD-L1, which is regulated by the COX2/mPGES1/PGE_2 pathway, pSTAT1-IRF1 axis and hypoxia-induced HIF-1α [80–82]. Consistent with an active role of these pathways in MDSC function, PD-L1 blockade under hypoxia diminished the ability of MDSCs to suppress T cell activation by reducing the production of IL-10 and IL-6 [82]. Notably, conditional deletion of PD-1 in the myeloid compartment in tumor-bearing mice, resulted in diminished NO production and attenuated suppressive capacity of M-MDSCs indicating that PD-1 blockade or deletion counteracts the generation of immunosuppressive immature myeloid cells [83].

Due to their potent immunosuppressive properties, MDSCs have been associated with the limited efficacy of immunotherapy and standard chemotherapy in mouse tumor models and cancer patients. Conversely, selective depletion of MDSC reversed resistance to anti-CTLA4 Ab treatment in tumor bearing mice and increased the numbers of cytotoxic $CD8^+$ T cells in tumor-deraining lymph nodes and TME [84]. Doxycyline-induced suppression of MDSCs improved the efficacy of PD-1 inhibitors [85]. Similarly, in a mouse model of gastric cancer, 5-flurouracil and oxaliplatin combination, which decreased MDSCs, augmented $CD8^+$ tumor infiltrating T cells and improved the therapeutic efficacy of anti-PD-1 treatment [86]. Furthermore, epigenetic targeting of MDSC in mice bearing immunotherapy-resistant tumors resulted in tumor eradication after anti-PD-1/anti-CTLA4 combined immunotherapy [87].

4. Generation of pro-tumorigenic tumor-associate macrophages (TAMs)

After egress from the bone marrow, monocytes (or M-MDSCs) are recruited to the TME via chemokines of the CC and CXC families, such as CCL2, CCL5, and CXCL12 [88] produced by cancer cells at early stages of the cancer immunity cycle and convert to bone marrow-derived tumor associated macrophages (Fig. 1), which, together with embryonically-derived tissue resident macrophages, form the population of TAMs. TAMs represent the most abundant immune population of the TME, consisting ~50% of hematopoietic cells and have predominantly pro-tumorigenic functions [89]. Pro-tumorigenic TAMs are immunosuppressive, lose the natural ability of macrophages to mediate phagocytosis and antigen presentation [83,90,91] and are characterized by expression of inhibitory molecules such as PD-1, PD-L1, VISTA, B7-H4 and TIM-3 [83,90–96].

The TME has a causative role in promoting the differentiation of pro-tumorigenic TAMs in a stepwise manner [97]. As a consequence of the malignant transformation of normal epithelial cells into cancerous cells by oncogenes and driver mutations, soluble factors, such as hematopoietic growth factors (e.g., M-CSF, GM-CSF) cytokines (e.g., IL-6, IL-1, and IL-8) and chemokines (e.g., CCL2, CCL5, CXCL12), are produced during cancer evolution (Fig. 1). For example, in pancreatic adenocarcinoma, $KRAS^{G12D}$ mutation induces secretion of GM-CSF, which is correlated with an increased tumor infiltration by myeloid cells and immunosuppressive function [98,99]. p53 mutation has been documented to induce cancer-related inflammation by suppressing the production of IL-1 receptor antagonist [100]. In human melanoma, $BRAF^{V600E}$, a highly oncogenic mutated form of BRAF, and STAT3, a potent transcription factor often linked to oncogenic signaling, have been shown to drive expression of IL-6, IL-10 and VEGF, which promote a tolerogenic differentiation of bone marrow derived monocytes [101]. BRCA1-associated triple negative breast cancer induces pro-tumorigenic TAMs, by reprogramming glycolysis and SREBP1-mediated lipid metabolism, a metabolic signature associated with resistance to PARP inhibitors [102]. These mechanisms induce a unique differentiation program of TAMs, which lose the normal properties of healthy macrophages and obtain pro-tumorigenic features.

The immunophenotypic and metabolic properties of TAMs were previously streamlined in the simplified concept that TAMs have a differentiation program resembling M2 macrophages, characterized by expression of CD206, CD204, VEGF, CD163 and Arg-1 [103]. This is in contrast to the expression of classical markers of M1 polarized macrophage including CD80, CD86, MHC-II, iNOS and CD68, which correlated with effector function of macrophages and tumoricidal function of TAMs [103]. The M1/M2 programs rely mainly on metabolism, since proinflammatory M1 macrophages are supported by glycolysis, whereas anti-inflammatory M2 macrophages utilize mainly fatty acid oxidation (FAO)[104]. This concept is no further considered appropriate, but such differentiation profiles remain useful in the characterization of TAMs because there is extensive experience regarding the correlation between the expression of such immune marker combinations and prognosis in various cancers [97,105].

Live cell imaging allowing assessment of the spatial interaction of TAMs with other cells of the TME has significantly improved our understanding about the role of TAMs in tumor

evolution. In a breast cancer mouse model, multiphoton microscopy studies showed that TAMs interact with mammary cancer cells, facilitating their intravasation and subsequent metastasis. Tumor cells closer to TAMs are more motile and are directed toward TAMs of the perivascular area where they interact with blood vessels and enter the blood stream [106, 107]. Real time imaging provided evidence about the role of perivascular Tie2^{hi} TAMs in promoting transient vascular permeabilization and tumor intravasation, thereby facilitating metastasis [108]. In a model of GBM, intravital 2-photon microscopy showed two distinct TAM subtypes with morphological and functional differences: microglial cells which are large, highly branched and less motile, and bone marrow-derived macrophages which are smaller, less branched and less motile [109]. The differential properties of the spatially distinct subsets of TAMs might form attractive targets for therapeutic intervention.

Beyond the impact of oncogenes in regulating the production of tumor-derived soluble factors that regulate monocyte recruitment and TAM generation, the mutational landscape of tumor cells alter other immune cell types that are recruited in the TME. Colorectal cancer (CRC) serves as a paradigm for this process. Based on gene expression studies, CRC can be classified into four molecular subtypes (CMS1–4) [110]. CRC-CMS1 has DNA mismatch-repair defects, which cause microsatellite instability and hypermutation. These tumors are densely infiltrated by $CD4^+$ and $CD8^+$ T cells with high expression of immune checkpoint inhibitors including CTLA-4, PD-1, and PD-L1 and display favorable responses to checkpoint immunotherapy [111]. In contrast, CRC-CMS4 is characterized by tumor cells with a mesenchymal-like phenotype, an RNA sequencing profile dominated by signatures of the TGF-β pathway, Th17 pathway, monocyte/macrophage infiltration, and resistance to checkpoint immunotherapy [111]. Thus, cancer-specific properties have a significant impact on the quantitative and qualitative inflammatory infiltrate of the TME and in regulating the crosstalk between myeloid cells and the adaptive immune system.

5. Tumor-associated T cells and signaling pathways shaping their function

A critical determinant of tumor containment and progression over time is the number and function of T cells within the TME. For this reason, T cells are the key targets of antibody-based and cell-based immunotherapies in cancer. During the phase of cancer escape from immune control, T cells that can recognize tumor-associated antigens and control tumor growth, lose the ability to mediate this function due to mechanisms related with tumor-induced tolerance and immunosuppression (Fig. 1) [112]. These dysfunctional T cells are characterized by features of T cell exhaustion (T_{EX}) observed in chronic viral infections [113] such as high surface expression of inhibitory receptors CTLA-4, PD-1, TIM-3, LAG-3, loss of expansion ability, and impaired effector function as determined by the diminished production of cytokines such as IFNγ and TNF-α (Fig. 3) [114]. The T_{EX} state might be reversible or irreversible [115] and a central goal of novel immunotherapies is to achieve re-invigoration of tumor-specific T_{EX} cells. The development of T_{EX} state is mediated primarily by T cell-extrinsic factors that lead to persistent T cell activation by tumor antigens [116].

The co-inhibitory molecules such as PD-1 (CD279) and CTLA-4 (CD152) are induced during physiologic T cell stimulation to tame activation signals. CTLA-4 being upregulated and acting early during T cell activation, is the high affinity receptor of CD80/CD86. CTLA-4 directly competes with CD28 for binding on CD80/CD86, and its binding on CD80/CD86 ligands on APC can result in the depletion of CD80/CD86 by trans-endocytosis [117] while simultaneously releasing free PD-L1 by eliminating availability of CD80 for PD-L1 engagement in PD-L1: CD80 interaction [118]. PD-1 is also induced upon TCR-mediated activation. Engagement of PD-1 by its ligands PD-L1 (B7-H1 or CD274) and/or PD-L2 (B7-DC or B7-H3) counteracts TCR signaling and CD28-mediated co-stimulation [119,120]. PD-1 and CTLA-4 are the prototype inhibitory receptors, known as immune checkpoints, and are the most extensively utilized therapeutic targets in cancer immunotherapy [121].

After engagement with its ligands PD-L1 or PD-L2, but also via tonic signaling [122], PD-1 inhibits T cell functions by recruiting phosphatases, predominantly SHP-2 but also SHP-1, to the immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM), expressed in PD-1 cytoplasmic tail, which antagonize TCR and CD28-mediated activation signals [123–126]. This TCR proximal signaling blockade results in inhibition of key downstream pathways including PI3K/Akt and Ras leading to altered biochemical, transcriptional and metabolic T cell reprograming [127–129]. Constitutive PD-1 expression on T cells induced by persistent antigen stimulation during cancer promotes immune evasion, and blockade of the PD-1 pathway can improve T cell function and reduce tumor burden in multiple experimental tumor models and multiple types of human cancers [130,131].

The expression status of PD-L1 on tumor cells has served as a factor for patient stratification for anti-PD-1 therapy. However, in several clinical studies, there were patients with undetectable PD-L1 on tumors, who also responded to anti-PD-1 therapy [132]. This effect can be explained by the fact that, other cell types in the TME, such as macrophages, DCs, tumor-associated fibroblasts and myeloid cells also express PD-L1, creating an immunosuppressive TME for T cells and supporting tumor cell growth. Therefore, the relative contribution of PD-L1 on tumor cells and other cell types in limiting anti-tumor responses in the TME remains under investigation. PD-L1 expression on tumor cells can locally inhibit $CD8^+$ T cell activation and protect $PD\text{-}L1^+$, but not $PD\text{-}L1^-$ tumors from eradication by the immune system, indicating a critical role for tumor PD-L1 in suppressing antitumor immunity [133]. By genetic deletion of PD-L1 in tumor cells and host mice, a different study showed comparable contribution of PD-L1 on each of these compartments to immune suppression, suggesting that PD-L1 expression in either of these compartments can be predictive of responses to PD-1/PD-L1 blockade therapy [134]. However by using conditional deletion of PD-L1 in tumor cells and dendritic cells-, transplantation chimeras, as well as various approaches of antibody blockade, it was determined by several investigators that PD-L1 expressed in APC, rather than tumor cells, has a causative role in compromising anti-tumor T cell responses and this APC-mediated PD-1 ligation and T cell immunosuppression is the dominant interaction targeted by checkpoint blockade therapy [95,135]. Since DCs mediate $CD4^+$ T cell priming and cross-presentation of tumor antigens

to CD8 $^{+}$ T cells, upregulation of PD-L1 on dendritic cells can attenuate cytotoxic T cell activity and compromise antitumor responses [136,137].

Recently, in *cis* interaction of PD-L1 and B7–1, on the same cell surface of APC, has been reported by several studies [51–54]. The in *cis* interaction of B7–1 with PD-L1 impairs the interaction of PD-L1 with PD-1 in *trans* (Fig. 3) resulting in reduced PD-1 signaling (reviewed in [138]). Blockade of the in *cis* interaction of PD-L1/B7–1 by B7–1-specific antibodies could increase PD-1-mediated T cell suppression and alleviate autoimmunity in several mouse models [139]. Among tumor-infiltrating myeloid cells, PD-L1-expressing DCs have a dominant role in regulating antitumor T cell responses via in *cis* interaction of PD-L1/B7–1 on DCs and PD-L1/PD-1 interactions between DCs and T cells. These findings suggest a new therapeutic mechanism of PD-L1 checkpoint blockade acting on DCs to induce enhanced antigen presentation for T cell priming and simultaneous clonal expansion [137]. A more comprehensive understanding of these mechanisms will be necessary to optimize PD-1/PD-L1 targeting immunotherapy.

While the PD1/PD-L1 axis has been extensively investigated, other inhibitory pathways are also exploited by tumor cells, contributing to the generation of an immunosuppressive TME and escape of immunosurveillance [140]. VISTA (B7-H5, PD-1 H, Gi24, Dies1, SISP1 and DD1α) has increasingly become a promising target for overcoming resistance to anti-PD-1 immunotherapy [141]. In addition to the ability of VISTA to regulate the immunosuppressive function of MDSC under hypoxia, as mentioned above [78], VISTA can function as an inhibitory regulator of naïve T cells, critical for steady-state maintenance of immune quiescence and peripheral tolerance (Fig. 3) [142]. Notably, VISTA can mitigate pathogenesis and progression of murine lupus by transmitting inhibitory signals on both T cells and myeloid cells [143, 144].

Although few studies have reported VISTA expression on tumor cells [145,146], VISTA expression on intratumoral myeloid cells and stromal cells has been recognized as a potential mediator of acquired resistance to anti-PD-1 and anti-CTLA-4 immunotherapies in patients with metastatic melanoma and pancreatic ductal adenocarcinoma [147–149]. Therapeutic blockade of the VISTA pathway has been limited by its unknown binding partners and their function. A recent study identified VSIG-3 as a putative ligand of VISTA to inhibit T cell activation and cytokine production [150]. While at physiological pH VISTA interacts mainly with VSIG-3, in acidic pH, VISTA serves as a selective ligand for PSGL-1 receptor on T cells suppressing T cell function (Fig. 3) [151]. This unique pH-dependent VISTA/PSGL-1 interaction suggests that engineering pH-sensitive antibodies might enable selective targeting of VISTA within the acidic TME to reverse cancer-mediated immune suppression without compromising self-tolerance and inducing autoimmunity [151]. Thus, targeting the VISTA pathway can be achieved in a context-dependent manner to overcome the immunosuppressive TME and reinvigorate responses of tumor-specific T cells.

5.1. T regulatory cells of the TME and implications in cancer immunotherapy

It has been previously established that inhibition of Akt and its downstream target mTOR is required to induce Treg differentiation and sustain Treg suppressor function [152,153]. PD-1 ligation inhibits Akt activation [127] and synergizes with TGF-β to induce FoxP3^{+} iTreg

cells with potent suppressive capacity [154]. Moreover, PD-1 promotes fatty acid oxidation [128], a metabolic program that supports the differentiation, survival and function of Treg cells [155]. Based on these, one would anticipate that PD-1 promotes Treg generation and suppressor function. However, recent studies revealed an unexpected connection between PD-1 expression in Treg cells and the outcome of PD-1 pathway blockade [156]. In humans, $FoxP3^{+}CD4^{+}$ Treg cells represent a heterogenous population that can be subdivided into three functionally and phenotypically distinct subsets [157]. Thymus derived naïve Tregs (nTreg: $CD45RA^{+}$ $FOXP3^{lo}$) and activated effector-Tregs (eTreg: $CD45RA^{-}$ $FOXP3^{hi}$) are highly suppressive while the $CD45RA^{-}$ $FOXP3^{lo}$ Treg cells represent a non-Treg population with a potential of inflammatory cytokine production. Among these Treg subsets, $FoxP3^{+}$ Tregs infiltrating the TME in most cancers are predominantly the eTreg cells [158]. Expression of PD-1 was found in eTreg cells and the frequency of PD-1^{+} eTreg cells was higher in tumor samples from patients who did not respond to PD-1-blocking immunotherapy. Notably, blockade of the PD-1: PD-L1 pathway induced activation of eTreg in TILs, as determined by upregulation of CTLA-4, GITR and ICOS and these Treg obtained a more potent suppressor function. These results highlight a previously unappreciated role of PD-1 signaling in Tregs and suggest that PD-1 blockade enhances the suppressive activity of Tregs that express high levels of PD-1. While blocking PD-1 in PD-1^{+} $CD8^{+}$ T cells converts them to $CD8^{+}$ Teff cells that have potent effector function leading to tumor regression, blocking PD-1 in PD-1^{+} Treg converts them to activated eTreg that have potent suppressor function leading to tumor progression. Thus, PD-1 expression balance between $CD8^{+}$ Teff cells and eTreg in the TME that might predict the clinical efficacy of PD-1 blocking immunotherapy.

6. T cell differentiation and cancer immunosurveillance

$CD8^{+}$ T cells play an important role in the adaptive immune response to intracellular pathogens and cancer [159]. Therapeutic responses to PD-1 checkpoint immunotherapy correlate with expansion of $CD8^{+}$ memory T cells in mouse tumor models and patients [160]. Immunometabolic programs have a causative role in T cell differentiation and their immune function [161,162]. This is particularly important in the metabolically stressful TME where nutrient competition, hypoxia, excess ROS and metabolic byproducts of cancer cells create uniquely hostile conditions under which T cell activation by and cytotoxic function should be operated [163–165]. Although glycolysis has been intimately linked to effector function [166,167], augmenting glycolytic flux drives $CD8^{+}$ T cells toward a terminally differentiated state, while its inhibition preserves the formation of long-lived T_M $CD8^{+}$ T cells [168]. Thus, multiple signaling pathways and mediators of metabolic reprogramming, such as mTOR, SREBP, Myc [169–172], have important roles in immune-mediated tumor control by guiding differentiation and function of T cells in the context of cancer [172–175]. After stimulation with antigen, $CD8^{+}$ naive T cells expand and differentiate into T_{EFF} cells and distinct T_M cell subsets, including T_{SCM}, T_{CM}, and T_{EM} [176]. Preclinical studies using adoptive transfer of purified $CD8^{+}$ T cell populations revealed that less-differentiated T cells with features of T_{SCM} and T_{CM} mediate enhanced antitumor and antiviral responses compared with more-differentiated T_{EM} and T_{EFF} cells [177–179]. Preservation of T_{SCM} and T_{CM} cells with a quiescent phenotype and increased

proliferative and survival capacities enhance T cell ability to maintain sustained anti-tumor control [180]. For example, Wnt signaling prevents T_{EFF} differentiation, while promoting the generation of T_{SCM} that maintain stemness and pluripotency and display long-lasting potent anti-tumor properties [179].

7. Tissue resident memory cells (T_{RM}) are novel regulators of anti-tumor immunity

Memory T cells have been classically divided into two subsets [181]. T_{EM} cells create immediate effector function to inflamed tissues whereas central memory T cells (T_{CM}) accumulate in secondary lymphoid organs and generate protective immunity [181,182]. Recently, a distinct T memory cell population, named tissue-resident memory T cells (T_{RM}) were identified and classified by their unique phenotype and properties. These cells reside permanently in peripheral non-lymphoid tissues and provide a protective effect against infections and cancer [183,184]. T_{RM} cells have been identified and investigated both in mice and humans in many tissues including skin, gut, brain and liver [185–187]. Although T_{RM} cells were defined as either $CD4^+$ or $CD8^+$, only $CD8^+$ T_{RM} play a major role in cancer immunosurveillance and tumor prognosis [188–190]. Traditionally, $CD8^+$ T_{RM} are defined by the co-expression of CD103, CD69 and/or CD49 [191], and downregulation of receptors that promote T cell recirculation including S1pr1, CD62L (L-selectin) and CCR7 [192–194]. CD103 integrin, formed by αE (CD103) and β7 subunits, promotes T_{RM} cells retention and homing to epithelial tissue and tumors [195–197]. T_{RM} cells have an established role in protective immunity in bacterial, viral infections and autoimmune diseases, including *Listeria monocytogenes* and Herpes Simplex infections and vitiligo.

An important role of T_{RM} in cancer is currently evolving. In a model of melanoma-associated vitiligo (MAV), using adoptive pmel cells which have a TCR that can detect melanoma gp100 antigen, it was shown that adoptively transferred pmel T cells acquired a T_{RM} immunological profile with a $CD103^+CD69^+CD62L^{lo}$ phenotype in the skin, draining lymph nodes, lung, and liver [198]. In this context, T_{RM} cells had a protective function against metastasis and expressed transcripts different from T_{CM} cells, characterized by features of effector function and lipid metabolism. In a mouse model of epicutaneous melanoma, it was also determined that T_{RM} have a role in promoting melanoma immune equilibrium [189]. Mice that did not develop melanoma (non-developer group) had higher numbers of $CD69^+CD103^+$ T_{RM} cells than tumor-developer mice. In addition, intact skin from the non-developer group, peritumoral area, and tumoral area showed higher number of T_{RM} cells. Notably, both CD69 and CD103 knock-out mice are more susceptible to melanoma formation than wild type counterparts, indicating the causative role of CD69 and CD103 proteins in regulating T_{RM} development and anti-tumor function [189]. Other studies have shown that $CD103^+$ T_{RM} cells can protect against melanoma re-exposure providing evidence for the long-lasting ability of T_{RM} to provide cancer immunosurveilance [199]. In many types of cancer, $CD8^+$ T_{RM} cells display robust production of cytokines such as granzyme B, granzyme A, perforin, and IFN-γ [198,200–203]. Importantly, recent work, using a VHL deficiency mouse model, provided evidence for a link between HIF-1α and CD103 expression and showed that HIF-1α expression supported enhanced differentiation

of T_{RM} cells with cytotoxic function [200,203]. These findings link HIF-1α, a key signature molecule of the TME, with T_{RM} differentiation and function and underline the direct relevance of T_{RM} in anti-tumor immunity.

T_{RM} cells express checkpoint receptors, and might serve as markers for prognosis prediction In melanoma patients, T_{RM} express PD-1, TIM-3, and PD-L1 at higher levels than peripheral blood T cells. Single-cell RNA sequencing of T cells isolated from human breast cancer demonstrated a high number of T_{RM} cells with a high level of TIM-3, PD-1, CTLA-4, TIGIT, and LAG-3 expression [190]. In lung cancer, PD-1, TIM − 3, and CD137 are highly expressed [202,204]. After ex vivo pharmacologic stimulation of PD-$1^{+}$$CD103^{+}$ CD8 TILs from ovarian cancer, these T_{RM}-like cells highly expressed TIM-3, CTLA-4, and LAG-3 [205, 206]. In lung cancer patients, after anti-PD-1 treatment, T_{RM} cells upregulated PD-1, CTLA-4, TIM-3, TIGIT, and CD39 [207], whereas in melanoma patients PD-1 checkpoint immunotherapy resulted in T_{RM}-specific upregulation of PD-1 and LAG-3 [208]. Notably, the number of T_{RM} cells is correlated with a better response to PD-1 checkpoint immunotherapy and a favorable prognosis in multiple cancers including melanoma, breast cancer, lung cancer, urothelial cancer, ovarian cancer and cervical cancer [205,208–215]. The unravelling role of T_{RM} cells in cancer evolution and response to immunotherapy suggest that T_{RM} might be a promising novel target for therapeutic exploitation in cancer.

8. Innate lymphoid cells and their role in cancer immunotherapy

Innate lymphoid cells (ILCs), which lack antigen-specific receptors, are considered as the innate equivalents of T cells. The most recent nomenclature classifies ILCs into five groups, namely ILC1s, ILC2s, ILC3s which are the equivalent of Th1, Th2, and Th17 $CD4^{+}$ T helper (Th) cells, respectively, natural killer (NK) cells, which are the equivalent of $CD8^{+}$ cytotoxic T cells, and lymphoid tissue-inducer cells (LTis), a unique subset involved in the development of secondary lymphoid organs [216]. With the exception of the highly mobile and constantly circulating NK cells, ILCs are largely tissue-resident cells. Besides their main role of immune surveillance and tissue homeostasis, ILCs have emerged as critical players in cancer growth and therapy [217]. ILCs express a plethora of activating and inhibitory receptors [218]. Among the latter, checkpoint inhibitory receptors increasingly raise attention due to their role in tumor immunotherapy. Here, we summarize the current knowledge on the role of checkpoint inhibitory receptors in ILCs in the context of cancer.

8.1. Natural Killer (NK) cells

NK cells belong to a highly diverse subset of ILCs that circulate between peripheral organs, exerting cytotoxic activity that can directly eliminate cancer cells or cells undergoing microbial infections [219–221]. In healthy adults, NK cells comprise about 5–15% of circulating lymphocytes with the majority of healthy tissues mainly consisting of the mature highly cytolytic $CD56^{dim}$ NK cell subset, while the immature and poorly cytolytic $CD56^{bright}$ subset, although also present in healthy tissues, it becomes significantly enriched in cancer [222]. A plethora of activating and inhibitory receptors regulate NK cell development and function, with the dominant signal being inhibitory, resulting primarily from the interactions of certain NK cell inhibitory receptors with major

histocompatibility complex class I (MHC-I) molecules [223,224]. These “classical” NK inhibitory receptorsinclude the group of Killer Ig-like receptors (KIRs) in humans and the LY49 group of receptors in rodents, as well as the CD94/NKG2A receptor (Fig. 4A), which also recognizes HLA-E in humans and Qa-1b in mice [218, 223–228]. According to the “missing self” hypothesis [229], these inhibitory receptors have the unique ability to prevent NK cell responses against self, and allow NK cells to exert their cytotoxic activity against cells that have reduced or absent expression of MHC-I molecules. However, in the context of cancer, these NK inhibitory receptors hamper NK cell responses and contribute to tumor cell escape from elimination. Blocking antibodies, usually termed “immune checkpoint inhibitors” against KIRs (lirilumab) [230] and NKG2A (monalizumab) [231], which aim to improve NK cell function, are in phase I/II clinical trials against a range of hematologic malignancies and solid tumors either as mono- or combination therapy [232,233]. In addition, adoptive transfer of NK cells engineered to downregulate NKG2A in immunodeficient mice, efficiently prevented HLA-E^{+} tumor-mediated suppression leading to increased anti-tumor activity, suggesting this as a promising approach to be tested in a clinical setting [234].

Besides the classical inhibitory receptors, NK cells also express checkpoint inhibitory receptors (Fig. 4A). Expression of programmed cell death 1 (PD-1) was found on NK cells from healthy individuals correlating with prior HCMV infection [235]. Notably, PD-1 expression has been found to be restricted to the fully mature $CD56^{dim}$ or to the $CD56^{neg}$ NK compartment and not to the immature $CD56^{bright}$ NK cell subset [236]. PD-1 is also expressed on NK cells in the context of cancer. PD-1^{+} NK cells were detected in the peripheral blood of patients with multiple myeloma, Kaposi sarcoma and lung cancer patients [237–239], in pleural effusions from primary mesothelioma or metastatic adenocarcinoma patients [240], in the peritoneal fluid of high-grade peritoneal carcinomatosis patients [241], and in the peripheral blood and ascitic fluid of ovarian carcinoma patients, with both mRNA and protein PD-1 levels to be higher in the $CD56^{dim}$ NK cell subset [242]. In contrast, PD-1 expression was found to be higher in $CD56^{bright}$ than $CD56^{dim}$ mature NK cells from blood and tissues from patients with Hodgkin lymphoma [243]. As in T cells, blockade of the PD-1/PD-L1 axis in NK cells could improve anti-tumor activity [243,244]. Interestingly, glucocorticoids present in the TME were found to be indispensable for PD-1 induction on human NK cells, particularly when combined with the inflammatory cytokines interleukin (IL)− 12, IL-15 and IL-18, which are abundant at the tumor site [245]. In addition, expression of the PD-1 ligand, PD-L1, on NK cells has been associated with a regulatory function, limiting DC-mediated tumor antigen cross-presentation to $CD8^{+}$ cells and resulting in impaired memory responses [246].

The inhibitory receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) has also been detected on NK cells from mice [247], in the blood from healthy donors and in the blood and tissues from cancer patients [248,249]. Similarly to PD-1, CTLA-4 was found to be predominantly expressed on $CD56^{dim}$ NK cells from the blood of healthy individuals and was associated with decreased NK cell functionality [249]. These findings explain why CTLA-4 blockade, besides T cells, was also found to favor NK cell infiltration and antitumor function [250]. Of note, treatment with anti-CTLA-4 antibody could favor NK cell

anti-tumor function indirectly, by depleting CTLA-4-expressing intratumoral regulatory T cells (Treg) [247].

Other checkpoint inhibitory receptors such as TIM-3, LAG-3, TIGIT), CD96 (TACTILE) have also been identified in NK cells (Fig. 4A). Although mostly considered as an inhibitory receptor [251,252], TIM-3 has also been reported to have an activation role in NK cells [253]. Higher TIM-3 expression has been observed on the $CD56^{dim}$ NK cell subset, while on the $CD56^{bright}$ cell subset it may be upregulated upon cytokine stimulation [251]. In the context of cancer, TIM-3 has been detected in NK cells from patients with advanced melanoma [254,255], gastric cancer [256], lung adenocarcinoma [257], and bladder cancer [258]. Increased TIM-3 expression was found in both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, correlating with shorter overall survival [257]. Conversely, blockade of TIM-3 signals with TIM-3-specific antibodies improved NK cell functions in vitro [254,257].

LAG-3 acts synergistically with PD-1 and contributes to T cell and NK cell dysfunction, exhaustion [259] and tumor escape [260]. Several years of efforts testing LAG-3 as promising checkpoint blockade therapy recently led to the first approval of LAG-3 blocking antibody (relatlimab-rmbw) for combination therapy together with PD-1 antibody nivolumab (Opdualag) for the treatment of patients with unresectable or metastatic melanoma. An alternative approach used a LAG-3-Ig fusion protein (IMP321) as a soluble high affinity MHC-II agonist to enhance antigen presenting function. Treatment with IMP321 as monotherapy enhanced IFN-γ and TNF-α production in NK cells from control donors and cancer patients *ex vivo* [261], and was also shown to increase the number and activation of NK cells in the blood of breast cancer patients in combination with standard chemotherapy [262].

A separate group of receptors, also referred to as novel NK cell immune checkpoints, includes the inhibitory receptors TIGIT and CD112R [263] as well as the costimulatory receptor CD226 (DNAM-1) [264], and CD96 (TACTILE) for which both inhibitory and costimulatory functions have been reported [265,266]. These receptors interact with molecules of the nectin family CD155 (PVR) and CD112 (Nectin-2) (Fig. 4A) [267], two ligands with ubiquitous low level expression, often up-regulated in cancer cells [268,269] and myeloid suppressor cells within the TME [270,271]. TIGIT is expressed by activated T cells and NK cells and binds its ligands CD155 and CD112 expressed on antigen presenting cells (APC) [272]. Expression of these ligands by several cancer types results in T cell and NK cell exhaustion, impaired tumor immune infiltration and compromised antitumor function [273–275]. The TIGIT pathway and its related receptors and ligands offer new options for antibody-based blocking strategies, which have shown to improve tumor control in mice particularly when combined with PD-1/PD-L1 or CTLA-4 blockade [276].

8.2. Helper ILCs

Recent studies suggest that checkpoint receptors are involved in the immune responses of helper ILC subsets and have important implications in anti-tumor immunity (Fig. 4B). However, little information is currently available regarding the mechanisms by which these receptors regulate helper ILC functions. Although not detected in helper ILCs from healthy human tissue samples [277], PD-1 is expressed on tumor-associated helper ILC populations

[278]. It was recently reported that helper ILC subsets are present in human malignant pleural effusions, with a notable PD-1 expression level on the ILC3 subset [240]. Another study, using single-cell RNA sequencing and a CRC mouse model associated with AOM/DSS-induced colitis, identified high-PD-1-expressing ILC2 infiltrates and demonstrated the importance of this specific subset in tumor progression [279]. Furthermore, PD-1^+ ILC2 cells were present in most human PDAC tissues as well as in orthotopic PDAC tumors in mice, in which PD-1 blockade, resulted in ILC2 expansion and improved antitumor immunity [280]. These findings suggest the potential role of ILC2 cells as tissue-specific enhancers of cancer immunity that may amplify the efficacy of anti-PD-1 immunotherapy.

Similarly to PD-1, CTLA-4 expression was found to be very low in helper ILCs from healthy donors [281], but is increased in helper ILC subsets within tumor tissues [278]. High CTLA-4 expression has also been reported on intratumoral ILC1 subsets in mice [282]. Although TIM-3 has been detected on ILC3s from human decidua during pregnancy and inhibiting IL-22 production [283], currently very limited information is known regarding TIM-3 expression on helper ILCs in the context of cancer. Transcriptomic and immunophenotyping analyses in mouse and human cSCCs identified infiltration of functionally impaired NK and ILC1 cells characterized by reduced cytotoxicity and IFN-γ secretion, which correlated with decreased expression of activating receptors and increased expression of exhaustion markers including TIGIT on NK cells, and PD-1 and TIM-3 on ILC1s [284]. LAG-3 expression has not been reported in resting ILCs, but in the context of cancer and upon TGF-β-mediated conversion of NK to ILC1 cells, several checkpoint receptors were upregulated, among which LAG-3, TIGIT and CD96 expression has been documented [282]. The functional effects of blocking these receptors on helper ILCs remain to be determined.

9. Concluding remarks

Profiling the immune cells in the TME of patients with evolving techniques has revealed significant information regarding the immune cell composition of the TME in experimental animal models and patients with various types of cancer. It is increasingly understood that the immune cellular components of the TME, including DCs, TAMs and MDSCs have decisive roles in regulating cancer evolution and immune escape but also the outcomes of cancer therapy including chemotherapy, checkpoint immunotherapy and cancer vaccines. Although in healthy tissues these myeloid cells provide defense against insults mediated by pathogens, in the TME these cells lose their protective immune functions and convert to pro-tumorigenic mediators that support cancer growth and metastasis. Via such changes, myeloid cell populations fail to recruit T cells, present tumor antigens and mediate anti-tumor T cell responses. Instead, these myeloid cells suppress T cell activation by blunting recognition of tumor antigens, eliminating engagement of costimulatory pathways, upregulating the expression of inhibitory receptors and their ligands, and producing inhibitory soluble mediators thereby creating an immunosuppressive milieu. Engagement of inhibitory receptors on T cells of the TME impairs signaling events mediated by T cell receptor and costimulatory pathways and promotes generation of T_{EX} tumor-specific cells that are unable to mount anti-tumor responses. The TME also promotes differentiation of iTreg by T cell receptor engagement by tumor antigens and concomitant ligation of

coinhibitory receptors, and production of Treg-promoting soluble factors such as TGF-β and IL-10. Together these mechanisms abrogate cancer immunosurveillance and support cancer evolution and immune escape. Although checkpoint blockade and cell-based therapies have achieved significant progress, challenges ahead include advancing the outcome of immunotherapy by targeting changes of the TME that compromise the function of key immune populations and preclude their ability to recognize and respond to tumor antigens. Such tentative targets for intervention include metabolic alterations, vasculature abnormalities and soluble inhibitors produced in the TME. Such efforts might repurpose available drugs previously used in the clinic, which can be administered together or sequentially with immunomodulating immunotherapies and cell-based therapies. In doing so, an additional challenge will be to achieve cancer elimination while preserving self-tolerance and preventing autoimmunity.

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Abbreviations:

HLA	human leukocyte antigens
IDO	indoleamine 2,3-dioxygenase
VEGF	vascular endothelial growth factor
TGF-β	transforming growth factor-β
PD-1	programmed cell death protein 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
RORγt	retinoic acid-related orphan receptor γ
FLT3L	FMS-like tyrosine kinase 3 ligand
IRF4	interferon regulatory factor 4
CXCL9	CXC chemokine ligand 9
CXCL10	CXC chemokine ligand 10
CXCL11	CXC chemokine ligand 11
CCL2	C-C motif chemokine ligand 2
CCL5	C–C motif chemokine ligand 5
XCL1	X-C motif chemokine ligand 1
COX1	cyclooxygenase 1
COX2	cyclooxygenase 2

CXCR3	CXC chemokine receptor 3
G-CSF	granulocyte colony-stimulating factor
PGE_2	prostaglandin E_2
CXCL1	CXC motif chemokine ligand 1
TNFα	tumor necrosis factor alpha
NF-κB	nuclear factor-kappaB
SHP-2	Src homology domain-containing phosphatase-2
TIM-3	T-cell immunoglobulin mucin-3
TLR3	Toll-like receptor 3
TLR7	Toll-like receptor 7
TLR9	Toll-like receptor 9
TIM-4	T-cell immunoglobulin mucin-4
IRF8	interferon regulatory factor 8
Th17	T helper 17 cell
MIP-1b	macrophage inflammatory protein-1β
CCL4	C–C motif chemokine ligand 4
RANTES	regulated on activation, normal T cell expressed and secreted
IFN-α	interferon alpha
ICOS-L	inducible costimulator ligand
AML	acute myeloid leukemia
VISTA	V-domain Ig suppressor of T-cell activation
mPGES1	microsomal prostaglandin E synthase-1
pSTAT1-IRF1	phosphorylated signal transducer and activator of transcription 1- interferon regulatory factor 1
HIF-1a	hypoxia-inducible factor 1
CXCL12	CXC chemokine ligand 12
B7-H4	B7 homolog 4
SREBP1	sterol regulatory element-binding protein-1
PARP	poly adenosine diphosphate-ribose polymerase

Arg-1	arginase 1
iNOS	inducible nitric oxide synthase
GBM	glioblastoma multiform
LAG-3	lymphocyte activation gene-3
VSIG-3	V-set and immunoglobulin domain containing 3
PSGL-1	P-selectin glycoprotein ligand-1
GITR	glucocorticoid-induced tumor necrosis factor receptor family-related receptor
mTOR	mammalian target of rapamycin
CCR7	C-C chemokine receptor type 7
VHL	von Hippel Lindau
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domain
HCMV	human cytomegalovirus
AOM/DSS	azoxymethane/dextran sodium sulfate
PDAC	pancreatic ductal adenocarcinoma
cSCCs	cutaneous squamous cell carcinomas

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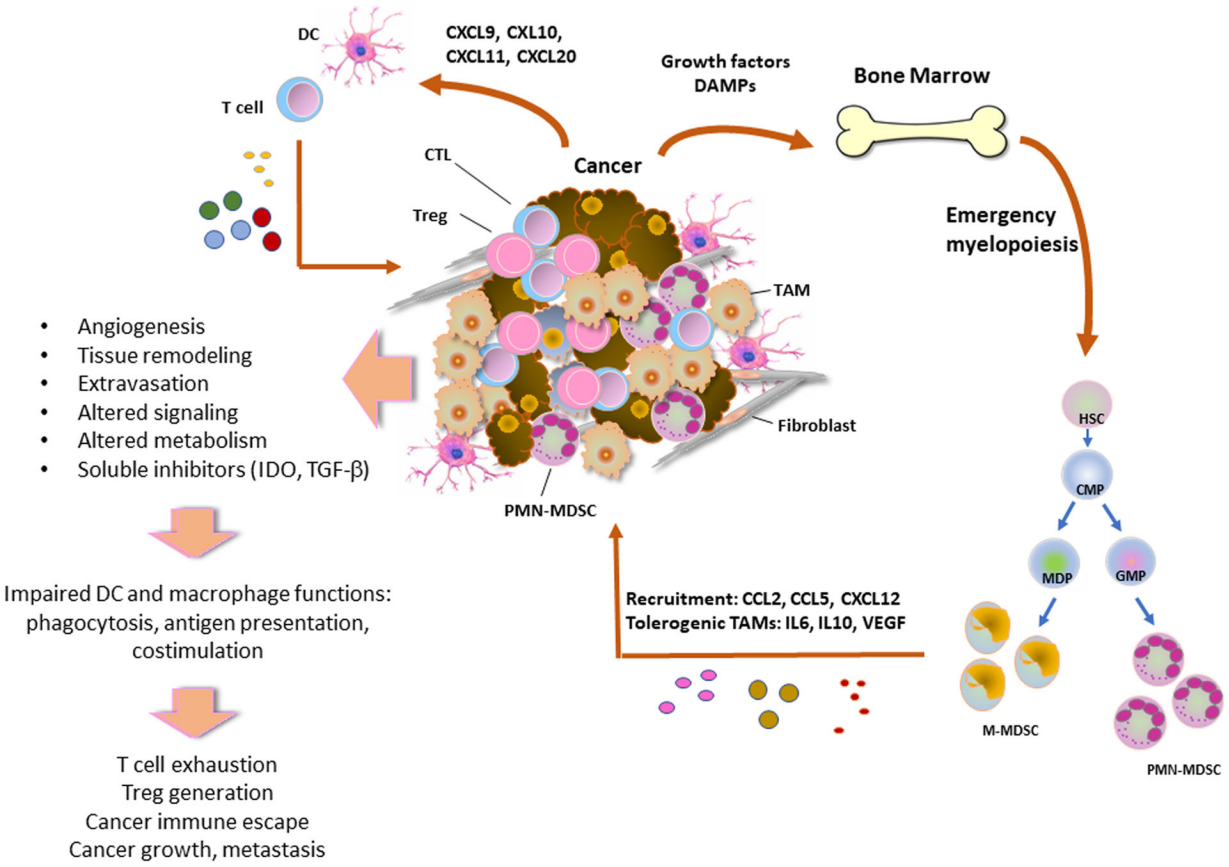

Fig. 1.

During tumorigenesis, growth factors, such as M-CSF and GM-CSF, and damage-associated molecular patterns (DAMPs) produced by cancer cells due to rapid replication and apoptosis, act on bone marrow myeloid progenitors inducing emergency myelopoiesis and output of immature myelosuppressive PMN-MDSCs and M-MDSCs, which are recruited to the tumor via chemokines such as CCL2, CCL5 and CXCL12. In the TME, PMN-MDSCs and M-MDSCs directly induce immunosuppression, whereas M-MDSCs are also converted into tolerogenic and pro-tumorigenic TAMs by the function of soluble factors such as IL-6, IL-10 and VEGF. TAMs lose the physiologic properties of macrophages such as phagocytosis and together with MDSCs and soluble factors produced in the TME suppress the antigen presenting function of DCs, leading to impaired activation of tumor-specific T cells and generation of Treg cells. These orchestrated changes in the properties of immune cells promote cancer immune escape.

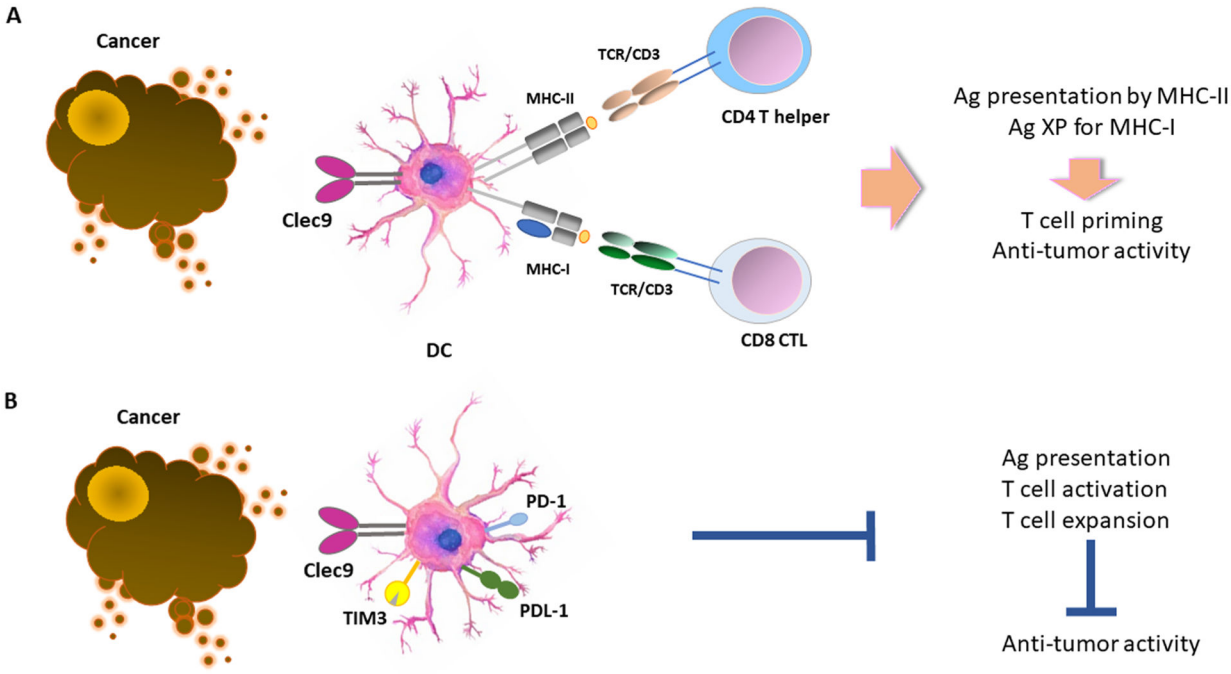

Fig. 2.

DCs, particularly the cDC1 subset, can capture and process tumor-associated antigens and present them to $CD4^+$ T cells by MHC-II molecules. cDC1 can also cross-present (XP) tumor-associated antigens to $CD8^+$ T cells by MHC-I molecules (A). Expression of checkpoint inhibitors such PD-1, PD-L1 and TIM-3, compromise these functions leading to impaired T cell activation, T cell expansion and anti-tumor immunity.

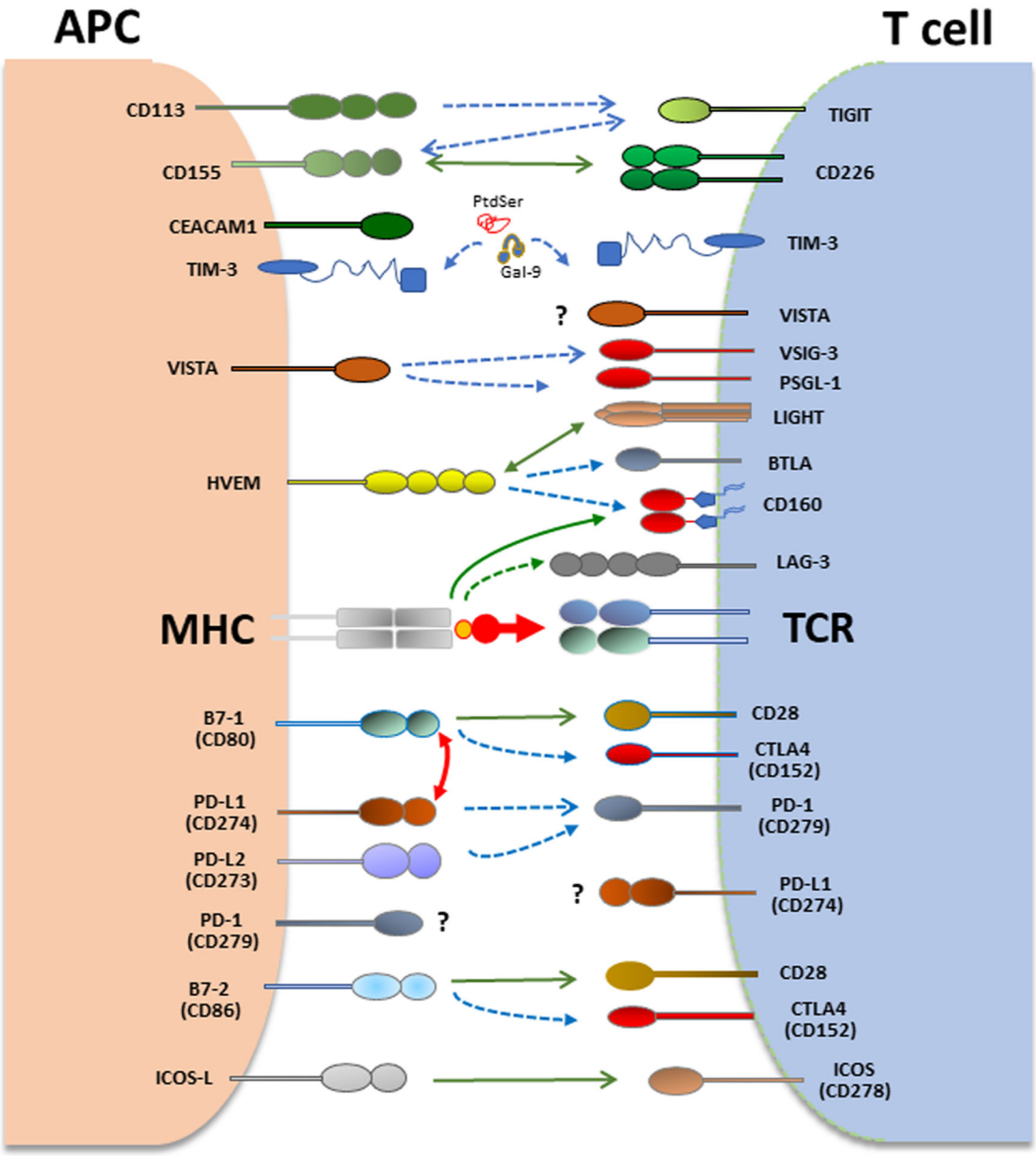

Fig. 3.

Coinhibitory pathways in T cells and APC. T cell activation is initiated by recognition of antigens presented by antigen-presenting cells (APCs) to the T cell receptor (TCR)/CD3 complex. CD28 is the prototype costimulatory receptor in T cells and interacts with CD80 and CD86. Many coinhibitory receptors are upregulated upon T cell activation and can attenuate TCR and costimulatory signals. CTLA4 and PD-1 are the prototype co-inhibitory receptors expressed in T cells. CTLA-4 interacts with B7–1 (CD80) and B7–2 (CD86) whereas PD-1 (CD279) interacts with PD-L1 (CD274) and PD-L2 (CD273) to inhibit T cell responses. In addition to canonical interaction of PD-L1 with PD-1 in *trans*, PD-L1 interacts with B7–1 (CD80) *in cis*, when co-expressed on the same APC leading to diminished availability of PD-L1 for canonical interaction with PD-1 in *trans*. VISTA, TIM-3 and PD-1

are expressed both in T cells and APC and can regulate immune responses by altering the properties of myeloid cells and T cells.

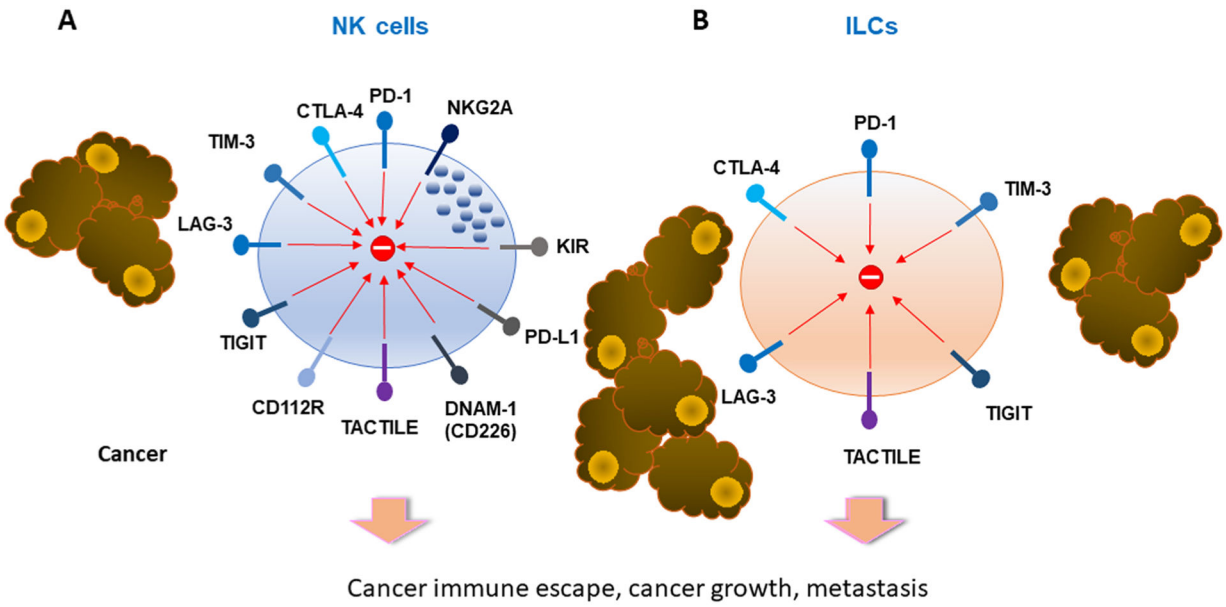

Fig. 4.

NK cells and ILCs express multiple inhibitory receptors. NK cells express the classical inhibitory receptors KIR and NKG2A. Both NK cells and ILC express multiple checkpoint inhibitors. These receptors and their ligands offer new therapeutic opportunities to improve NK cell functions for cancer immunotherapy.