NK-MYELOID CELL INTERACTIONS IN THE TUMOR MICROENVIRONMENT: IMPLICATIONS FOR CANCER IMMUNOTHERAPY

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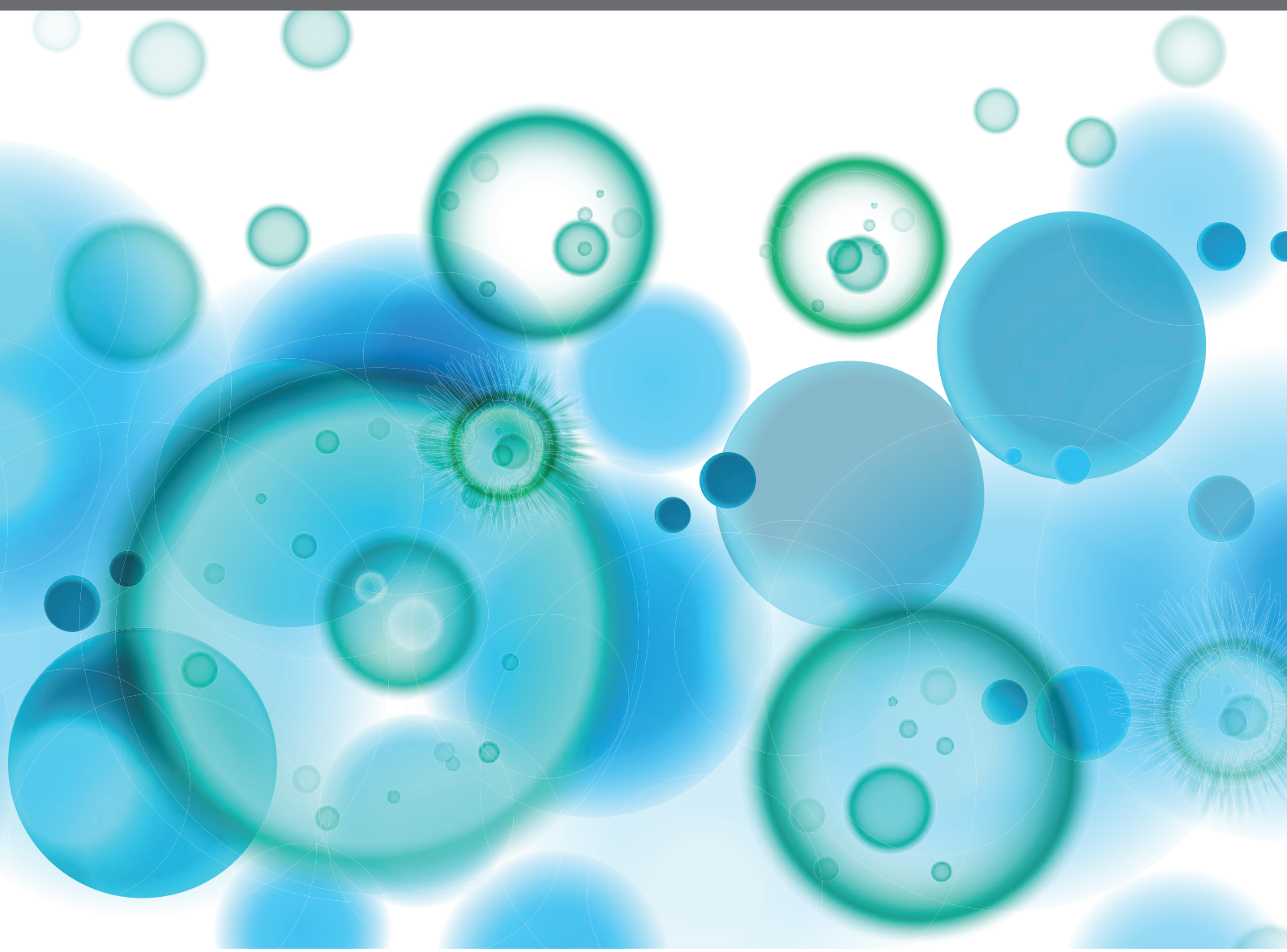

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NK-MYELOID CELL INTERACTIONS IN THE TUMOR MICROENVIRONMENT: IMPLICATIONS FOR CANCER IMMUNOTHERAPY

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EDITORIAL
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Editorial: NK-Myeloid Cell Interactions in the Tumor Microenvironment: Implications for Cancer Immunotherapy

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Keywords: NK cells, myeloid cells, dendritic cells, MDSCs (myeloid-derived suppressor cells), cancer immunotherapy, platelets, cytokines

Editorial on the Research Topic

NK-Myeloid Cell Interactions in the Tumor Microenvironment: Implications for Cancer Immunotherapy

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Over the last decades, immunotherapy has revolutionized cancer treatment. The emergence of immune checkpoint inhibitors (ICI) targeting PD-1 and CTLA-4, has rendered many aggressive cancers treatable and even curable. However, ICI often fails to generate sustained responses, particularly in patients with low or absent pre-existing T cell immunity. In order to turn these "cold" tumors "hot", initiation of *de novo* tumor-specific immune responses is required, a process which is dependent on the actions of innate immune cells, and whose infiltration and function is highly impacted by the composition of the tumor microenvironment (TME).

Natural killer (NK) cells are innate lymphoid cells that play essential roles in cancer immunosurveillance and anti-tumor immunity due to their unique ability to identify and kill tumor cells by recognizing missing-self and induced stress ligands. The cytolytic potential of NK cells can be harnessed for treatment of advanced cancers through adoptive cell transfer therapies and by augmenting their function and persistence *in vivo*. Recently discovered bi-directional crosstalk between NK cells and certain subsets of dendritic cells (DCs), which are the key orchestrators of initiating, maintaining and regulating anti-tumor immunity, has shed new light on the role of NK cells in shaping adaptive immune responses in cancer. Importantly, emerging evidence of how NK cell function is suppressed by myeloid cells in the TME, warrants a re-examination of how cancers evolve to specifically evade NK cell killing *via* recruitment and polarization of tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs).

Here, we present a collection of reviews, mini-reviews and original research articles which discern the interactions between NK cells and myeloid cell subsets, how their crosstalk regulates the anti-tumor immune response, and how it can be manipulated to potentiate cancer immunotherapies.

NK-DC INTERACTIONS

Recent work has highlighted that NK cells and DCs, and in particular conventional type 1 DC, (cDC1) engage in an intercellular crosstalk to coordinate adaptive immunity against cancer. Furthermore, this crosstalk has also been linked to increased survival and responses to anti-PD-1 immunotherapy in patients with metastatic melanoma. In this edition, Peterson and Barry review recent findings on the role of NK cells and cDC1s in protective immune responses to cancer and immunotherapy, as well as current therapies targeting this NK cell–cDC1 axis. In addition, Bödder et al. discuss the function of cDC1s and NK cells, their bidirectional crosstalk and potential strategies to improve cancer immunotherapy. In the TME, different DC subsets can display tumor-promoting or -inhibiting characteristics. To this end, Jacobs et al. discuss recent findings on the interaction of DCs and NK cells under physiological conditions and within the TME. In addition, they discuss potential strategies to overcome the immunosuppressive outcome of the DCs and NK cells interaction within the TME.

NK-PLATELET INTERACTIONS

Emerging evidence reveal that the interaction between platelets and tumor cells in peripheral blood is an important factor to support dissemination of cancer. Platelets can also produce high levels of TGF-β to support an immunosuppressive TME. Schmied et al. review the interplay between platelets and NK cells in solid tumors and also discuss how these could apply to hematological malignancies. They furthermore explore the possible implications for NK cell therapy in patients with solid tumors and patients who depend on frequent platelet transfusions. The activating receptor NKG2D induces NK cell-mediated killing of tumor cells by recognition of stress-induced ligands. Platelets can shield tumor cells directly from engagement of NK cell-mediated attack by cleaving such stress-induced ligands from tumor cells. To this end, Maurer and de Andrade discuss the underlying mechanisms of NK cell control by platelets with a focus on NKG2D and its ligands, providing a perspective to exploit these pathways with novel immunotherapeutic approaches.

NK-MDSC INTERACTIONS

MDSC is a heterogeneous population that contains a range of immature cells with potent inhibitory functions against anti-tumor immunity. MDSC can be divided broadly into monocytic and polymorphonuclear (PMN) subsets according to the lineage origin and cell morphology. A wealth of literature evidence demonstrates that MDSC can regulate NK cell function in health and disease. In this special issue, Pelosi et al. investigated the effects of G-CSF mobilization treatment on NK cells and PMN-MDSC in healthy individuals. The authors demonstrated that G-CSF mobilization reduced the cytolytic functions of NK cells against a leukemia cell line. Marked changes of gene expression profiles were observed in NK cells and PMN-MDSC isolated from G-CSF mobilized individuals, as compared to the non-mobilized counterparts. PMN-MDSC showed prolonged survival and better migration in response to chemo-attractants. This study provides an important data source to dissect the immune regulatory effects of G-CSF mobilization during leukemia treatment. In a separate study, Gallazzi et al. reported elevated exhaustion markers on peripheral NK cells from prostate cancer patients, as compared to the healthy controls. Similar changes were observed when NK cells were treated with TGF-β or IL-6. Using *in vitro* co-culture models, the authors showed that these patient-derived NK cells could activate the inflammatory responses in endothelial cells and reshape the functions of monocytes and macrophages. Altogether, this paper highlights the functional interactions between NK cells and other cell compartments in the prostate cancer microenvironment.

NK CELL CROSSTALK IN CANCER THERAPY

Adoptive NK cell transfer for treating hematological malignancies led to promising results and was followed by a substantial number of studies in solid tumors. Genetically modified NK cells carrying tumor antigen-targeting chimeric antigen receptors (CARs) are currently under clinical investigation. Overall, clinical efficacy has been limited in particular in solid tumors thus highlighting the need to better understand the hurdles within the permissive TME. Myeloid cells are characterized by an exceptional plasticity and represent a key cell population within the TME. They have been shown to readily obtain tumor-promoting properties as M2-type macrophages, TAMs, and MDSCs. Zalfa and Paust comprehensively describe the mechanisms leading to MDSC accumulation (e.g., induction by cytokines or chemokine-mediated recruitment) in cancer and how those heterogeneic cells interact with NK cells thereby limiting their anti-tumor activity. Finally, the authors offer an extensive overview on strategies to interfere with MDSCs and to thereby harness the efficacy of NK cell-based immunotherapies. Tumino et al. look at the interaction of NK cells and MDSCs from the perspective of the allogeneic hematopoietic stem cell transplantations (allo-HSCTs). In fact, allo-HSCT remains one of the most successful immunotherapies and is for some malignant diseases the only therapy with a curative potential. The therapeutic success is based on the so-called graft versus leukemia (GvL) effect and donor NK cells are important mediators of the GvL. The authors explain how reconstituting monocytic or neutrophilic MDSCs counteract the NK cells' GvL activity especially during the early post-transplant period. Furthermore, they describe pharmacological interventions that could reduce the immunosuppressive activity of MDSCs thus improving the outcome of allo-HSCTs. Carnevalli et al. focus in their review on current and future therapeutic approaches to target the detrimental interaction between myeloid cells (including

MDSCs and tolerogenic macrophages) and (anti-tumor) NK cells. The strategies that are introduced comprise amongst others blockade of inhibitory checkpoints (on myeloid cells), triggering of co-stimulatory receptors (on NK cells), application of myeloid-cell inhibitors (e.g., CSF1R inhibitors), and interference with intracellular myeloid cell signaling (e.g., STAT3). Finally, Gaggero et al. give an overview on cytokines that antagonize (e.g., TGF-β) and that promote (e.g., IL-15) NK-cell function. They emphasize how remodeling of the TME cytokine milieu could skew the balance between anti-tumor reactivity and tumor tolerance towards the former. They also speculate about the generation of genetically engineered CAR NK cells that carry cytokine signaling elements or dominant negative cytokine receptors and are therefore resistant towards the TME, which would ensure their longevity and persistence.

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

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MINI REVIEW
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NK Cell Interaction With Platelets and Myeloid Cells in the Tumor Milieu

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Natural killer (NK) cells recognize and kill tumor cells *via* germ-line encoded receptors and polarized degranulation of cytotoxic molecules, respectively. As such, NK cells help to inhibit the development of cancers. The activating receptor NKG2D induces NK cell-mediated killing of metastasizing tumor cells by recognition of the stress-induced ligands MICA, MICB, and ULBP1-6. However, platelets enable escape from this immune surveillance mechanism by obstructing the interactions between NK cells and tumor cells or by cleaving the stress-induced ligands. It is also being increasingly appreciated that NK cells play additional roles in cancer immunity, including chemokine-mediated recruitment of antigen presenting cells in the tumor microenvironment that is followed by generation of adaptive immunity. However, the NK cell interplays with dendritic cells, and macrophages are extremely complex and involve molecular interactions *via* NKG2D and cytokine receptors. Specifically, NKG2D-mediated chronic interaction between NK cells and tumor-infiltrating macrophages causes immune suppression by differentiating NK cells toward a dysfunctional state. Here we discuss the underlying mechanisms of NK cell control by platelets and myeloid cells with focus on NKG2D and its ligands, and provide a timely perspective on how to harness these pathways with novel immunotherapeutic approaches.

Keywords: NK cells, platelets, myeloid cells, NKG2D, proteolytic shedding

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INTRODUCTION

Natural Killer (NK) cells are innate lymphocytes that recognize and kill abnormal cells, such as tumor cells and cells infected by viruses (1). NK cell activation is guided by "missing self" and "induced self" recognition of abnormal cells. "Missing self" implies that NK cells kill target cells with low/absent major histocompatibility complex (MHC) class I expression (2). "Induced self" is the expression of ligands for activating NK receptors, such as NK group 2D (NKG2D) that promotes NK cell-mediated cytotoxicity (3). NKG2D recognizes proteins upregulated by cells in response to stress, such as DNA damage, hypoxia or accumulation of unfolded proteins (4). NKG2D is present in both mice and humans, but the ligands are distinct between the two species. The human NKG2D ligands (NKG2DL) comprise MHC class I polypeptide-related sequence A (MICA), MHC class I polypeptide-related sequence B (MICB), and the six different types of

UL16-binding proteins (ULBP1-6). In contrast, the murine NKG2DL are retinoic acid early inducible-1 (Rae-1) α-ϵ proteins, murine UL16-binding protein-like transcript 1 (MULT1), and H60a-c proteins (5).

NKG2D-mediated recognition of malignant cells is a major mechanism of cancer immunosurveillance (6, 7). However, as discussed in more details below, malignant cells frequently evade the recognition by NK cells via, for example, abnormal interactions with platelets. Furthermore, myeloid cells express NKG2DL in response to specific types of stimulations and NKG2D drives the interactions between NK cells and myeloid cells. Although the importance of NKG2DL on tumor cells for NK cell-driven immunity is well appreciated, how platelets and myeloid cells affect NK cell functions represent new and paradigm-shifting research fields. Here we discuss some of the recent and impactful studies that substantiate the importance of platelets and myeloid cells for effective NK cell-driven antitumor immunity.

PLATELETS IN-BETWEEN TUMOR CELLS AND NK CELLS

Malignant tumors frequently use the blood stream to metastasize (8). However, the blood is a hostile environment because it is highly enriched in NK cells (9). To bypass them, metastatic cells take advantage of platelets, which are small non-nuclear, megakaryocyte-derived cell fragments of the myeloid lineage that, in normal conditions, form clots to stop bleedings upon injury (10). Metastatic cells mimic injury-related clots by activating and forming hetero-aggregates with platelets. These unusual cellular clusters frequently cause thrombophlebitis, which is a medical term for inflammatory blood clots that block veins, and Trousseau's syndrome, i.e., recurring thrombophlebitis (11). This, together with the fact that thrombocytosis (enhanced platelet count) associates with poor outcome in several solid tumor entities, suggests a causal relationship between the coagulation system and malignant dissemination (12, 13). In the following two sub-sections, we review some of the cellular and molecular mechanisms underlying the platelet-mediated immune escape of metastatic cells.

Platelets Shield Metastatic Cells and Enable Immune Escape

Depletion of platelets directly inhibits metastases in the lungs of immunocompetent mice upon intravenous inoculation of cell lines of fibrosarcoma, melanoma, and lymphoma, whereas this effect is reversed upon additional depletion of NK cells. This indicates that platelets guard circulating tumor cells from NK cell immunosurveillance (14). Platelet activation and aggregation are mainly mediated by G-protein-coupled receptors, which upon stimulation by their respective ligands transduce intracellular signals by activating heterotrimeric G proteins (15). Platelets lacking Gαq, a subunit of heterotrimeric G proteins, are irresponsive to adenosine diphosphate, thrombin, collagen, and thromboxane, which are well known clot-forming stimuli (16). Gαq-deficient mice display substantially lower levels of metastases in the lungs after being intravenously inoculated with melanoma and lung cancer cells, and this protection was dependent on NK cells (17). Therefore, platelets contribute to dissemination of metastases. These studies predominantly utilized experimental metastasis models, with injection of tumor cells directly into the blood circulations. Further studies are needed to clarify the potential role of platelets in the context of spontaneous metastases, which also address the early steps of dissemination during tumor cell intravasation into the blood stream.

The molecular mechanisms relevant for platelet-mediated immune escape are only partially understood. Palumbo and colleagues reported that tumor cells evade NK cell-mediated surveillance *via* fibrin deposition, which enhances platelet aggregation on the tumor cell surface (17). Furthermore, the aggregated platelets transfer MHC class I molecules to tumor cells (18). MHC class I is frequently downregulated on tumor cells to evade T cell immunity, which in contrast enables recognition by NK cells *via* the 'missing self' mechanism (19). Surface expression of platelet-derived MHC class I complexes inhibits NK cell antitumor reactivity (20). Since platelet-derived MHC class I molecules present self-antigens, they do not induce T cell responses against metastatic cells. This intriguing mechanism of immune escape has been confirmed by a study by Placke and colleagues, who found in an *in vitro* model using shear stress that platelet-derived human leukocyte antigen A variant 2 (HLA-A*02) is transferred from platelets to tumor cells *via* trogocytosis. Therefore, platelets interfere with the "missing self" recognition of metastatic cells and dampen NK cell-driven anti-tumor immunity *via* pseudo-expression of "non-malignant" MHC class I.

Trogocytosis is frequently observed between physically interacting cells. For example, MICA/B and ULBP1-3 can be transferred from the target cell surface to NK cells at the immunological synapse (21–23). Surface molecules from antigen presenting cells are also transferred to T cells in the immunological synapses (24). Since platelets physically interact with metastasizing cells, it is possible that a plethora of other molecules with putative or confirmed roles in modulating NK reactivity can also be transferred to tumor cells in addition to MHC class I molecules (25–27).

Platelets Promote the Shedding of NKG2D Ligands by Tumor Cells

High levels of NKG2DL tip the balance toward NK cell activation (28, 29). However, certain ligands are subjected to proteolytic cleavage, which interferes with NKG2D recognition. It is well known that tumor cells cleave their own NKG2DL *via* expression of 'a disintegrin and metalloproteinase domain-containing protein' (ADAM) 10 and ADAM17 (5, 30, 31). Interestingly, recent studies also suggested platelet-mediated cleavage of NKG2DL since platelets express both proteases (32, 33) that mediate NKG2DL shedding on tumor cells (34–37). We recently

discovered that tumor cell-associated NKG2DL, predominantly MICA and MICB, were cleaved following interaction with platelets or platelet releasate. We also demonstrate that platelet-mediated shedding of NKG2DL dampens NK cell antitumor immunity by reducing the activating signals. Of note, expressions of both proteolytic enzymes are increased on platelets from patients with non-small cell lung cancer, thus suggesting that cancer patients-derived platelets have enhanced proteolytic cleavage capacity (38). Furthermore, platelets express NKG2DL, in particular ULBP2, which may be released as soluble form (39). The biological activity of soluble ULBP2 is not well known, but ULBP2 shedding may also inhibit recognition of platelet-tumor aggregates by NKG2D. Altogether, platelets modulate the expression and release of NKG2DL and thereby inhibit NKG2D-mediated NK cell recognition of abnormal cells (**Figure 1A**).

Platelets also inhibit NK cells by inducing the NKG2D, downregulation, thus hindering "induced self" recognition. Upon stimulation with agonists or interaction with tumor cells, platelets release a variety of factors, the collectivity of which is herein referred to as "releasate". Platelet releasate includes large amounts of active transforming growth factor β (TGF-β) (40). TGF-β impairs NK effector function by downregulating NKG2D, as determined with samples from cancer patients (41–43). Salih and colleagues have also demonstrated that TGF-β is released by platelets upon interaction with tumor cells, and it impairs NK cell cytotoxicity and production of interferon-γ *via* NKG2D downregulation (44). Others reported that TGF-β directly inhibits expression of NKG2DL on solid tumors (45, 46). Whether this is also true for platelet-derived TGF-β is yet to be shown. Altogether, platelets promote dissemination of metastases by obstructing NK cells and producing immunosuppressive molecules (**Figure 1A**).

THE INTERACTION BETWEEN NK CELLS AND MYELOID CELLS

The antitumor immunity is a coordinated interplay of multiple leukocyte populations, including NK cells and myeloid cells (e.g. dendritic cells, macrophages, etc) (47). NK cells can recruit (see below) and interact with myeloid cells *via* chemokines and cytokines, respectively. For example, interleukin-15 is a well-known cytokine produced by dendritic cells (DC) that stimulates NK cells (48). Interestingly, myeloid cells can also express NKG2DL, but they are not killed by NK cells. As discussed below, the NKG2D-driven interplays between NK cells and myeloid cells cause profound impacts on the antitumor immunity by dictating the functionality of NK cells. Here we review some of the key and paradigm shifting studies that revealed several mechanisms of interactions between NK cells and myeloid cells.

NK Cell-Mediated Recruitment of Dendritic Cells Into Tumors

The effector functions of NK cells are not restricted to cytotoxicity. As revealed by gene expression analyses, specialized NK cell populations infiltrate into melanoma metastases. Although blood and tumor-infiltrating NK cells express predominantly perforin and granzyme genes that are related to cytotoxicity, tumor-infiltrating NK cells upregulate *XCL1*, *XCL2*, *CCL3*, *CCL4*, *CCL4L2*, and *CCL5* that are chemokine genes. Tumor-infiltrating NK cells differentiate into two main populations with distinct profiles of chemokine gene expression: $XCL1^{+}$ $XCL2^{+}$ NK cells and $CCL3^{+}$ $CCL4^{+}$ $CCL4L2^{+}$ $CCL5^{+}$ NK cells. The first NK cell population also upregulates *TIGIT*, an inhibitory receptor, whereas the later one also upregulates *IL7R*, a cytokine receptor gene (49).

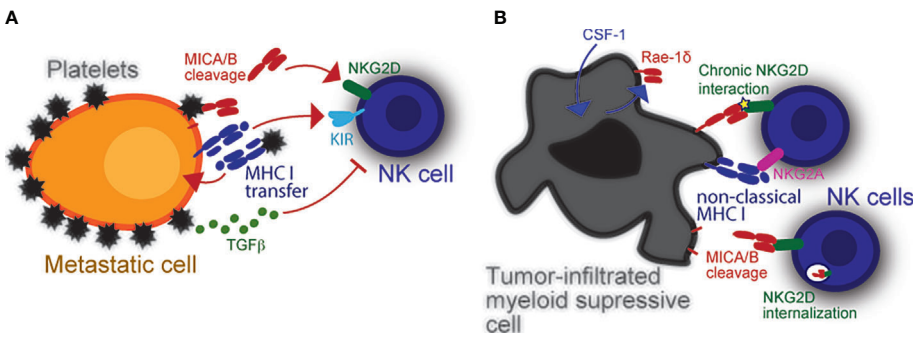

FIGURE 1 | Modulation of NK cell reactivity by platelets and myeloid cells. **(A)** Platelets obstruct NK cells and enable escape of metastasizing tumor cells. Platelets also provide specific immune modulatory molecules like MHC class I which inhibits NK cells. The latter can be transferred into the tumor cell membrane *via* trogocytosis to inhibit missing self-driven NK cell cytotoxicity. Their cognate Killer-cell immunoglobulin-like receptors (KIRs) inhibit NK antitumor responses upon stimulation. Platelets can also dampen induced self recognition of tumor cells *via* NKG2D. Platelet-derived metalloproteases (i.e., ADAM10 and ADAM17) cleave NKG2DL from the tumor cell surface. Platelet-released TGF-β also causes NKG2D downregulation, thereby further hindering NK cell antitumor response. **(B)** The expression of NKG2DL is not restricted to malignant cells. In fact, DC and macrophages can also express NKG2DL upon stimulation or infection, which in-turn induces NK cells to proliferate and produce interferon-γ (IFNG). Virus-infected myeloid cells may become targets and be killed by NK cells upon NKG2D recognition. Intratumoral myeloid cells also express NKG2DL. It is currently unknown what induces NKG2DL expression in intratumoral myeloid cells, yet a potential mechanism is *via* cellular stress inside the hypoxic tumor microenvironment. These cells benefit tumors by inhibiting NK cells *via* chronic NKG2D interaction with low affinity ligands, which cause NKG2D internalization.

Conventional type-1 dendritic cells (cDC1) express XCR1, which is the receptor for XCL1. This DC population takes up particles of dead tumor cells, migrates to draining lymph nodes, and presents tumor cell-derived antigens to $CD8^+$ T cells (50). The XCR1 – XCL1 axis promotes recruitment of cDC1 into tumors and promotes adaptive immunity against cancers. CCL5 also contributes to cDC1 recruitment into tumors. In a mouse $BRAF^{V600E}$-mutant melanoma model, intratumoral NK cells upregulate *Xcl1* and *Ccl5* whereas NK cell depletion lowered the numbers of tumor-infiltrating cDC1. Antibody-mediated blockade of XCL1 and CCL5 also inhibited tumor-infiltrating cDC1, thus leading to the conclusion that intratumoral NK cells produce XCL1 and CCL5 which in turn recruit cDC1 (51). Another study also showed that NK cell depletion inhibits the cDC1 infiltration into mouse B16F10 melanoma tumors, whereas melanoma patients who responded to checkpoint blockade immunotherapy have higher levels of intratumoral NK cells (52). Therefore, the NK cell-mediated recruitment of cDC1 is an additional role, beyond cytotoxicity, played by NK cells in the antitumor immunity.

The Interaction Between NK Cells and NKG2DL$^+$ Dendritic Cells Has Two Distinct Outcomes: Activation or Inhibition

Myeloid cells express NKG2DL in response to certain stimuli. For example, interferon-α triggers MICA expression on human monocyte-derived DC, which in turn promotes NK cell-mediated cytotoxicity against K562 myeloid leukemia cells *in vitro*. A MICA antibody that blocks interaction with NKG2D consequently inhibits DC stimulation of NK cells (53). LPS, Poly I:C, and virus infections (e.g. measles virus, influenza virus) also trigger the expression of several ULBP molecules and MICB on DC. Virus-infected DC promote, in a NKG2D-dependent manner, NK cell proliferation and trigger interferon-γ production (54). However, the NKG2D-driven crosstalk with DC does not always benefit NK cells. To study the interaction between NK cells and DC *via* NKG2D *in vivo*, Morvan et al. developed a mouse strain in which Rae-1ϵ, a murine NKG2DL, is constitutively expressed by $CD11c^+$ DC. Although the numbers of NK cells are apparently normal in these mice, these NK cells downregulate NKG2D. NK cells from CD11c-Rae-1ϵ mice allowed the expansion of Rae-1ϵ -expressing splenocytes in a model of NKG2D-dependent lysis of target cells *in vivo* (55). Therefore, NKG2D-driven chronic interaction with DC inhibits NK cells whereas the expression of NKG2DL on DC in response to acute infection and interferon-α promotes NK cell functions.

NK Cells Downregulate NKG2D Upon Interaction With NKG2DL+ Macrophages

Macrophages also express Rae-1α, Rae-1β, and Rae-1γ upon treatment with LPS, poly I:C, or E. coli. Rae-1δ and Rae-1ϵ were also expressed by murine macrophages that were treated with LPS, but were not expressed by macrophages from *Myd88*$^{-/-}$ mice. MyD88 is an adaptor molecule for toll like receptors (TLR), thus suggesting that the upregulation of NKG2DL by macrophages is downstream of TLR signaling. Of note, NK cells co-cultured with Rae-1^+ macrophages downregulate the surface expression of NKG2D (56). However, macrophages upregulate Qa-1 to protect themselves from NK cell-mediated attack. Qa-1 binds to the NK group 2A receptor, which inhibits NK cells (57). Furthermore, intratumoral macrophages also express Rae-1δ in response to tumor-derived colony-stimulating factor-1, and Rae-$1\delta^+$ macrophages caused NKG2D downregulation upon co-culture with NK cells (58).

Human macrophages upregulate MICA upon treatment with LPS or CL097, which are TLR4 and TLR7/8 agonists, respectively. CL097 also triggers the expression of MICB (59). Even monocytes express MICA in response to LPS or poly I:C. These $MICA^+$ monocytes are not lysed by NK cells, but promote the interferon-γ production. A MICA antibody partially inhibited the interferon-γ production by NK cells; this effect was partial likely because LPS also triggers the expression of interleukin-12 that potently promotes interferon-γ production by NK cells. The $MICA^+$ monocytes also caused a modest NKG2D downregulation on the surface of NK cells (60). However, an independent study did not observe NKG2D downregulation on NK cells that were co-cultured with LPS-treated monocytes, macrophages, or DC. This work confirmed that LPS triggers MICA expression in macrophages and found that these cells also express ULBP3. NK cells lysed the LPS-treated macrophages in a NKG2D-dependent manner (61).

MULT1 is an intriguing NKG2DL that has unique properties. A recent study showed that B16F10 melanoma cells engineered to secrete a truncated MULT1 protein, which lacks the transmembrane domain, form small subcutaneous tumors following inoculation into C57BL/6 mice. NKG2D was upregulated on intratumoral NK cells, whereas NK cell depletion with anti-NK1.1 enabled the growth of B16F10 tumors secreting MULT1. Soluble MULT1 interfered with NKG2D – Rae-1 interactions between intratumoral NK cells and myeloid cells, likely because MULT1 has higher affinity to NKG2D compared to Rae-1. Therefore, soluble MULT1 displaces the NKG2D – Rae-1 chronic interaction and consequently restores NK cells (62).

NKG2D downregulation upon chronic interaction was observed in several of the studies cited above and is frequently assumed to represent a mechanism of immune suppression. However, NKG2D is also internalized upon ligand interaction at the immunological synapse between NK cells and myeloid cells. This enables physical approximation between NKG2D and its associated intracellular signaling pathways that trigger NK cell-mediated cytotoxicity (63). Therefore, NKG2D can be downregulated following the interactions between NK cells and myeloid cells also to enable intracellular signaling, which will ultimately dictate the NK cell functionality.

Myeloid Cells Shed MICA and MICB

The proteolytic shedding of MICA and MICB by tumor cells causes immune escape *via* downregulation of these NKG2DL (30, 35, 36, 64, 65). The shedding of MICA and MICB are multi-step processes that start with the removal of disulfide bonds in the MICA and MICB alpha-3 (α3) domains by disulfide

isomerases followed by cleavage by metalloproteases (64, 66). In contrast, monoclonal antibodies against the α3-domain inhibit the MICA and MICB shedding, while enabling NKG2D recognition and triggering antibody-dependent cellular cytotoxicity by NK cells. These antibodies inhibit the outgrowth of syngeneic melanoma in immunocompetent mice and human melanoma in NSG mice reconstituted with human NK cells (67). Even tumors with mutations associated with resistance to T cell checkpoint blockade were effectively treated by these α3-domain-specific antibodies (68). Of note, macrophages treated with acetylated low-density lipoproteins, an *in vitro* model of foam cells present in atherosclerotic lesions, upregulate MICA and MICB expression (69). The α3-domain-specific antibodies stabilized MICA and MICB on the surface of these macrophages (67). Therefore, macrophages, like tumor cells, proteolytically shed MICA and MICB.

In summary, NKG2D mediates the interplay between NK cells and myeloid cells. Myeloid cells upregulate NKG2DL in response to acute infection or detection of pathogen-associated molecular patterns, which in turn stimulate NK cells *via* NKG2D (**Figure 1B**). In contrast, intratumoral myeloid cells also upregulate NKG2DL in response to unknown stimuli, but NKG2DL$^+$ myeloid cells inhibit intratumoral NK cells. Macrophages shed MICA and MICB, but how MICA and MICB shedding by macrophages influences NK cells remains unknown. Therefore, the NKG2D-driven interaction between NK cells and myeloid cells is an intriguing research area that challenges current paradigms and offers opportunities to develop therapeutic approaches.

CONCLUSION AND PERSPECTIVE

NK cells are being increasingly appreciated and exploited by new immunotherapeutic modalities for cancers. New insights about how platelets and myeloid cells affect NK cells may help to develop cancer immunotherapies. We envision that platelets and myeloid cells can be harnessed to enable NK cell recognition of metastasizing cells and promote NK cell functions in the tumor environment, respectively, with novel therapeutic approaches. This may include targeting immune checkpoints involved in tumor cell - platelet - NK cell interaction or by already approved drugs (70). Beyond that, adaptive immunity may be promoted by mimicking the NK cell-mediated recruitment of cDC1s into tumors *via* local inoculation of XCL1 and CCL5. We here reviewed current knowledge on the cellular interplays of platelets - myeloid and NK cells with a focus on the NKG2D/NKG2DL system and provided a systematic overview on the rationale to target this axis, which may include but is not limited to prevention of NKG2DL shedding by a blocking antibody. While therapeutic targeting of these axes in the tumor microenvironment appears to be promising, further investigations are warranted to study the pathophysiologic role of the here summarized mechanisms in the context of different tissues, tumor entities and disease stages.

AUTHOR CONTRIBUTIONS

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ORIGINAL RESEARCH
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Prostate Cancer Peripheral Blood NK Cells Show Enhanced CD9, CD49a, CXCR4, CXCL8, MMP-9 Production and Secrete Monocyte-Recruiting and Polarizing Factors

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Natural killer (NK) cells, effector lymphocytes of the innate immunity, have been shown to be altered in several cancers, both at tissue and peripheral levels. We have shown that in Non-Small Cell Lung Cancer (NSCLC) and colon cancer, tumour associated circulating NK (TA-NK) and tumour infiltrating NK (TI-NK) exhibit pro-angiogenic phenotype/functions. However, there is still a lack of knowledge concerning the phenotype of peripheral blood (PB) NK (pNK) cells in prostate cancer (PCa). Here, we phenotypically and functionally characterized pNK from PCa patients (PCa TA-NKs) and investigated their interactions with endothelial cells and monocytes/macrophages. NK cell subset distribution in PB of PCa patients was investigated, by multicolor flow cytometry, for surface antigens expression. Protein arrays were performed to characterize the secretome on FACS-sorted pNK cells. Conditioned media (CM) from FACS-sorted PCa pTA-NKs were used to determine their ability to induce pro-inflammatory/pro-angiogenic phenotype/functions in endothelial cells, monocytes, and macrophages. CM from three different PCa (PC-3, DU-145, LNCaP) cell lines, were used to assess their effects on human NK cell polarization *in vitro*, by multicolor flow cytometry. We found that PCa pTA-NKs acquire the $CD56^{bright}CD9^{+}CD49a^{+}CXCR4^{+}$ phenotype, increased the expression of markers of exhaustion (PD-1, TIM-3) and are impaired in their degranulation capabilities. Similar effects were observed on healthy donor-derived pNK cells, exposed to conditioned media of three different PCa cell lines, together with increased production of pro-inflammatory chemokines/chemokine receptors CXCR4, CXCL8, CXCL12, reduced production of TNFα, IFNγ and Granzyme-B. PCa TA-NKs released factors able to support inflammatory angiogenesis in an *in vitro* model and increased the expression of CXCL8, ICAM-1, and VCAM-1 mRNA in endothelial cells. Secretome

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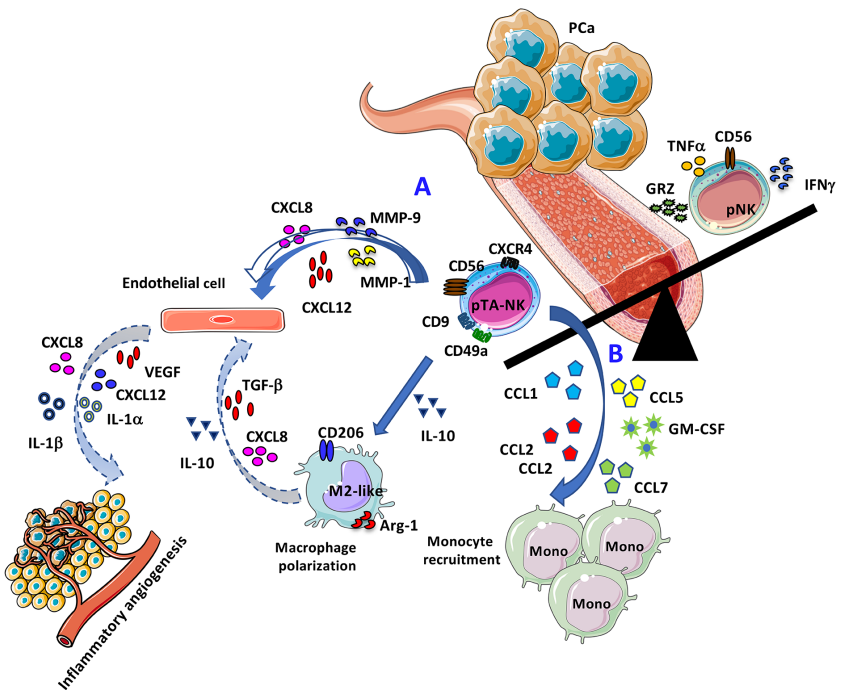
analysis revealed the ability of PCa TA-NKs to release pro-inflammatory cytokines/chemokines involved in monocyte recruitment and M2-like polarization. Finally, CMs from PCa pTA-NKs recruit THP-1 and peripheral blood CD14$^+$ monocyte and polarize THP-1 and peripheral blood CD14$^+$ monocyte-derived macrophage towards M2-like/TAM macrophages. Our results show that PCa pTA-NKs acquire properties related to the pro-inflammatory angiogenesis in endothelial cells, recruit monocytes and polarize macrophage to an M2-like type phenotype. Our data provides a rationale for a potential use of pNK profiling in PCa patients.

Keywords: natural killer cell, myeloid cells, monocytes, macrophages, immune cell polarization, inflammation, angiogenesis, prostate cancer



GRAPHICAL ABSTRACT | Representative cartoon illustrating the pro-angiogenic features of PCa pTA-NKs. **(A)** Direct effects of PCa pTA-NKs in supporting angiogenesis by interacting with endothelial cells. **(B)** Proposed model for PCa pTA-NK pro-angiogenic activities via monocyte recruitment and macrophage polarization.

INTRODUCTION

Prostate carcinoma (PCa) is the one of most commonly diagnosed cancer in males worldwide (1). Surgery and radiation therapy (2) are still important treatment options, as well as chemotherapy (3) and hormonal therapy (4). Recently, immunotherapy came of age as a possible effective strategy for PCa therapy (5). Several immunotherapeutic approaches have been proposed for PCa, that include dendritic-cell based vaccines, whole tumor cell vaccines, vector-based vaccines and antibodies. Currently FDA-approved immunotherapy approaches for PCa include the Sipuleucel–T (a dendritic-cell-based agent) and pembrolizumab (a checkpoint inhibitor that targets the PD-1/PD-L1 axis), while others are in clinical trials.

Evasion from immune system surveillance and induction of an inflammatory microenvironment are among host-dependent biological features, widely accepted as cancer hallmarks, as defined by Hanahan and Weinberg (6) and which play a role in prostate cancer. Based on their extreme cell plasticity, inflammatory cells from innate and adaptive immunity can acquire tumor-promoting phenotypes and functions in cancer patients. Acquisition of a tolerogenic state, anergy/exhaustion and induction of inflammatory angiogenesis are some of these aberrant functions (7–11).

Natural killer (NK) cells are large granular lymphocytes endowed with an inherent capability to kill virally infected and malignant cells, also participating to the modulation of the immune system, through their production of numerous cytokines and chemokines.

NK cells have been included in the Type-1 Innate Lymphoid cell group (ILC-1), based on their capability to produce IFNγ, following T-bet and EOMES expression by the $ID2^+$ ILC precursor (12). A study by the group of Eric Vivier, placed NK cells as cell subset originating from a cell lineage different from ILC-1 (13). While ILC-1 and NK cells share the ability to produce IFNγ in a T-bet dependent manner, NK cells functionally differ from ILC-1 for their cytotoxic abilities, via IFNγ and perforin production (13).

NK cells constitute approximately 5–15% of circulating lymphocytes in healthy adults, representing one of the three major lymphocyte population. Although lymphocytic in origin, NK cells are considered part of the innate immune system, since they do not require antigen presentation for target recognition. They exert effector functions that include cytotoxic activity and cytokine production, during antiviral and antitumor responses (14). Similarly to several immune cells (7–11), NK cells have been described to acquire a tolerogenic behavior and to be altered in their cytotoxic activities in different cancer types (7, 9–11, 15–20). However, pro-inflammatory, pro-tumor NK cells still represent an under investigated cell type; only few studies focused on the ability of polarized NKs to support cancer by acquiring pro-angiogenic phenotypes and functions (7, 10, 15, 16, 18). Major mechanisms associated with impaired NK cell function in cancer patients, are downregulation of lytic perforin and granzyme production, accompanied with reduction of degranulation capabilities, together with reduction of NKG2D (a relevant NK cell activation receptor) expression (21–23). The ligands for NKp30 and NKp46 have been found to be expressed in prostate cancer cell lines, and the blockade of the interaction between the Natural Cytotoxicity Receptors (NCR) with their ligand, can inhibit tumor cell growth (24).

However, studies on prostate cancer associated NK cell phenotype and functions remain limited (20, 25, 26). Isolation of tumour-infiltrating immune components is challenging, due to the small size of prostate biopsies, and the absence of stromal compartments. A study by Daniel Olive laboratory showed that inherent and tumour-driven immune tolerance in the prostate microenvironment impairs NK cell antitumor activity (20). Interestingly, this study also showed enrichment of $CD56^{bright}$ NK cells in tumor tissue, together with impaired NK cell functions, both in tumor tissues and in the peripheral blood (20). Here, we focused on peripheral blood NK cells in PCa patients, with the aim to evaluate their different phenotype and functional profiles in a perspective of a potential liquid biopsy-based procedure.

Two major subsets of NK are mostly present in the peripheral blood (pNK): the cytotoxic $CD56^{dim}CD16^+$ NK cell subset, (90–95% of pNK) and the low cytotoxic, highly cytokine producing NK cell subset, $CD56^{bright}CD16^{-/low}$ (14).

Our research group has identified a new pro-angiogenic NK cell subset in non-small-cell lung carcinoma (NSCLC), described as $CD56^{bright}CD16^-VEGF^{high}PlGF^{high}CXCL\text{-}8^+IFN\gamma^{low}$ NK cells (10, 16), supporting a role of NK cells in the inflammatory pro-angiogenic switch in solid tumors. These NK cell population is similar to a peculiar NK subset that has been found within the developing decidua, the decidual NK cell (dNK). dNK cells exhibit the $CD56^{superbright}CD16^-CD9^+CD49a^+$ phenotype and are closely linked with vascularization of the decidua and embryo implantation, in both humans and mice (27, 28). dNK cells produce VEGF, PlGF, and CXCL8, are poorly cytotoxic and are associated with induction of $CD4^+$ T regulatory (Treg) cells (27, 28). We characterized pro-angiogenic NK cells also in the peripheral blood (tumour associated NK cells, pTA-NKs) and tissue infiltrate (tumour infiltrating NK cells, TI-NKs) in colorectal cancer patients. These NK cells also display pro-angiogenic features as those in NSCLC patients (16). NK cells in the peripheral blood of NSCLC and CRC, in particular the $CD56^{bright}CD16^{low/-}$, share some similarities with the respective TI-NKs, and although they can be defined as decidual NK-like, feature of pregnant women, a similar population is present in both male and female cancer patients (10, 16, 29, 30). We identified TGFβ, a major immunosuppressive cytokine in the tumour microenvironment (TME) (31, 32), as an inducer of the inflammatory/pro-angiogenic switch of cytolytic NK, cells both at tissue and peripheral levels (16). Also, we found that STAT3/STAT5 activation regulates the polarization in CRC NK cells and that STAT5 chemical inhibition, with the anti-psychotic agent Pimozide, interferes with this process (15, 30).

Here, we show, for the first time, that NK cells isolated from peripheral blood of patients with PCa (PCa pTA-NKs), acquire a pro-inflammatory and pro-angiogenic phenotype, characterized

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ADK, adenocarcinoma; ANG, angiogenin; ANGIOP1, angiopoietin 1; ANOVA, analysis of variance; CCL, chemokine ligand (C-C motif); cDNA, complementary DNA; CM, conditioned media; CRPC, castration-resistant prostate cancer; CXCL, chemokine Ligand (C-X-C motif); DNAM-1, DNAX accessory molecule 1; dNK cells, decidual Natural Killer cells; EBM, endothelial cell basal medium; EGM, endothelial cell growth medium; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FC, flow cytometry; FSC, forward scatter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBV, hepatitis B virus; HC, healthy control; HCC, hepato cellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HUVEC, human umbilical vein endothelial cell; ICAM, inter cellular adhesion molecule-1; IFN- γ, interferon γ; IHC, immuno histo chemistry; IL-, interleukin-; I-TAC, interferon-inducible T-cell alpha chemoattractant; mAbs, monoclonal antibodies; MCP-1/CCL2, monocyte chemoattractant protein-1; MMPs, matrix metallo proteinases; MNCs, mono nuclear cells; NK, natural killer; NKG2D, natural killer receptor group 2 D; NSCLC, non-small cell lung cancer; PAI, plasminogen activator inhibitor; PB, peripheral blood cells; PBMCs, peripheral blood mononuclear cells; PCa, prostate cancer; PD-L1, programmed death receptor ligand 1; PGE2, prostaglandin E2; PlGF, placental growth factor; PMA, phorbol merystate acetate; P/S, penicillin/streptomycin; PTA-NKs, prostate tumor-associated natural killer cells; RANTES, regulated upon activation, normal t cell expressed and presumably secreted; SSC, side scatter; STAT, signal transducer and activator of transcription; TAMs, tumor-associated macrophages; TGFβ, transforming growth factor-β; TIMP, tissue inhibitor of metallo-proteinase; TME, tumor microenvironment; TNF α, tumor necrosis factor-α; Treg, T regulatory cells; uPAR, urokinase-type plasminogen activator receptor; VCAM, vascular cellular adhesion molecule-1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

by increased expression of the surface antigens CD56, CD9, and CD49a. Analysis of CM of FACS-sorted NK from PCa blood samples, allowed the identification of pNK signatures, characterized by up-regulation of cytokines and chemokines with pro-inflammatory and pro-angiogenic (CXCL8/IL-8) properties, as well as factors involved in the extracellular matrix (ECM) remodeling cascade (MMP-1, MMP-9, uPAR); pro-monocyte recruiting features (CCL1, CCL2, CCL5) and properties involved in M2-like macrophage polarization (IL-10). CM of FACS-sorted pNK cells from PB of PCa patients were able to recruit THP-1 and peripheral blood $CD14^+$ monocytes and to polarize THP-1 differentiated macrophages and PB CD14+ place at apex monocyte-derived macrophages towards M2-like/TAM, at transcript level.

Increasing evidence suggests that polarized NK cells are present in the peripheral blood of patients with several types of cancer (9, 10, 15, 16, 29, 30, 33) and their altered profile could be a relevant feature. The idea that NK cells can be envisaged as biomarkers for PCa have been previously explored (26, 34–36). Also, a clinical trial is exploring the significance of circulating NK cells in metastatic PCa (https://clinicaltrials.gov/ct2/show/NCT02963155). Our results provided the characterization of PCa pTA-NK cells, focusing on their polarization state, pro-inflammatory and pro-angiogenic features, for possible NK cell tracing and profiling in PCa patients.

MATERIALS AND METHODS

Sample Selection and Patient Characteristics

Peripheral blood (PB) samples (15–20 ml of whole blood, EDTA) were obtained from patients with prostate adenocarcinomas (ADK, n = 35). Controls (HC, n=27) included peripheral blood of healthy, tumor-free, male individuals. Patients with diabetes, human immunodeficiency virus (HIV)/hepatitis C virus (HCV)/hepatitis B virus (HBV) infection, chronic inflammatory conditions, treated with chemotherapy or radiotherapy, iatrogenically immunosuppressed or subjected to myeloablative therapies, were excluded to the study. The study was approved by the institutional review board ethics committees (protocol no. 0024138 04/07/2011 and protocol no.10 2 10/2011, within the study PROSTATEST) and according to the Helsinki Declaration of 1975 as revised in 2013. All patients enrolled in the study signed the informed consent, in accordance to the Helsinki Declaration of 1975 as revised in 2013. Demographic features of the cohort of PCa patients and controls are showed in **Supplementary Table 1**. Monocytes used for migration studies and monocyte-derive macrophages for polarization experiments were obtained from mononuclear cells from 4 different healthy subjects.

Cell Culture and Maintenance

The human prostate cancer (PCa) cell lines PC-3, DU-145, LNCaP (all purchased by ATCC) were maintained in RPMI 1640 medium, supplemented with 10% Fetal Bovine Serum (FBS) (Euroclone), 2 mM L-glutamine (Euroclone), 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone), at 37°C, 5% CO_2. Cells were routinely screened for eventual mycoplasma contaminations. CM were collected following 72 h of starvation in FBS free RMPI 1640 (Life Technologies), supplemented with 2 mM L-glutamine (Euroclone), 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone), at 37°C, 5% CO_2. PCa cell line CMs were used for NK cell polarization as detailed below.

Human umbilical vein endothelial cells (HUVEC, Lonza) were maintained in endothelial cell basal medium (EBM, Lonza) supplemented with endothelial cell growth medium (EGM™ SingleQuots™, Lonza), 10% of FBS, 2 mM L-glutamine (Euroclone), 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone). HUVECs were used between the three and five passages.

The human monocytic THP-1 cell line (ATCC) was cultured in RPMI 1640 medium, supplemented with 10% FBS, 2 mM L-glutamine (Euroclone), 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone), at 37°C, 5% CO_2. Differentiation of adherent THP-1 macrophages was obtained following 48 h of treatments with phorbol-merystate-acetate (5 ng/ml, PMA, Sigma Aldrich) (37).

$CD14^+$ monocytes were isolated from PB samples of healthy controls and used as $CD14^+$ monocytes or $CD14^+$ monocyte-derived macrophages, for cellular and molecular studies. Briefly, total PBMCs were isolated by density gradient stratification with Ficoll Histopaque-1077 (Sigma-Aldrich) and $CD14^+$ cells were immediately isolated using the $CD14^+$ cell isolation kit (Miltenyi Biotec). $CD14^+$ monocyte-derived adherent macrophages were obtained, following $CD14^+$ monocyte culture in RPMI 1640 medium, supplemented with 10% Fetal Bovine Serum (FBS), (Euroclone), 2 mM L-glutamine (Euroclone), 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone), 50 ng/ml M-CSF (Miltenyi Biotec), at 37°C, 5% CO_2, for 7 days.

Natural Killer Cell Isolation by FACS Sorting

pNK cells were isolated from peripheral blood mononuclear cells (PBMCs) of PCa ADK and HC subjects. Whole blood was diluted with PBS 1:1 (v/v), then subjected to a density gradient stratification with Ficoll Histopaque-1077 (Sigma-Aldrich), at 500xg for 20 minutes. The white ring interface, composed of total mononuclear cells (MNCs), was collected, washed twice in PBS, then used for subsequent experiments or for pNK isolation. Total MNCs were subject to cell sorting, using a BD FACS-AriaII instrument. Following 30 min of staining with anti-human FITC-conjugated CD45, anti-human PE-conjugated CD14, anti-human PerCP-conjugated CD3 and anti-human APC-conjugated CD56, NK cells was sorted as $CD45^+CD14^-$ $CD3^-$ $CD56^+$ (gating strategy is showed in **Supplementary Figure 1A**). For details of antibodies used, see **Supplementary Table 2**.

FACS-sorted NK cells (2×10^5 cells/ml) were used, following 24 h of culture in serum-free RPMI, for molecular analysis (qPCR) and to collect conditioned media for functional and secretome studies. Following 24 h, supernatants were collected, centrifuged to remove residual dead cells and debris and concentrated using Concentricon (Millipore) with a 3kDa membrane pore cut-off, to obtain concentrated supernatants.

Cell Treatment with Conditioned Media and Cytokines

For NK cell polarization, total PBMCs (1×10^6 cells/ml) were polarized with 30% of PC-3 or DU-145 or LNCaP CMs (v/v), or TGFβ (10 ng/ml) or IL-6 (25 ng/ml), in RMPI 1640 (Euroclone), supplemented with 10% FBS (Euroclone), 2 mM L-glutamine (Euroclone), 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone), 100 U/ml IL-2 (R&D), at 37°C, 5% CO_2, for 72 h. Cells were pulsed with fresh complete RPMI (30%, v/v), alone or with CM or cytokines, at day 0 and at 48 h, during the polarization schedule.

Conditioned media from FACS-sorted NK cells were used to detect the production of pro-inflammatory factors by endothelial cells. 2×10^5 HUVE cells were seeded into six well plates and exposed for 24 h to CM (50 µg/ml of total protein) of PCa pTA-NKs or pNK cell from HC. HUVECs were then harvested and used for real-Time PCR analysis.

THP-1 or peripheral blood $CD14^+$ monocytes were used to detect PCa pTA-NK-induced migration, while THP-1 differentiated and peripheral blood $CD14^+$ monocyte-derived macrophages were used to investigate pNK-induced polarization, via soluble factors. THP-1 differentiated or $CD14^+$ monocyte-derived macrophages were pulsed with CMs (50 µg of total protein) from FACS-sorted pNK cells (either from PCa patients of controls) for 72 h. Cells received CM at day 0 and 48 h of stimulation. Expression of M1-like or M2-like/TAM markers, following polarization, was detected by Real-Time PCR.

Phenotype Characterization of Conditioned Media-Polarized Peripheral Blood Natural Killer Cells and Prostate Cancer pTA-NKs

The polarization state of either pNK cells exposed to PCa cell line (PC-3, DU-145, LNCaP) conditioned media or pNK cells from PCa patients (PCa pTA-NKs), was assessed by flow cytometry for surface antigen expression. Briefly, 2.5×10^5 of total PBMCs per FACS tube were stained for 30 min at 4°C with anti-human monoclonal antibodies (mAbs) as follows: PerCP-conjugated anti-CD3, APC-conjugated anti-CD56, FITC-conjugated anti-CD16, PE-conjugated anti-CD9, PE-conjugated anti-CD49a, PE-conjugated anti-NKG2D, PE-conjugated anti-PD-1, PE-conjugated anti-TIM-3 (all purchased by Miltenyi Biotec). Following Forward/Side Scatter setting, NK cells were identified as $CD3^-$ and $CD56^+$ cells (total NK cells). CD16 and NKG2D expression was evaluated on $CD3^-CD56^+$ (total NK) gated cells. Finally, CD56 brightness, the expression of the dNK markers CD9, CD49a, expression of CXCR4 and the expression of the exhaustion markers PD-1 and TIM3, were evaluated on total $CD3^-CD56^+$NK cells. For details on antibodies used, see **Supplementary Table 2**.

Degranulation Assay

NK cell degranulation ability, as detected by CD107a production, was evaluated both in NK cells from PCa and HC clinical samples or in HC NK cells pre-polarized by 72 h of exposure to PC-3 and DU-145 cell conditioned media. Total MNCs (1×10^6 cells/ml), isolated from PCa patients and controls were cultured, overnight, in RMPI 1640 (Euroclone), supplemented with 10% FBS (Euroclone), 2 mM L-glutamine (Euroclone), 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone), 100 U/ml IL-2 (R&D), at 37°C, 5% CO_2.

2×10^5 MNCs were co-incubated with 2×10^5 K562 (E:T ratio of 1:1), in the presence of anti-CD107a- FITC (BD Bioscience) MNCs or K562 alone were used as controls for basal degranulation activities on effector and target cells. Cells were stimulated for 6 h with PMA (10 ng/ml) and ionomycin (500 ng/ml) (both from Sigma), in the presence of GolgiStop plus GolgiPlug (both from BD Biosciences), for 5 h. Finally, the expression of CD107a was detected on $CD3^+CD56^+$ NK cells, by flow cytometry. To determine the degranulation efficiency, the basal levels of NK cell degranulation was subtracted from the NK cells/K562 co-culture.

Intracellular Staining for Cytokine Detection of Conditioned Media-Polarized Peripheral Blood Natural Killer Cells

For intracellular cytokine detection, 2×10^6 PBMCs from PCa-ADK patients or HC were cultured, overnight, in RPMI 1640 (EuroClone) supplemented with 10% FBS (Life Technologies,) 1% (v/v) L-Glutammine (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (Euroclone) and IL-2 (100 U/ml; R&D Systems) at 37°C and 5% CO_2. For intracellular staining, the third day of polarization, cells were stimulated for 6 h with PMA (10 ng/ml) and ionomycin (500 ng/ml) (both from Sigma), in the presence of GolgiStop plus GolgiPlug (both from BD Biosciences). Cells were collected and stained for NK cell surface markers, as previously described, washed with PBS and treated with Cytofix and Cytoperm fixation and permeabilization kit (BD) for 10 min at 4°C. Cells were then washed in PBS and stained with PE-conjugated anti human CXCL8, CXCL12 (R&D System), IFNγ, TNFα, GranzymeB (Myltenyi Biotec) for 30 min. For indirect staining, cells were incubated for 1 h at 4°C with primary unlabelled antibodies anti-human Angiopoietin 1, anti-human Angiogenin, (all purchased from Abcam), washed and then stained with secondary PE-conjugated antibody anti-mouse IgG, for 30 min, at 4°C. Cytokines production was detected by flow cytometry, using a BD FACS CantoII analyzer. Isotype control and the secondary antibody alone were used as staining controls. For details on antibodies used, see **Supplementary Table 2**.

Secretome Analysis of Prostate Cancer pTA-NKs

The secretome of conditioned media (50 µg of total protein) of FACS-sorted pNK was assessed, using the Human Angiogenesis Array C1000 (RayBiotech, Inc., Norcross GA) to detect cytokines and chemokines release, as detailed before (30). A pool of three ADK or HCCMs was used. Chemiluminescent signals (revealed as black dots) were captured by membrane exposure to Amersham Hyperfilm. Arrays were computer scanned using the Amersham Imager 680 Analyzer and optical density was determined using the ImageJ software.

Network Formation Assay on Endothelial Cells

HUVEC cells (1.5 x10^4 cells/well) were seeded in a 96 well plate, previously coated with 50 µL of 10 mg/ml polymerized Matrigel (BD). After exposure to conditioned media (50 µg/ml total protein, pools of CM from 3 different ADKs or HCs), in serum-free EBM medium, HUVECs were then incubated at 37°C, 5% CO_2 for 24 h. The formation of capillary-like structures was detected by microphotographs, using an inverted microscope (Zeiss). The number of master segment and master segment length, as indicators of tube formation efficiency, were determined, using ImageJ software and the Angiogenesis Analyzer tool.

Detection of THP-1 Cell and Peripheral Blood CD14+ Monocyte Recruitment by Prostate Cancer pTA-NKs

Migration assay was performed using modified Boyden chambers. 5×10^4 THP-1 or $CD14^+$ monocytes were resuspended in 500 µl of serum-free RPMI and loaded into the upper compartment of the Boyden chamber. The lower chambers were filled with 250 µl of serum-free RMPI medium, supplemented with conditioned media (50 µg/ml total protein, pools of CM from 3 different ADKs or HCs), ADK or HC pNK cells. 5 µm pore-size polycarbonate filters (Whatman, GE Healthcare Europe GmbH, Milan, Italy) previously pre-coated with 2 µg/ml of fibronectin, were used as interface between the two chambers. The Boyden chambers were incubated for 6 h at 37°C. Filters were recovered, cells on the upper surface mechanically removed with a cotton swab. Cells migrated toward the filter surface, were fixed with ethanol at serial percentage (70%, 100%), finally rehydrated in water. Filters were stained with 10 µg/ml DAPI (Vectashield, Vector Laboratories,) and incubated at room temperature, protected from light, for 10 min. Cells in the filters were counted in a double-blind manner in five consecutive fields/filter, with a fluorescent microscope (Nikon Eclipse).

Quantitative Real-Time PCR

Total RNA was extracted from HUVECs, THP-1 macrophages or peripheral blood $CD14^+$ monocyte-derived macrophages, exposed to CM from FACS-sorted PCa pTA-NKs or HC pNK cells, using the small RNA miRNeasy Mini Kit (Thermo Fisher) and quantified by Nanodrop Spectrophotometer. Following genomic DNA removal, by DNase I Amplification Grade (Thermo Fisher) treatment, reverse transcription was performed on 500 ng of total RNA using SuperScript VILO cDNA synthesis kit (Thermo Fisher). Real-time PCR was performed using SYBR Green Master Mix (Thermo Fisher) on QuantStudio 6 Flex Real-Time PCR System Software (Applied Biosystems, Thermo Fisher Scientific, USA). All reactions were performed in triplicate. The GAPDH gene was used as housekeeping and results were showed as $2^{\wedge -\Delta Ct.}$ HUVECs or THP-1 macrophages in their respective basal medium alone, were used as baseline controls. Primer sequences are provided in **Supplementary Table 3**.

Statistical Analysis

Statistical differences between two datasets were determined using two tailed t-test. For multiple datasets, analysis of variance (ANOVA) followed by Tukey's post-hoc test was used. P values (p) ≤ 0.05 will be considered statistically significant. Data were analysed using the GraphPad Prism8 (San Diego, CA). Flow cytometry data were analysed using the BD FACS-Diva and FlowJo-v10 software.

RESULTS

pTA-NKs From Prostate Cancer Patients Exhibit a Pro-inflammatory, Pro-angiogenic, Exhausted Phenotype

We investigated whether pNK from PCa patients are characterized by a pro-inflammatory and pro-angiogenic phenotype. Flow cytometry analysis of CD56 and CD16 surface antigen expression revealed that the $CD56^+CD16^+$ NK cells are the predominant subset in the peripheral blood in PCa-ADK and HC samples (**Supplementary Figure 1A**). We found increased frequency of $CD56^{bright}$ NK cells in the peripheral blood of patients with PCa ADK (****p ≤ 0.0001) (**Figure 1A**). Peripheral blood NK cells from PCa-ADK samples express also higher levels of the decidual-like markers CD9 (**Figure 1B**) (****p ≤ 0.0001), CD49a (**Figure 1C**) (***p ≤ 0.001), as compared with those isolated from healthy controls. We also found increased expression of CXCR4 on NK cells from PCa-ADK samples (**Figure 1D**) (*p ≤ 0.05). We observed that PCa pTA-NKs have reduced expression of the NKG2D activation marker (*p ≤ 0.05), together with increased levels of the exhaustion markers PD-1 (****p ≤ 0.0001) and TIM-3 (**p ≤ 0.01), as compared to those from HC (**Figures 2A–C**). Also, PCa TA-NKs exhibit reduced ability to degranulate against K562 cells (****p ≤ 0.0001), as compared to those from HC (**Figure 2D**).

Real-time PCR results showed that TA-NKs cells, FACS-sorted from PCa-ADK, have increased expression of mRNA for the pro-inflammatory factors *CXCL8* (**p ≤ 0.01), *CXCL12* and *PAI* and confirmed the increased RNA expression of *CXCR4*, as well as *VEGF* (****p ≤ 0.0001), as compared to NK isolated form the peripheral blood of healthy controls (**Figure 2E**).

pTA-NKs From Prostate Cancer Patients Exhibit a Secretome Profile Enriched in Pro-Inflammatory, Pro-Angiogenic Cytokines, and Chemokines Involved in Monocyte Recruitment and Polarization

To investigate whether the acquisition of the pro-inflammatory phenotype in PCa pTA-NKs would correlate with their capability to release soluble factors involved in direct and indirect induction of inflammatory-angiogenesis, we investigate the contents of CM from PCa TA-NKs. We characterized the production of secreted proteins from PCa TA-NKs using a commercially available angiogenesis-membrane array kit. The overall secretome analysis (**Supplementary Figure 2**) revealed signatures characterizing PCa-ADK pTA-NKs

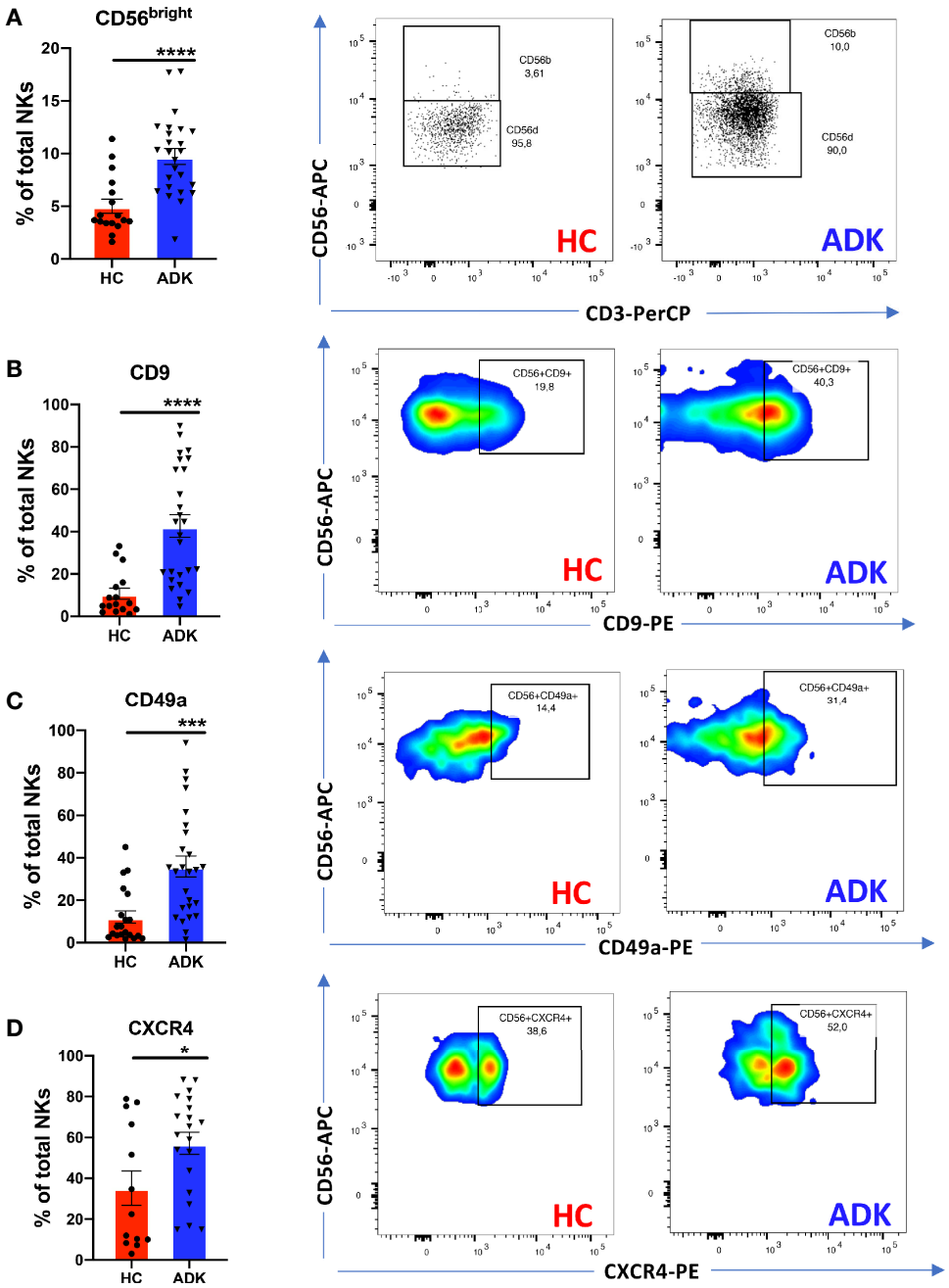


FIGURE 1 | pNK cell polarization in peripheral blood of PCa patients. PCa TA-NKs have increased numbers of CD56^{bright} NKs as compared with those from HC **(A)**. Peripheral blood NK (pTA-NKs) from PCa patients significantly express higher levels of the dNK cell markers CD9 **(B)** CD49a **(C)** and CXCR4 **(D)**, as compared with those from HC. Every dot in dots/bars graph refers to single patients or control. Representative dot plots show the specific antigen expression (as % of total pNK cells). Data are showed as mean ± SEM, t-student test, *p<0.05, ***p<0.001, ****p<0.0001. HC, healthy controls; ADK, prostate cancer adenocarcinoma.

involved in inflammation and angiogenesis (CXCL8) (**Supplementary Figure 2**, **Figure 3A**), tissue remodelling (MMP-1, MMP-9, uPAR) (**Supplementary Figure 2**, **Figure 3A**), monocyte recruitment (CXCL1, CCL2, as the most up-regulated) (**Supplementary Figure 2**, **Figure 4A**) and M2-like macrophage polarization (IL-10) (**Supplementary Figure 2**, **Figure 4A**).

pTA-NKs From Prostate Cancer Patients Functionally Support Inflammatory Angiogenesis In Vitro

We further investigated whether PCa-ADK pTA-NKs, expressing pro-inflammatory cytokines (also involved in angiogenesis), chemokines and chemokine receptors, were also effectively able to

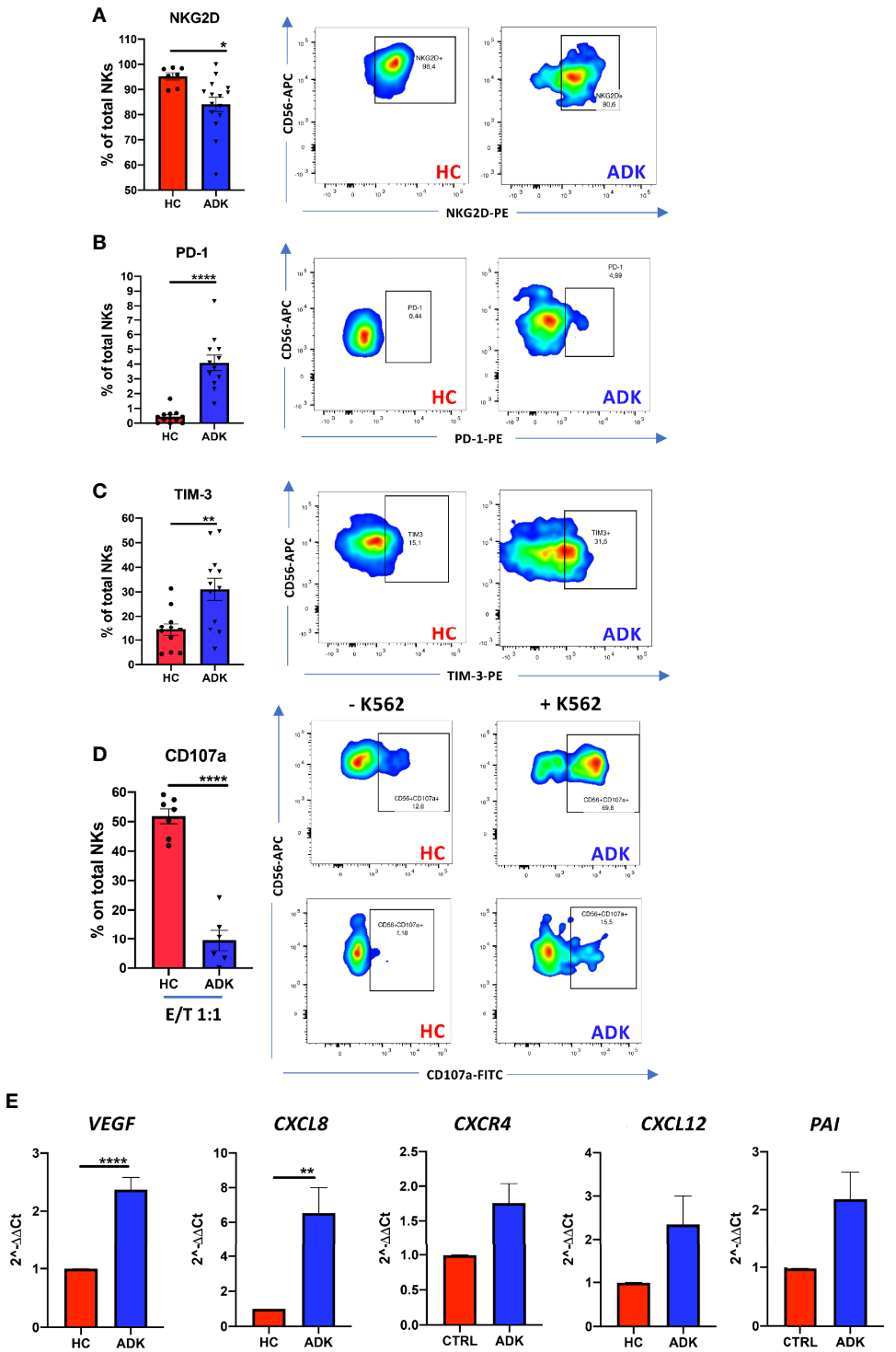


FIGURE 2 | pNK cell exhaustion and degranulation activities in peripheral blood of PCa patients. PCa pTA-NKs have decreased levels of the NKG2D activation markers **(A)**, increased expression of the PD-1 **(B)** and TIM-3 **(C)** exhaustion markers and impaired degranulation abilities against the K562 cells **(D)**. panel D shows NK cell degranulation capabilities, alone or co-incubated with K562 cells in PCa p-TA-NKs and NK cell from healthy controls. Every dot in dots/bars graph refers to single patient or control. Representative dot plots show the specific antigen expression (as % of total pNK cells). pNK cells, FACS-sorted from patients with ADK-PCa have increased expression of the pro-inflammatory factors *VEGF, CXCL8, CXCR4, CXCL12, PAI* **(E)**. qPCR have been performed using pNK cell from 3 PCa patients and 3 controls, in triplicate. Data are showed as mean ± SEM, t-student test, *p<0.05, **p<0.01, ****p<0.0001. HC, healthy controls; ADK, prostate cancer adenocarcinoma.

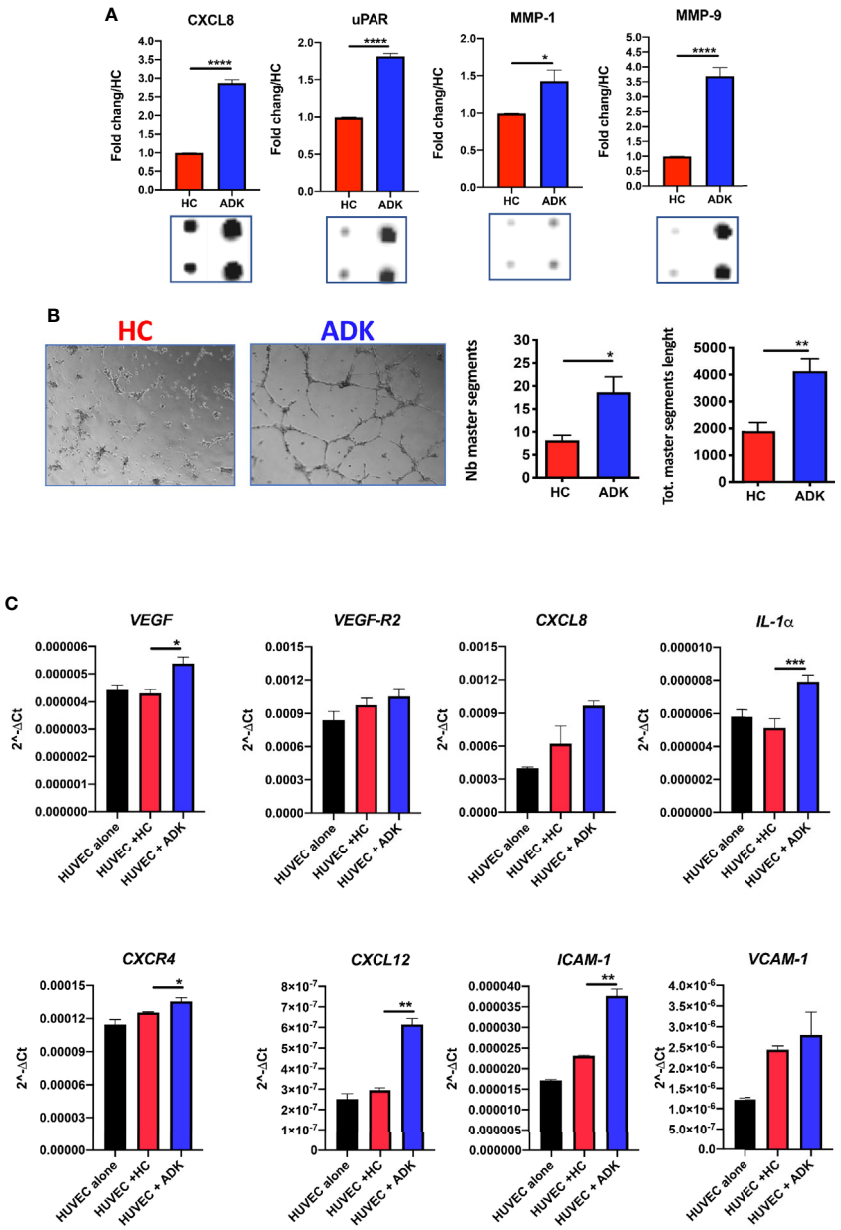


FIGURE 3 | Pro-inflammatory activities of pTA-NKs from PCa patients on endothelial cells. Conditioned media (CM) form FACS-sorted PCa pTA-NKs are enriched in pro-inflammatory and tissue-remodelling factors, such CXCL8, uPAR, MMP-1, MMP-9 **(A)** and functionally support the formation of capillary like structures in human umbilical-vein endothelial cells (HUVEC) on matrigel **(B)**. HUVE cells exposed to conditioned media of PCa pTA-NKs express higher levels of pro-inflammatory factors like *VEGF, VEGF-R2, CXCL8, CXCR4, CXCL12, ICAM-1, VCAM-1, IL1-α*, as compared to those exposed to conditioned media released by healthy control NK cells **(C)**. Capillary like-structure formation and qPCR on HUVECs have been performed using CM of pNK cell from 3 PCa patients and 3 controls, in triplicate, on 4 different HC. Data are showed as $2^{\wedge-\Delta Ct}$ values, mean ± SEM, ANOVA, *p<0.05, **p<0.01, ****p<0.0001. The condition HUVEC (black bar) stands for HUVE cells alone, as baseline condition. HC, healthy controls; ADK, prostate adenocarcinoma.

induce network formation in HUVECs *in vitro*. We found that CM of pNK cells isolated PCa-ADK samples have higher contents of the pro-inflammatory and tissue-remodelling factors CXCL8/IL-8 (****p≤0.0001), MMP-1 (*p≤0.05), MMP-9 (****p≤0.0001), uPAR (****p≤0.0001) (**Figure 3A**, **Supplementary Figure 2**). To detect whether conditioned media of inflammatory NK cells isolated PCa-ADK samples were effectively able to induce network formation in HUVE cells, we treated HUVE cells with these conditioned media. We found that conditioned media of pNK cells isolated from PCa-ADK are able to induce the formation of capillary-like structures by HUVE cells, on a matrigel layer (*p ≤ 0.05; **p ≤ 0.01), as a consequence of their pro-inflammatory secretome (**Figure 3B**). Real-time PCR results showed that HUVE cells, exposed for 24 h to CM from PCa-ADK pTA-NKs have a pro-inflammatory

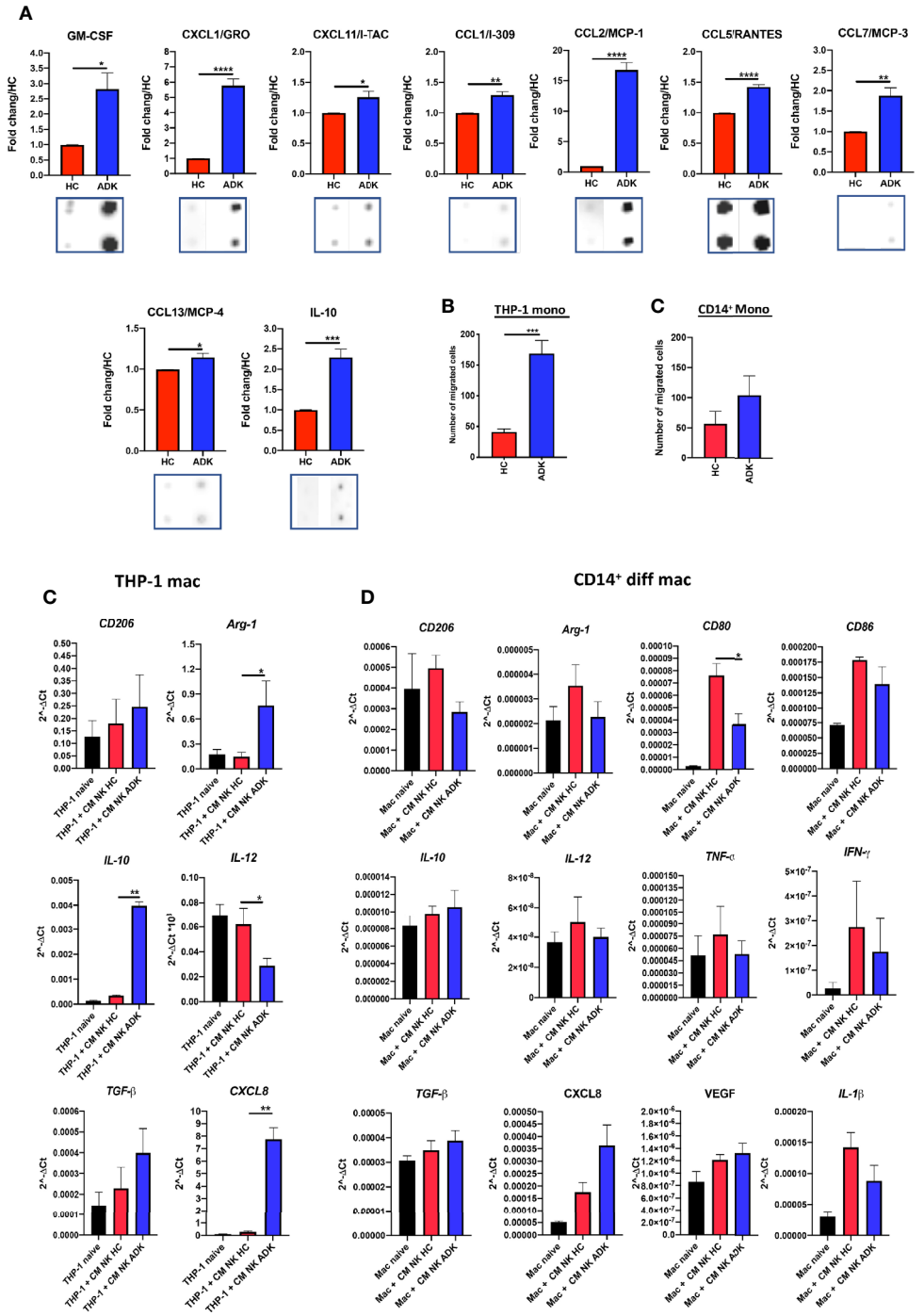


FIGURE 4 | Effects of PCa pTA-NKs on monocyte recruitment and polarization. Conditioned media from PCa pTA-NKs are enriched with factors involved in macrophage recruitment (GM-CSF, CXCL1/GRO, CXCL11/I-TAC, CCL1/I-309, CCL2/MCP-1, CCL5/RANTES, CCL7/MCP-3, CCL13/MCP-4, and polarization (IL-10) **(A)** and can recruit THP-1 and CD14$^+$ monocytes as compared with those from heathy controls **(B, C)**, as revealed by the migration assay (Boyden Chambers). Exposure to conditioned media of pNK from PCa patients for 72 h result in THP-1 ability to express higher levels of M2-like/TAM markers (*CD206, Arg-1*, *IL-10, ARG1, CXCL8, TGFβ*) and reduced expression of *IL-12* (M1-like marker) **(C)**. Data from THP-1 were extended, using a larger gene panel *(CD206, Arg-1, CD80, CD86, IL-10, IL-12, TNFα, IFNγ, TGFβ, CXCL8, VEGF, IL-1β*) on peripheral blood CD14$^+$ monocyte derived macrophages **(D)**. CMs were pooled from pNK cells FACS sorted from 3 different PCa patients or controls. Arrays were performed in duplicates. q-PCR were performed using CMs pooled from pNK cells FACS sorted from 3 different PCa patients or controls, and used for 4 different experiments (THP-1) and for 4 different donors of peripheral blood CD14$^+$ monocyte-derived macrophages, in triplicate. Data are showed as mean ± SEM, ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. HC, healthy controls; ADK, prostate adenocarcinoma; naive indicates THP-1 cells in control medium.

phenotype with increased mRNA expression of *VEGF* (*p ≤ 0.05), *VEGF-R2*, *CXCL8* and of factors involved in vascular inflammation and immune cells mobilization, such as *CXCR4* (*p ≤ 0.05), *CXCL12* (**p ≤ 0.01), *ICAM-1* (**p ≤ 0.01), *VCAM-1*, together with induction of *IL-1α* (***p ≤ 0.001) (**Figure 3C**).

pTA-NKs From Prostate Cancer Patients Can Recruit Monocytes and Induce an M2-Like/TAM Features In Vitro

Secretome analysis revealed that conditioned media of pNK cells isolated PCa-ADK samples are enriched in soluble factors involved in macrophage recruitment and polarization (**Supplementary Figure 2**, **Figure 4A**) such as GM-CSF (*p ≤ 0.05), CXCL1/GRO (****p ≤ 0.0001), CXCL11/I-TAC (*p ≤ 0.05), CCL1/I-309 (**p ≤ 0.01), CCL2/MCP-1 (****p ≤ 0.0001), CCL5/RANTES (****p ≤ 0.0001), CCL7/MCP-3 (**p ≤ 0.01), CCL13/MCP-4 (*p ≤ 0.05) and IL-10 (***p ≤ 0.001) (**Figure 5A**). Based on these results, we functionally investigated PCa-ADK NK cells ability to recruit THP-1 or $CD14^+$ monocytes, via soluble factors. We observed that CM from PCa pTA-NK cells, FACS sorted from the peripheral blood of PCa-ADK patients, promote the recruitment of THP-1

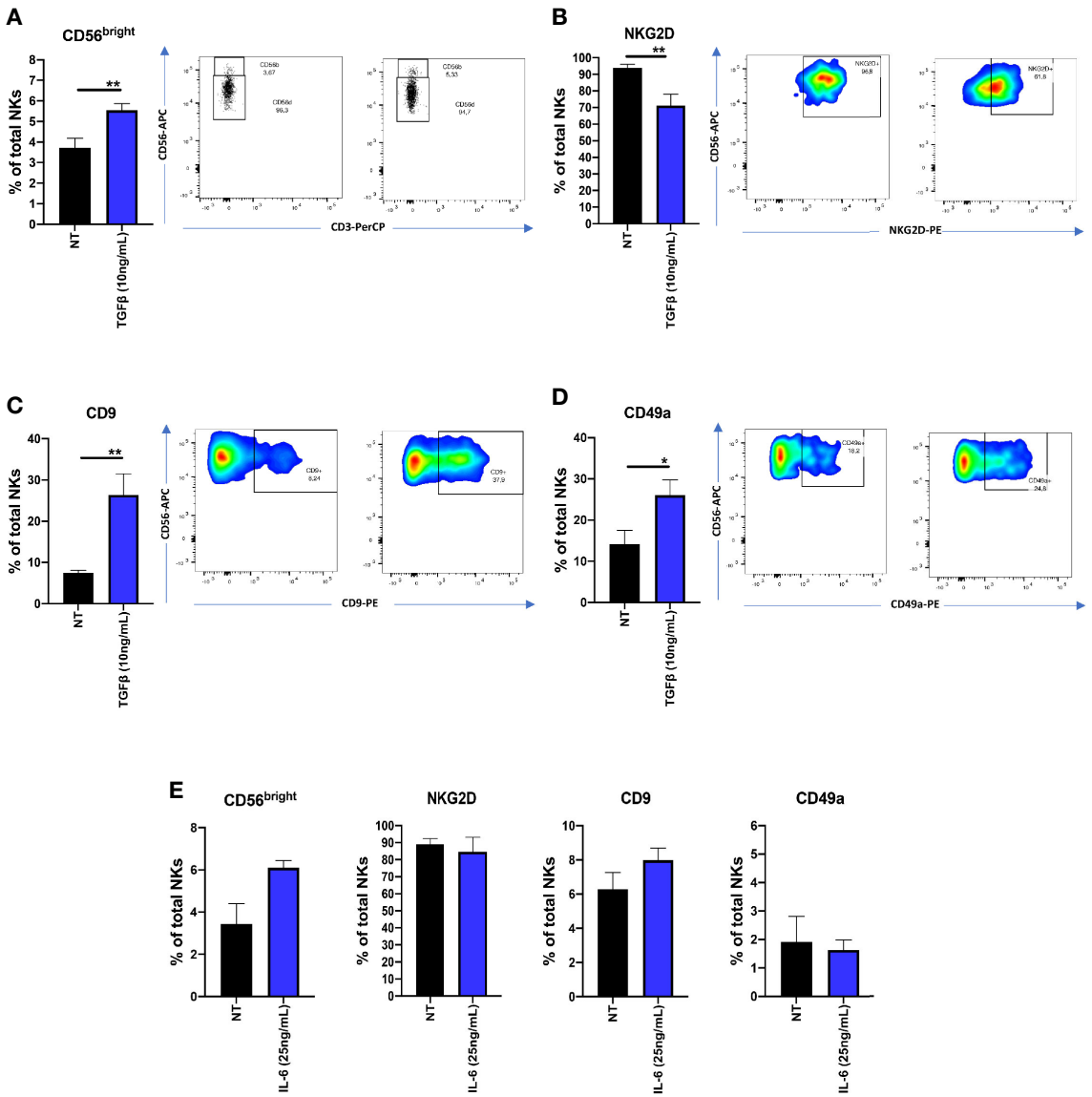


FIGURE 5 | Effects of TGFβ and IL-6 on NK cell polarization. NK cells from healthy donors, following 72 h of exposure to TGFβ (10 ng/ml) or IL-6 (25 ng/ml) were analyzed for their polarization state, by multicolor flow cytometry. TGFβ induced the $CD56^{bright}CD9^+CD49a^+NKG2D^{low}$ phenotype in cytolytic NKs **(A–D)**. The same effect was not observed on cells polarized with IL-6 **(E)**. Polarization experiments were performed on seven different healthy donors for TGFβ and four different healthy donors for IL-6. Data are shown as mean ± SEM, ANOVA, *p<0.05, **p<0.0. NT, not-treated cells.

monocytes (***p ≤ 0.001) as compared to CM of NK cells isolated from healthy controls (**Figure 4B**). A similar trend was observed in peripheral blood $CD14^+$ monocytes, exposed to CM from PCa pTA-NK as compared to CM of NK cells isolated from healthy controls (**Figure 4C**). We also observed that THP-1 differentiated macrophages, following 72 h of exposure to PCa pTA-NK CMs, displayed increased expression of the M2-like/TAM factors, such as CD206/Mannose receptor, Arg1 (*p ≤ 0.05), IL-10 (**p ≤ 0.01), TGFβ, CXCL8 (***p ≤ 0.001) and decreased expression of the M1-like cytokine IL-12 (*p ≤ 0.05) (**Figure 4C**). We extended this analysis on peripheral blood $CD14^+$ monocyte-derived macrophages, using a larger gene candidate panel, exposed for 72 h to CMs from FACS-sorted PCa pTA-NK or those from HC (**Figure 4D**). While data on CD206 and Arg1 expression seems to not reflect those observed in THP-1 macrophages, we observed decreased levels of CD80 (*p ≤ 0.05) and CD86 (M1-like markers). Results on IL-10 (M2-like) and IL-12 (M1-like), TGFβ and CXCL8 (both M2-like), show a trend similar to that observed in THP-1 macrophages (**Figure 4D**). In addition, we found that peripheral blood $CD14^+$ monocyte-derived macrophages, exposed for 72 h to CMs from FACS-sorted PCa pTA-NK or those from HC, have a trend in increased VEGF (M2-like) transcript, together with decreased expression of TNFα, IFNγ and IL-1β pro-inflammatory (M1-like) cytokines (**Figure 4D**).

TGFβ and IL-6 Effects on Natural Killer Cell Polarization

Since TGFβ and IL-6 have been found to be abundant in serum and plasma levels of PCa patients, we investigated TGFβ and IL-6 abilities to polarized cytolytic NK cells from heathy donors. We found that, following 72 h of TGFβ (10 ng/ml) exposure, NK cells increase the surface expression of CD56 (**p ≤ 0.01) (**Figure 5A**), decreased NKG2D expression (**p ≤ 0.01) (**Figure 5B**) and increased surface expression of CD9 (**p ≤ 0.01) and CD49a (*p ≤ 0.05) (**Figure 5C-D**). In contrast, IL-6 (25ng/ml), was not able to induce a similar effect (**Figure 5E**).

Prostate Cancer Cell Lines Conditioned Media Polarize pNK Cells Toward Pro-Inflammatory Angiogenic, Exhausted Natural Killer Cells

To verify the results obtained from PCa pTA-NKs, we used an *in vitro* model mimicking the interaction of the secretome of PCa cells with normal PBMC of healthy donors. Mononuclear cells from peripheral blood were exposed to soluble factors (CM) collected from three different PCa cell lines (PC-3, DU145, LNCaP) and assessed for their expression of decidual and pro-inflammatory, pro-angiogenic markers and polarization state. We found that pNK cells from healthy controls, following 72 hs of exposure to the CM of three different PCa cell lines (PC-3, DU-145, LNCaP) showed increase expression of the CD9, CD49a of CXCR4 (*p ≤ 0.05, **p ≤ 0.01) (**Figures 6A, B**). We also found that 72 h of stimulation with CM from the three PCa cell lines resulted in pNK enhanced ability to produce pro-inflammatory and pro-angiogenic factors, such as Angiogenin, Angiopoietin-1, CXCL8 (**p ≤ 0.01) and CXCL12 (*p ≤ 0.05, **p ≤ 0.01), and decreased ability to produce IFNγ, TNFα and Granzyme-B (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001), as detected by flow cytometry (**Figures 6C, D**). Finally, we observed that pNK cells from healthy controls, following 72 h of exposure to the CM of PC-3 and DU-145 cell lines are exhausted, as revealed by the trend of increased levels of PD-1 and TIM-3, together with decreased degranulation capabilities against K562 cells (*p ≤ 0.05) (**Figures 6E, F**).

DISCUSSION

Although immunotherapy has emerged as the "next generation" cancer treatment (38), it is not always successful in the treatment of patients with PCa, for whom the preferential therapeutic options still remain radiotherapy, chemotherapy and androgen deprivation therapy (3, 39–41). This clearly suggests that, to address more efficient immune therapeutic approaches against PCa, a better understanding of how the PCa is able to subvert the host immune system, still remains a major issue and a clinical unmet need. Preclinical and clinical evidences suggest that chronic inflammation plays a crucial role in multiple stages of prostate cancer development (42–44).

The polarization of the immune inflammatory cells in peripheral blood is directed by specific chemokines and cytokines that can shape their state and make them acquire altered phenotype and functions, depending on tumour scenario (7, 10, 11, 45–47).

NK cells have been found to be compromised in several cancers (7, 10, 11, 14–17, 19, 22, 23, 29, 30, 48). Skewed NK cell contribution to tumour progression goes beyond tumour escape and immunosuppression (7, 10, 15, 16, 29, 30). We demonstrated that NK cells in NSCLC cancer (16), colorectal cancer (7) and in malignant pleural effusions (29), acquire pro-angiogenic, pro-inflammatory phenotype and functions, identified as $CD56^{bright}CD16^{-}VEGF^{high}CXCL8^{+}IFN\gamma^{low}$ and share several features and behaviours with the highly pro-angiogenic decidual NK (dNK) cells. This was observed also by other groups in breast and colon cancers (49).

NK cell scenario in PCa is less investigated. Here, we characterized pNK cells isolated from the PB of patients with PCa, in the framework of approved clinical protocols. We found that PB NK cells from PCa patients (PCa pTA-NKs) show a pro-inflammatory and pro-angiogenic polarization, by acquiring the $CD56^{bright}CD9^{+}CD49a^{+}CXCR4^{+}$ phenotype. Our results on increased $CD56^{bright}$ frequency are in line with those showed by Pasero et al. in prostate cancer tissues (20). Similar to these results, we observed that PCa pTA-NKs, have impaired degranulation capabilities. In addition, we also found that PCa pTA-NKs exhibit down-regulation of NKG2D and increased markers of exhaustion, such as PD-1 and TIM-3, as compared with those from healthy controls. Increased expression of PD-1 and TIM-3 have been reported as markers of NK cell exhaustion also in other cancer types (50–55). We found that circulating PCa pTA-NKs were able to express larger amount of the pro-inflammatory and pro-angiogenic factors VEGF, CXCL8, CXCL12, PAI, as compared

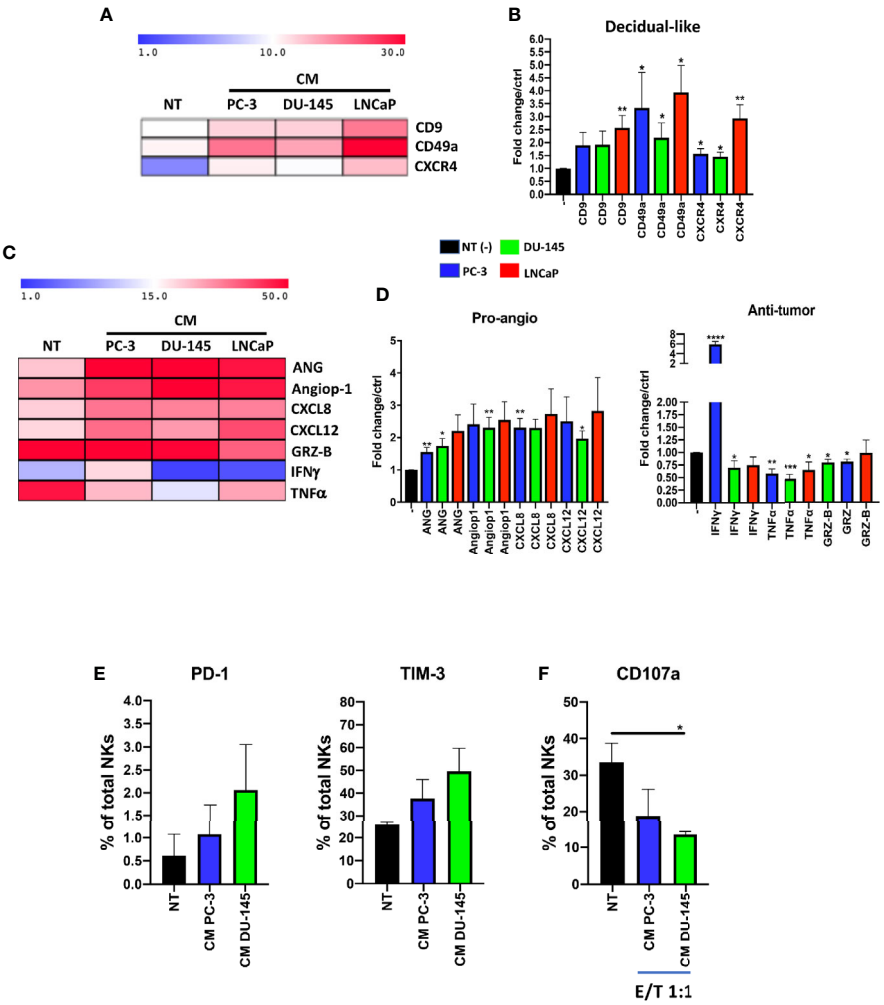


FIGURE 6 | Effects of prostate cancer cell line conditioned media (CM) on pNK cell polarization and functions. NK cells from healthy donors, following 72 h of exposure to conditioned media (CM) from PC-3, DU-145 and LNCaP PCa cell lines, exhibit a pro-inflammatory angiogenic decidual-like phenotype, as revealed by the increased levels of the dNK-like markers CD9, CD49a, CXCR4 **(A, B)**, enhanced production of pro-inflammatory factors (angiogenin, ANG; angiopoietin-1, Angiop1; CXCL8) and reduced production of cytolytic factors (granzyme B, GRZ-B; TNFα; IFNγ) **(C, D)**, as revealed by flow cytometry analysis. Experiments were performed using peripheral blood samples of 5-to-9 independent healthy donors. NK cells from healthy donors, following 72 h of exposure to conditioned media (CM) from PC-3, DU-145 cell lines increase the expression of PD-1 and TIM-3 exhaustion markers **(E)**, together with decreased degranulation capabilities against K562 cells **(F)**. Experiments were performed using peripheral blood samples of 3 independent healthy donors. Data are shown as mean ± SEM, ANOVA, *p<0.05, **p<0.01, ***p<0.001. CM, conditioned media/conditioned media from 72 h of SFM PCa cell lines.

to those from controls, introducing a new scenario for the possible pro-inflammatory and pro-angiogenic activities of circulating NK cell in PCa.

Based on these first results, we investigated whether soluble-related factors, released by PCa pTA-NKs, might support pro-inflammatory and pro-angiogenic-like behaviour, acting on endothelial cells and cellular components of the innate immune system, such as monocyte or macrophages. NK cells can interact with most of the innate and adaptive cellular components of the immune system (7, 11, 15, 56, 57). Monocytes are the second most represented phagocytes in circulation and in established progressing tumours, were they display an M2-like/TAM phenotype (58–60). M2-like macrophages, induced *in vitro*, have been shown to decrease the susceptibility of tumour cells to NK cell cytotoxicity, with increased PD-L1 and decreased NKG2D ligands in castration-resistant prostate cancer cells (61).

Here, we analysed the PCa pTA-NK production of pro-inflammatory/pro-angiogenic factors, using commercially available protein membrane arrays. We found elevated release of CXCL8/IL-8 by PCa pTA-NK, which can be responsible for the PCa pTA-NK soluble-factor mediated induction of HUVEC capillary-like structures on matrigel. These results support the hypothesis that PCa pTA-NK can potentially promote inflammatory angiogenesis. A number of studies have linked higher serum levels or expression of CXCL8/IL-8 with aggressive prostate cancer. Elevated CXCL8/IL-8 has been reported to

correlate with high Gleason score and with AR loss in metastatic disease (62–65). Interestingly, CXCL8/IL-8 was the most abundant factor that we found to be released by PCa pTA-NK.

We also found that PCa pTA-NK can produce factors involved in tissue remodelling and metastasis, such as MMP-1, MMP-9, uPAR. Other studies reported that MMP-1, MMP-9, uPAR play important roles in tissue remodelling with prognostic implication in PCa (66–68). In previously published results, we have shown that MMP-9 is upregulated in peripheral blood NK cells of colon cancer patients and the TIMP-1/MMP-9 axis, as well as uPAR, are altered, as compared to normal circulating NK cells (30).

The crosstalk between NK cells and M1 macrophages plays a crucial role in the protection against infections and tumour development (69–71). In hepatocellular carcinoma (HCC), tumour-derived monocytes have been found to induce dysfunctions in NK cells that were impaired in their ability to produce TNFα and IFNγ (71). CM of M2 type macrophages have been found to decreases the susceptibility of tumour cells to NK cell cytotoxicity, as a result of increased PD-L1 and decreased NKG2D ligands in prostate cancer cells. This has been reported to be mediated through the IL-6 and STAT3 pathway (61).

While macrophage-NK cell crosstalk has been investigated in different cancers (69, 70, 72–74), less studies have investigated the crosstalk in the opposite direction. We assessed the ability of PCa TA-NKs to recruit THP-1 and $CD14^+$ monocytes *in vitro*. We found that PCa pTA-NKs have increased ability to stimulate migration of THP-1 and $CD14^+$ monocytes, as compared to pNK cells from healthy controls. We also tested whether the PCa pTA-NK released products may impact on macrophage polarization state. We found that THP-1-differentiated and peripheral blood $CD14^+$ monocyte-derived macrophages, exposed for 72 h to conditioned media from PCa pTA-NK cells, acquire increase the expression of M2-like/TAM genes (*CD206, ARG-1, IL10, TGFβ, CXCL8, VEGF)*, while decreasing the expression of M1-like factors *CD81, CD86*, *IL-12, TNFγ, IFNγ*. These results provide the rational to propose that pro-inflammatory, pro-angiogenic activities by PCa pTA-NKs may also act by shaping monocyte and macrophage polarization and functions. M2-like macrophages/TAMs have been associated with increased tumour angiogenesis and poorer survival in PCa patients (75–77).

IL-2 priming of NK cells from patients with PCa, has been reported to result in distinct NK cell phenotypes and correlates with different NK cytotoxic activities (48). Once again, these cited results, together with our study, point out the important role of the phenotype and functions of NK cells in PCa patients, that could be used, in the future, for immune-profiling of NK cells in PCa.

Based on our previous studies (16, 30), we tested, *in vitro*, the possible contribution of major cytokines, TGFβ and IL-6 in supporting the pro-inflammatory and pro-angiogenic polarization of NK cells from healthy controls.

TGFβ has been largely reported as an inducer of immunosuppression and immune cell escape in diverse cancers (31), including PCa (78). TGFβ is largely present in plasma/serum samples of PCa patients (78). We found that TGFβ induced the $CD56^{bright}CD9^+CD49a^+NKG2D^{low}$ phenotype in healthy donor derived NK cells.

IL-6 has been reported to be produced by several cancer types, including PCa (79), endowed with pleiotropic effects (80). IL-6 has been reported to impair NK cell functions by activating the STAT3 pathway (80–82). Also, inhibition of IL-6-JAK/STAT3 signalling result in the enhancement of NK cell-mediated cytotoxicity via alteration of PD-L1/NKG2D ligand levels, in castration-resistant prostate cancer cells (82). In our study, we found that IL-6 was not able to induce the $CD56^{bright}CD9^+CD49a^+NKG2D^{low}$ phenotype in healthy donor derived NK cells.

We validated our findings from clinical samples using an *in vitro* model, mimicking the interaction of PCa soluble factors on cytolytic NK cells, by exposing NK cells from healthy donors to conditioned media of different PCa cell lines (PC-3, DU-145, LNCaP). Pasero et al previously showed that the PC-3 cell line can alter the expression of activation receptors in NK, such as NKG2D, DNAM-1, NKp46, NKp30, together with decreased degranulation capabilities (20). Using three different PCa cell lines, PC-3, DU-145 and LNCaP, respectively, we observed that their conditioned media were able to induce the $CD56^{bright}CD9^+CD49a^+CXCR4^+$ phenotype in NK cells derived from healthy controls, together to the capability to produce Angiogenin, Angiopoietin-1, CXCL8, CXCL12. We confirmed that CM from PCa cell lines induce anergy in healthy donor derived NK cells, with reduced capability to produce the anti-tumour cytokines IFNγ, TNFα and the cytotoxic factor granzyme-B. As a further proof anergy, PC-3 and DU-145 CM-polarized NK cells increase the expression of the PD-1 and TIM-3 exhaustion markers and exhibit reduced degranulation activities.

CONCLUSIONS

Limited data are available of PCa pTA-NKs. A pivotal study by Pasero et al. showed enrichment of $CD56^{bright}$ NK cells in tumour tissue, together with impaired NK cell functions both in tumour tissues and in the peripheral blood (20). Here, we focused on the characterization and phenotyping of peripheral blood NK cells from PCa patients, with the aim to evaluate their different phenotype and functional profiles, as compared to those from heathy controls.

We show that PCa pTA-NKs have a pro-inflammatory and pro-angiogenic phenotype, are endowed with the ability to support angiogenesis, *in vitro*, stimulating endothelial cell activation and functions, are able to recruit monocytes and polarize macrophages via soluble factors. Since obtaining PB NK cells is a relatively easy and poorly invasive procedure, our data provide a rationale for the future use of the pNK profiling in PCa patients to monitor NK cell polarization state and for designing approaches to restore pNK lytic activity in PCa patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committees of the University of Insubria (protocol no. 0024138 04/07/2011), Viale Borri, 57-21100 Varese (VA), Varese, Italy, ethics committees IRCCS MultiMedica (protocol no 10 2 10/2011), Via Milanese 300-20099, Sesto San Giovanni (Milan), Milan, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MG, DB, LM, ABo, GB, and ABr: performed the experiments in a previous submission. MG, LM, GB, and ABr performed the experiments in this revision. DB and ABr analyzed the data, performed the statistical analysis, and prepared the figures. FD, PC, AN, and AG provided and collected the clinical samples and provided the clinical support. DB, LM, DN, AA, and ABr conceived the experiments and analyzed the data. DB, LM, DN, AA, and ABr wrote the manuscript. DN and ABr provided funds. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.586126/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Gating strategy and expression of CD16 in PCa pTA-NKs and controls. **(A)** Gating strategy used for NK cell FACS sorting. **(B)** pTA-NKs from PCa patients share similar pNK cell subset frequency ($CD56^+CD16^+$ and $CD56^+CD16^-$ cells) as those from peripheral blood samples of healthy controls **(A)**. Data are showed as mean ± SEM, t-test student, **p<0.01. HC: healthy controls; ADK: prostate adenocarcinoma.

SUPPLEMENTARY FIGURE 2 | Secretome profiling of PCa pTA-NKs. Secretome profiling, using an antibody membrane array showed that conditioned media from pNK cells of PCa patients are enriched in several factors directly and indirectly associated with induction of angiogenesis, immunosuppression, M2-like polarization, macrophage recruitment, ECM/tissue remodelling. **(A)** Representative heatmap; **(B)** bar histogram showing the fold change for every modulated factor PCa over HC; **(C, D)** scan acquisition for the angiogenesis antibody-array (C1 and C2), showing overall dots, following exposure to conditioned media (CM) from FACS sorted pNK cells of PCa-ADK patients and controls. Green tables, to identify dots position for array C1 and C2, is provided. CMs were pooled from pNK cells sorted from 3 different PCa patients or HC. Arrays were performed in duplicates. Data are showed as mean ± SEM, t-test student, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ADK, prostate adenocarcinoma; HC, healthy controls.

SUPPLEMENTARY TABLE 1 | Demographic and clinical features of our cohort of PCa patients and controls. Table summarizing the features of our cohorts of patients, with relative sample size. Average of age is showed as mean ± SD. N: sample size, ADK, prostate adenocarcinoma; HC, healthy controls.

SUPPLEMENTARY TABLE 2 | Antibodies used in flow cytometry experiments. The table summarizes the antibodies (primary conjugated, primary not-conjugated, secondary conjugated) used in flow cytometry analysis.

SUPPLEMENTARY TABLE 3 | Primer sequences for oligos used for Real-time PCR. Sequences for forward and reverse oligos used for real-time PCR are showed.

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Cytokines Orchestrating the Natural Killer-Myeloid Cell Crosstalk in the Tumor Microenvironment: Implications for Natural Killer Cell-Based Cancer Immunotherapy

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Natural killer (NK) cells are endowed with germline-encoded receptors that enable them to detect and kill malignant cells without prior priming. Over the years, overwhelming evidence has identified an essential role for NK cells in tumor immune surveillance. More recently, clinical trials have also highlighted their potential in therapeutic settings. Yet, data show that NK cells can be dysregulated within the tumor microenvironment (TME), rendering them ineffective in eradicating the cancer cells. This has been attributed to immune suppressive factors, including the tumor cells *per se*, stromal cells, regulatory T cells, and soluble factors such as reactive oxygen species and cytokines. However, the TME also hosts myeloid cells such as dendritic cells, macrophages, neutrophils, and myeloid-derived suppressor cells that influence NK cell function. Although the NK-myeloid cell crosstalk can promote anti-tumor responses, myeloid cells in the TME often dysregulate NK cells *via* direct cell-to-cell interactions down-regulating key NK cell receptors, depletion of nutrients and growth factors required for NK cell growth, and secretion of metabolites, chemokines and cytokines that ultimately alter NK cell trafficking, survival, and cytotoxicity. Here, we review the complex functions of myeloid-derived cytokines in both supporting and suppressing NK cells in the TME and how NK cell-derived cytokines can influence myeloid subsets. We discuss challenges related to these interactions in unleashing the full potential of endogenous and adoptively infused NK cells. Finally, we present strategies aiming at improving NK cell-based cancer immunotherapies *via* pathways that are involved in the NK-myeloid cell crosstalk in the TME.

Keywords: natural killer (NK) cells, myeloid cells, cytokines, tumor microenvironment, cancer immunotherapy, tumor immunity

INTRODUCTION

Natural Killer (NK) cells are cytotoxic lymphocytes that innately recognize their target cells based on signals from an array of germline-encoded inhibitory and activating cell surface receptors (1). While inhibition is mainly mediated by HLA class I-binding receptors such as KIR, LIR-1, and NKG2A, activation is triggered by the NKG2D, DNAM-1, NKp30, NKp46, and 2B4 receptors, among others (2). Experimental approaches delineating how NK cells target tumor cells have in more recent years been harmonized with studies evidencing their role in tumor immune surveillance (3) and clinical therapy to treat patients with cancer (4, 5). However, it has also become increasingly clear that NK cells are often dysfunctional in cancer patients (6, 7). This is most prominent in the tumor microenvironment (TME), although also observed in blood and other tissues in patients with advanced cancer (6–8). Factors suppressing endogenous or adoptively infused NK cells in the TME are likely limiting the full potential of NK cell-based cancer immunotherapies.

NK cells can be disarmed in the TME by both direct and indirect interactions with the tumor cells (6, 7, 9). However, several other cell types in the TME, such as stroma cells and immune cells, acting by direct interactions and *via* release of reactive oxygen species, growth factors, and cytokines, can also induce NK cell dysfunction (9–11). Both the degree and mode of NK cell suppression in the TME may dynamically vary from early to later stages of cancer development as well as between different tumor histotypes. Beyond reducing the anti-tumor cytotoxicity of NK cells *per se*, suppression of NK cells in the TME can also negatively impact their ability to recruit other immune cells (12–15), which is crucial for initiating and maintaining proper anti-tumor responses. In this regard, a pivotal interaction in the TME is the one between NK cells and myeloid cells, such as dendritic cells (DCs), macrophages, neutrophils, and myeloid-derived suppressor cells (MDSCs) (16–19). While NK cells positively promote DC infiltration and maturation *via* release of pro-inflammatory cytokines such as interferon (IFN)-γ (20), myeloid-derived cytokines, including interleukin (IL)-12, IL-15, and IL-18, critically promote NK cell maturation, proliferation, and anti-tumor functions (21). However, aggravation of the TME often observed in more advanced stages of cancer direct myeloid cells towards a suppressive phenotype that instead can impede NK cell functions *via* secretion of cytokines, such as transforming growth factor (TGF)-β, IL-1β, and IL-10 (22–24). Thus, the NK-myeloid crosstalk is intricate but critical for proper anti-tumor properties of NK cells in the TME.

Here we give an overview of the cytokines involved in the interplay between NK cells and myeloid cells in the general TME. We discuss how myeloid cells promote NK cell functions and vice versa, but foremost, how this interaction can hinder NK cell-mediated tumor rejection. We outline current methods and possible future approaches to enhance anti-tumor responses by NK cells *via* administration or manipulation of cytokines and cytokine signaling, as well as preventing myeloid cell infiltration into the TME. This review highlights that a better understanding of the crosstalk between myeloid cells and NK cells is likely critical to improve the efficacy of NK cell-based cancer immunotherapy.

MYELOID-DERIVED CYTOKINES PROMOTING NATURAL KILLER CELL RESPONSES TO CANCER

Several myeloid cells, exemplified by macrophages and DCs, are characterized by a pro-inflammatory phenotype and release cytokines such as type-1 IFNs, IL-12, IL-15, IL-18, IL-21 (**Table 1A**) upon recognition of damage-associated molecular patterns (DAMPs) on the transformed cells (70, 71). This pro-inflammatory cytokine milieu, together with key chemokines, aid in the recruitment of NK cells to the tumor site while promoting their persistence and anti-tumor effector functions (70, 72). Several of these cytokines have overlapping functions but also possess specific functions in the regulation of NK cell responses in cancer (35, 73–79) (**Figure 1** and **Table 1A**). In this section, we will present the key cytokines released by myeloid cells that promote anti-tumor cytotoxicity by NK cells but also give examples of how cytokines can have dual functions.

Direct and Indirect Contribution of Interferon-α and Interferon-β in Natural Killer Cell Activation

The type-1 IFNs, IFN-α, and IFN-β are secreted by activated myeloid cells and stimulate NK cell expansion while enhancing the effector functions upon stimulation of the IFN-α receptor (IFNAR) (80, 81). Inversely, as highlighted in experimental models using IFNAR-deficient NK cells and NK cells with defective downstream signaling molecule transducer and activator-1 (STAT1), impaired type-1 IFN signaling results in defective functional NK cell maturation and hampered anti-tumoricidal potential in sarcoma and lymphoma mouse models (82, 83). Importantly, while transient or intermittent type-1 IFN stimulation results in preferential phosphorylation of STAT4 than STAT1 and thereby increased IFN-γ production by NK cells promoting pro-inflammation, chronic stimulation coupled with increased levels of IFN-γ also bolster NK cell cytotoxicity (81, 84). This is due to increases basal levels of STAT1 protein expression following chronic IFN-γ stimulation that triggers preferential activation of STAT1 over STAT4 (81, 84). Additionally, autocrine type-1 IFN signaling in activated myeloid cells induces interleukin (IL)-15 production, which is a critical cytokine for NK cell development, proliferation and cytotoxic function (37, 85).

Regulation of Natural Killer Cell Activation and Effector Function by Interleukin-12 Family of Cytokines

The IL-12 family of heterodimeric cytokines, including IL-12, IL-23, and IL-27, critically regulate NK cell activation and effector functions (**Figure 1A** and **Table 1A**). Phagocytic macrophages and DCs produce these cytokines following the recognition of

TABLE 1 | Cytokines and chemokines involved in the NK-myeloid cell crosstalk and drugs directed to modulate these interactions.

A. Myeloid cell-derived cytokines and their effects on NK cells.

Cytokine	Produced by	Effects	Therapy	Ref.
TGF-β	MDSCs, TAMs, tumor cells, mast cells	↓activating receptor, cytokine production, cytotoxicity, proliferation	Fresolimumab, galunisertib, M7824 (clinical trial)	(25–30)
IL-10	MDSCs, TAMs, NK cells, DCs, macrophages	↓/↑ cytotoxicity, cytokine production	–	(24, 31–33)
IL-32α	DCs	↓perforin, granzyme B	–	(34)
TNF	Macrophages	↑ cytokine production	–	(35)
IL-12	DCs, macrophages, monocytes, neutrophils	↑ cytotoxicity, cytokine production, proliferation, survival	IL-12, IL-12 + pembrolizumab (clinical trial)	(21, 36)
IL-15	DCs, macrophages, monocytes	↑ cytotoxicity, cytokine production, proliferation, survival, activating receptors, KIR	ALT-803 (phase 1 and 2 clinical trial), IL-15 + Ipilimumab and Nivolumab (phase 1 clinical trial)	(37–39)
IL-18	M0 macrophages, TAMs, DCs,	↑ cytotoxicity, cytokine production, proliferation, survival	IL-18 (phase 1 and 2 clinical trial)	(40–44)
IL-21	DCs	↑ cytotoxicity, proliferation, activating receptors	IL-21, IL-21 + Ipilimumab and nivolumab (phase-I and –II clinical trial)	(45)
IL-6	MDSC, TAM, tumor cell, macrophages, monocytes, mast cells	↓/↑ cytotoxicity, ↓ cytokine production	Tocilizumab (clinical trial)	(46, 47)
IL-1α	Monocytes, DCs, macrophages	↓maturation	Anakinra, Canakinumab, Isunakinra (phase 1 and 2 clinical trial)	(23)
IL-27	DCs, macrophages, MDSCs	↓/↑ cytotoxicity, cytokine production	p28 peptide	(48–53)
IL-23	MDSC and TAM, DC and macrophage	↓/↑ cytotoxicity, cytokine production	–	(54, 55)
IL-17	Neutrophils	↑cytotoxicity, ↓maturation	–	(56, 57)
IFN-α/β	DC	↑ cytotoxicity, cytokine production, proliferation, survival, NKG2D	IFN-α/β approved	(58)

B. NK cell-derived cytokines and their effects on myeloid cells.

Cytokine	Target population	Effects	Therapy	Ref.
IFN-γ	DCs	maturation, activation	–	(59–61)
	TAMs	polarization towards pro-inflammatory Mφ	–	(62, 63)
	TANs	inhibition of pro-tumorigenic TANs	–	(64)
TNF-α	DCs	maturation, activation	–	(59–61)
	TAMs	polarization towards pro-inflammatory Mφ	–	(62)
HMGB1	DCs	activation	–	(43, 65)
GM-CSF	DCs	activation	–	(62)
	TAMs	polarization towards pro-inflammatory Mφ	–	(62)
	TANs	activation, promotes NETs	–	(66–68)
VEGF-A	Endothelial cells, tumor cells	proliferation, migration	–	(69)

A) Effects of myeloid cell-derived cytokines on NK cells and therapeutic targeting thereof. B) Effects of NK cell-derived cytokines on the maturation and differentiation of different myeloid cell subsets and therapeutic targeting thereof.

DAMPs on dying tumor cells. Despite sharing sequence similarly, these cytokines uniquely modulate NK cell function.

Interleukin-12

IL-12 (p35 and p40 complex) signals by engaging the heterodimeric receptor complex of IL-12Rβ1 and IL-12Rβ2 subunits that are readily expressed by mature activated but not immature NK cells. In NK cells, IL-12 signaling principally mediates STAT4 phosphorylation that is essential for both IFN-γ and perforin expression, as observed both in human NK cells *in vitro* and in murine NK cells *in vivo* (21, 86–89). Accordingly, blockade of IL-12 diminishes DC-induced IFN-γ production by NK cells, suggesting IL-12 is critical for optimal IFN-γ release by activated human NK cells (90). Additionally, IL-12 can work in concert with IL-15 and IL-18 to generate 'memory-like' NK cells, partly facilitated by epigenetic reprogramming of the CNS1 enhancer region of the *Ifng* locus in NK cells, which enables them to produce elevated levels of IFN-γ compared to conventional NK cells upon activation as shown by transferring IL-12/IL-15/IL-18-pretreated human NK cells in NSG mice (91).

Interleukin-23

IL-12p40 also interacts with p19 subunits forming the heterodimeric cytokine IL-23. Upon stimulation with IL-23, $CD56^{bright}$ NK cells release higher levels of IFN-γ as compared to $CD56^{dim}$ NK cells due to their higher expression levels of IL-23R. IL-23 also increases IL-18Rα expression, thus priming NK cells for IL-18-induced IFN-γ production. IL-23 stimulates human NK cell activation *in vitro* while inhibiting IL-15- and IL-18-induced NK cell proliferation (54). However, IL-23 reduces both cytotoxicity and IFN-γ production *in vivo*, indeed anti-IL-23 therapy synergizes with IL-2 or anti-erbB2 treatment

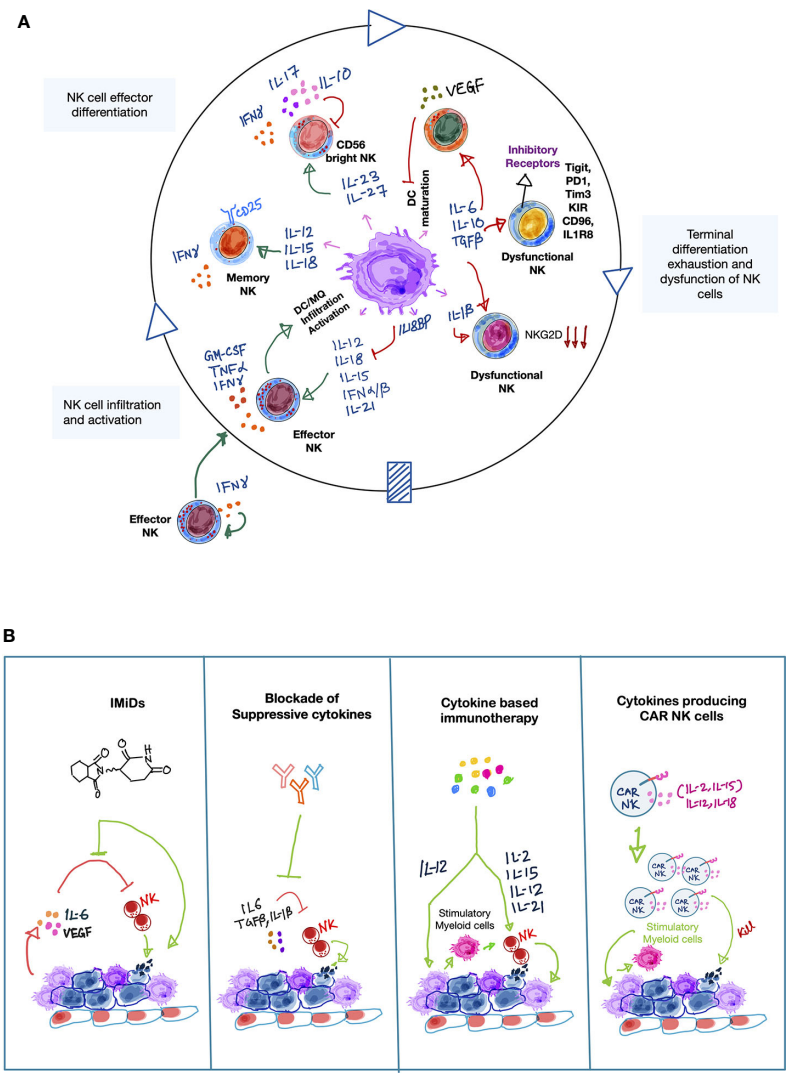

FIGURE 1 | Cytokines involved in the NK-myeloid cell cross talk in the tumor microenvironment. **(A)** A simplified schematic illustration showing the orchestra of myeloid- and NK cell-derived cytokines involved in forming anti-tumor immune responses by NK cells in the tumor microenvironment (TME). Activated NK cells produce IFN-γ which indirectly promotes recruitment of other NK cells from peripheral blood to the tumor sites. Upon recognition of tumor antigens, myeloid cells, especially DCs and macrophages produce inflammatory cytokines such as type-1 IFNs, IL-12, IL-15, IL-18, IL-21. These cytokines either alone or cooperatively promote NK cell survival, proliferation, maturation and production of spectrum of pro-inflammatory cytokines, including IFN-γ, TNF-α, GM-CSF, which further boost anti-tumor immune-activating potential of myeloid cells while recruiting additional inflammatory myeloid cells [including M1 macrophages (MQ), mature dendritic cells (DCs)] to sustain the anti-tumor immune response. Furthermore, myeloid-derived IL-23 and IL-27 cytokines can also promote NK cell activity by inducing IFN-γ production, but they can also negatively influence the NK-myeloid cell anti-tumor crosstalk by secretion of tumor-promoting cytokines such as IL-17 and IL-10, respectively. This suggests a dual role of IL-23 and IL-27 in NK cell-mediated tumor immunity. In line with the dual roles of certain cytokines, myeloid cells can become immune suppressive (myeloid-derived suppressor cells; MDSCs). This is frequently occurring during cancer progression. MDSCs secrete a plethora of immune suppressive cytokines that negatively influence the anti-tumor potential of NK cells *per se* but also impair anti-tumor responses normally resulting from the NK-myeloid cell crosstalk. For example, suppressive cytokines promote NK cell exhaustion and directly impair NK cell-mediated cytolytic activity, while limiting the ability of myeloid cells to produce NK cell stimulatory cytokines such as IL-12, IL-15, and IL-18. Green arrows indicate positive interactions and red arrows indicate negative interactions. **(B)** Therapeutic approaches that directly or indirectly modulate cytokine mediators that enhance NK cell-mediated anti-cancer responses in the TME. Simplified illustrations showing validated therapeutic approaches that can either restore or reinforce a stimulatory cytokine environment to augment NK cell-mediated tumor killing activity. On one hand, immunomodulatory drugs (IMiDs) such as lenalidomide can indirectly augment NK cell anti-tumor activity by reducing the levels of pro-tumorigenic factors, such as IL-6 and VEGF, while stimulating other immune cells to secrete IL-2. Accordingly, targeted blockade of immune-suppressive cytokines, such as IL-6, TGF-β can also positively impact NK-myeloid anti-tumor cross-talk in a similar manner. On the other hand, recombinant or synthetic cytokines as well as cell-based therapies such as cytokine-secreting CAR-NK cells can directly influence NK cell-mediated cancer cell killing. Importantly, cytokine-activated NK cells can further edit myeloid cells to enhance anti-tumor response *via* the production of inflammatory cytokines, such as IFN-γ. Light green arrows show the mode of therapeutic action.

in mammary and melanoma tumor models in an NK cell-dependent manner (55, 92).

Interleukin-27

The IL-27 heterodimer is composed of p28 and EBI3 and promotes pro- and anti-inflammatory functions mainly through STAT1 and STAT3 (93). Like IL-23, IL-27 shows differential activity on human $CD56^{bright}$ and $CD56^{dim}$ subsets, maybe related to the higher expression of IL-27Rα in the $CD56^{bright}$ subset (48). While $CD56^{dim}$ NK cells are not affected, $CD56^{bright}$ NK cells acquire a regulatory phenotype. Yet, *in vivo* studies on a murine squamous cell carcinoma model and *in vitro* analysis on human NK cells also demonstrated that IL-27 primes NK cells to respond to IL-18, while inducing perforin, granzyme B, NKp46, and NKG2D expression, as well as promoting antibody-dependent cellular cytotoxicity (ADCC) (49–52, 94, 95).

Critical Role of Gamma-Chain Cytokines Released by Myeloid Cells in Promoting Natural Killer Cell Survival, Proliferation, and Functions

The gamma-chain family consists of cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 that all bind the common gamma-chain receptor. Below we will focus the discussion on IL-15 and IL-21 that are commonly derived from myeloid cells.

Interleukin-15

IL-15/IL-5Rα induces NK cell survival and proliferation by acting as a soluble form or presented at the DC membrane. As mentioned above, it is also an essential driver of NK cell development and activation (37–39, 96). IL-15 expression correlates with NK cell infiltration in human tumor samples (97), and data from a murine melanoma model indicate $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ monocytic cells are the major source of this cytokine (78). IL-15, together with IL-12, also indirectly regulates NK cell functions by controlling metabolism *via* mTORC1 activation, which stimulates nutrient uptake, glycolysis, and OXPHOS, thereby providing the energy for NK cell proliferation, proper functions and enhanced persistance (36, 98). Interestingly, chronic IL-15 stimulation of NK cells results in exhaustion by reducing the mitochondrial respiratory capacity (99).

Interleukin-21

In addition to IL-15, DCs also release IL-21, which following STAT3 and STAT1 signaling in NK cells, promotes NK cell cytotoxicity *via* increased granzyme B and perforin expression. Moreover, STAT1 and PI3K pathways are essential for IL-21-mediated reversal of NK cell exhaustion in mice and for intratumoral human NK cells cultured *in vitro* (45). Interestingly, IL-21 differentially regulates the expression of activating receptors by inducing NKp30 levels while reducing NKG2D/DAP10 expression in human NK cell (100). However, IL-21 contributes to tumor rejection in an NKG2D-dependent manner in multiple mouse tumor models (101).

Interleukin-17 Can Promote Natural Killer Cell Cytotoxicity While Limiting Terminal Differentiation of Natural Killer Cells

Neutrophils are the major producers of IL-17, which binds to a dimeric receptor and mainly signals through the NF-kB and ERK pathways (102). IL-17 has been shown to enhance NK cell recruitment in human esophageal cancer through tumor-derived chemokines and NK cell cytotoxicity through the increased expression of activating receptors, perforin, granzyme B, TNF-α, and IFN-γ (103). However, IL-17 has also been reported to limit IL-15-mediated terminal murine NK cell differentiation *via* upregulation of suppressor of cytokine signaling (SOCS), which inhibits STAT5 phosphorylation, and reduces NK cell killing in the presence of IFN-γ (56).

Context-Dependent Role of Interleukin-18 in Cancer

Upon interaction with its heterodimeric receptor and the activation of the MyD88 signaling pathway, IL-18 primes NK cells to produce IFN-γ (104). *In vitro* data also show that IL-18 can favor the differentiation of human $CD56^{dim}$ $CCR7^{+}$ $CD25^{+}$ $CD83^{+}$ helper NK cells, which control tumor dissemination and $CD8^{+}$ T cell activation through the crosstalk with DCs in the lymph nodes (40, 41, 105). Similar to other activating cytokines, such as IL-17 above, also IL-18 can display immunosuppressive features by boosting TGF-β-mediated immunosuppression (106), formation of MDSCs, and induction of PD1 expression on NK cells in mouse models (42, 107, 108). Notably, the upregulation of IL-18 binding protein (IL-18BP), which sequesters IL-18 as a physiological negative feedback mechanism, has been reported as an immune escape strategy (42, 109).

NATURAL KILLER CELL DYSFUNCTION IN THE TUMOR MICROENVIRONMENT TRIGGERED BY MYELOID-DERIVED CYTOKINES

Although the NK-myeloid crosstalk stimulates anti-tumor immunity by NK cells in the early tumor development, immunosuppressive cytokines promoting NK cell dysfunction predominate in the aggravated TME of more advanced tumors. Tumor-associated macrophages (TAMs) and MDSCs are usually the main myeloid cell populations in such TME and represent the major producers of NK cell suppressive TGF-β and IL-10 (**Figure 1A** and **Table 1A**) (110).

Transforming Growth Factor-β

As mentioned above, TGF-β is a key suppressor of NK cell migration, cytotoxicity, and cytokine production *via* transcriptional and post-transcriptional control of receptor and effector molecule expression (111). As an example, TGF-β-mediated downregulation of CX3CR1 can limit NK cell migration towards the tumor site (25); similarly, the

downregulation of activating receptors including NKG2D and NKp30 as well as the adaptor proteins DAP10 and DAP12 triggered by TGF-β diminishes human NK cell cytotoxicity *in vitro* (26–28, 111). TGF-β-mediated NK cell conversion into Eomes$^-$ ILC1 with increased expression levels of inhibitory receptors may represent an additional mechanism to reduce NK cell cytotoxicity in mouse tumor models (29), while a third mechanism is the inhibition of signaling pathways downstream of pro-inflammatory cytokines (30, 36, 111, 112). TGF-β-induced miRNA targets STAT1, which is essential for perforin expression (111), whereas blockade of IL-15-mediated mTOR activity dampens NK cell metabolism. Beyond this, TGF-β also reduces NK cell-mediated IFN-γ and TNF-α production in both human and mouse (29, 30, 36, 111, 112).

Interleukin-10

Like TGF-β, IL-10 directly inhibits IFN-γ and TNF-α production by NK cells *in vitro*. This effect is also indirectly mediated *via* inhibition of IL-12, IL-15, and IL-18 production in myeloid cells (24, 58). Yet, in the presence of IL-12 and IL-18, IL-10 has also been shown to stimulate NK cell proliferation, cytotoxicity, and IFN-γ production *in vitro via* the STAT3 signaling pathway (24, 31, 113, 114). More studies are needed to determine this complex relationship between NK cell suppressive and possibly NK cell promoting properties of IL-10.

Interleukin-32α

Another myeloid-derived immunosuppressive cytokine that can characterize the NK cell suppressive TME is IL-32α. IL-32α, which is often found highly expressed in the TME (115), inhibits IL-15-induced upregulation of perforin and granzyme B *in vitro* (34). Interestingly, dysregulated levels of IL-32α impairs human NK cell functions in chronic myelomonocytic leukemia and myelodysplastic syndrome (116).

Interleukin-1β

IL-1β is released by monocytes, DCs, and macrophages and stimulates the expansion of CD11b$^+$Gr-1$^+$Ly6C$^-$ MDSCs, which are potent inhibitors of murine NK cells *in vivo* (117). This cytokine has also been reported to maintain human NK cells in an immature state in the presence of IL-15 in secondary lymphoid tissues (23). Similarily to several cytokines above, IL-1β can also have NK cell promoting effects. One example is by indirectly promoting NK cell IFN-γ release by inducing IL-21 production in Th9 cells in mice (118).

NATURAL KILLER CELL-DERIVED CYTOKINES REGULATING MYELOID CELLS IN THE TUMOR MICROENVIRONMENT

Upon cytokine stimulation and target cell encounter, NK cells themselves produce a range of cytokines such as IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and in some cases IL-10, that in turn modulate myeloid cells (119–121) (**Figure 1A** and **Table 1B**). In this section, we will summarize how cytokines released by NK cells affect the NK-myeloid cell crosstalk.

Dendritic Cells

DCs are central in triggering immune responses by T and NK cells. However, NK cells are also important for the DC function. In addition to cell-to-cell contact, NK cell-derived IFN-γ, TNF-α, and GM-CSF play a key role in the maturation and activation of human antigen-presenting DCs in the TME and lymphoid organs (59–61, 122). Further, activated human DCs maintain the IFN-γ production and induce high mobility group box 1 (HMGB1) production in NK cells *via* secretion of IL-12 and IL-18, resulting in further DC activation and maturation (43, 65). Hence, NK cells can be central in promoting DC maturation and activation, thereby feeding the positive loop of NK-myeloid crosstalk involving DCs in the TME.

Tumor-Associated Macrophages

TAMs are often immunosuppressive and known drivers of tumor progression (123). However, macrophages in the TME have a high degree of plasticity and can even display a pro-inflammatory phenotype (M1) and release IFN-γ and TNF-α (124). *In vitro* studies have shown that IFN-γ is the main cytokine that drives classical activation of macrophages and polarizes them towards an M1-like phenotype (62). Indeed, as highlighted in a murine sarcoma model, NK cell-derived IFN-γ promotes M1 polarization of macrophages (63). Additionally, TNF-α, and GM-CSF also support an inflammatory phenotype in macrophages (62). Hence, NK cells can also have a critical role in maintaining a pro-inflammatory phenotype of TAMs.

Tumor-Associated Neutrophils

Similar to TAMs, tumor-associated neutrophils (TANs) can be both tumor-promoting or pro-inflammatory and thereby counteract tumor growth. Recently, the first studies have investigated the interaction of NK cells with TANs in mouse models. NK cell-derived IFN-γ inhibits the tumor-promoting function of TANs in murine sarcoma and lung cancer models (64). NK cell-derived GM-CSF appears to promote Neutrophils Extracellular Trap formation by neutrophils, which can support tumor metastasis (66, 67).

Myeloid-Derived Suppressor Cells

MDSCs include a heterogenous group of myeloid cells that have a markedly strong immunosuppressive ability. While NK cell-derived GM-CSF results in DC activation and macrophage polarization towards an anti-tumorigenic phenotype, GM-CSF expands MDSCs in human and murine tumors (62, 125, 126). The exact role of NK cell-derived GM-CSF in promoting MDSC expansion in the TME remains unclear but should likely not be neglected. Importantly, it is clear that NK cells can promote MDSCs in the TME also by other mechanisms. A CD73$^+$ IL-10-secreting subset of NK cells was recently identified in human sarcomas (127). While IL-10 promotes regulatory T cells (Tregs), it has also been shown to maintain the immune-suppressive functions of MDSCs in ovarian cancer (128).

THERAPEUTIC APPROACHES TO DIRECTLY STIMULATE NATURAL KILLER CELLS OR TO REVERT THE TUMOR MICROENVIRONMENT TO FAVOR NATURAL KILLER CELL ANTI-TUMOR RESPONSES

As highlighted in the previous sections, controlling the cytokine milieu in the TME is likely key to unleash the full potential of NK cell-based immunotherapies for several malignancies. Below, we will discuss ongoing and future approaches to enhance NK cell cytotoxicity in the TME (**Figure 1B**).

Administration of Cytokines

Administration of cytokines to boost NK cell anti-tumor cytotoxicity has been widely explored in the recent years. IL-2, IL-12, IL-15, and IL-21 represent the most promising cytokines under investigation. While IL-2 acts on several immune cell populations, the effects of IL-15 is mainly limited to NK cells and $CD8^+$ T cells. Promising results have been obtained in phase I clinical trials for melanoma and hematologic malignancies using ALT-803 (an IL-15 mutein/IL-15Rα complex fused with the IgG1 Fc) (129, 130). However, side-effects observed following high-dose administration of both IL-2 and IL-15 represents a major challenge along with mobilization of Tregs (131, 132). An alternative strategy to reduce the risk of side-effects while stimulating NK cells more specifically is the use of IL-2 mutants, such as IL-2 F42K and IL-2 H9 that compared to wildtype IL-2 preferentially bind IL-2Rα and thereby increases NK cell activation without inducing Treg expansion (133–135). Likewise, although the use of IL-18 therapy did not show toxicity and was ineffective in several clinical trials, a novel IL-18 mutant has been recently shown to induce NK cell anti-tumor activity (136).

To avoid the toxicities of high-dose cytokines, investigators have also addressed the use of lower doses following adoptive NK cell transfer or combined with checkpoint blockade or fused to anti-tumor antibodies. Intermediate doses of IL-2 have been explored to support adoptively infused NK cells (5). In this context, IL-2 is intended to promote persistence and expansion of the infused donor NK cells, however, data also show IL-2 mobilizes Tregs which likely counteract the effect of the transferred NK cells. As clinical protocols on adoptive NK cell transfer where post-infusion IL-2 has been omitted report similar outcomes as those with IL-2 (137), it remains unclear as to whether post-NK cell infusion IL-2 is of benefit or not. Administration of IL-15 has also been used to support adoptively infused NK cells. However, although in initial trials report this cytokine may trigger better responses, intermediate doses of IL-15 were associated with cytokine-release syndrome (CRS) when administrated subcutaneously (138). Additional investigations are needed to identify which cytokines and the window in which they promote the effect of adoptively infused NK cells.

IL-15, IL-12, and IL-21 are under clinical investigation combined with anti-CTLA4, anti-TIM3, or anti-PD-1 (139, 140). Alternative approaches that are also currently explored are based on the generation of anti-tumor antibody-cytokine fusion molecules. Trispecific killer engagers (TriKEs) fused with IL-15 have demonstrated their ability to boost NK cell functions and persistence (141, 142), whereas IL-21 fused to anti-CD20 increases mouse survival in a lymphoma model (143). Future studies have to address the clinical efficacy of these approaches.

Modulation and Prevention of Cytokine Signaling

In addition to the more direct cytokine-based treatment approaches discussed above, there are also indirect strategies explored. Targeting pathways downstream of cytokine receptors may represent an alternative or complementary approach. Inhibition of SOCS proteins is promising since blockade of the STAT5 inhibitor CIS increases NK cell-mediated anti-tumor activity (144). In preclinical models, agonists of the stimulator of IFN genes (STING) pathway induce tumor regression by stimulating IL-15 production by infiltrating myeloid cells (145). Data also support the potential of GSK3 inhibitors in promoting maturation and cytotoxicity of NK cells following expansion *ex vivo* with IL-15 (146) GSK3 inhibition increased NK cell production of TNF and IFN-γ as well as bolstered the NK cytotoxicity *per se* and *via* ADCC, which translated into better tumor control of human ovarian cancer in a mouse model. An alterantive strategy is to alter the cytokine environment in the TME by neutralizing immunosuppressive cytokines. Ongoing trials with anti-TGF-β (Fresolimumab) and inhibitors of TGF-β signaling (140) will show if such approach has potential for the future. Depletion and/or prevention of the infiltration of suppressive cytokine-producing myeloid cells in the TME *per se* represents a tempting and yet incompletely explored alternative that indirectly would bolster the anti-tumor properties of NK cells. This approach needs further attention in future studies.

CONCLUDING REMARKS

The network of cells and signals in the TME is complex and not yet fully understood. However, ample evidence show that this environment most often is NK cell suppressive, especially in more advanced disease. Yet, the NK-myeloid cell crosstalk is central in shaping NK cell anti-tumor responses and that a better understanding of this crosstalk is required to improve outcomes of NK cell-based cancer immunotherapies. While strategies directed towards boosting NK cell cytotoxicity *per se* using cytokines or drugs that modulate cytokine signaling, other complementory approaches directed towards reverting the TME to favor anti-tumor immunity is likely required to promote long-term responses.

As pointed out in this mini review, challenges hindering prompt progress in the field include the multitude of different cell types in the TME along with the context-dependent functions of several cytokines derived from both the myeloid cells and the NK cells. We predict that recent developments related to genetic engineering of NK cells along with the arsenal of new cytokine mutants as well as targeted and immunomodulatory drugs, alone or combination, can facilitate progress in the field. We foresee the use of genetically engineered NK cells to help improve their efficacy *per se* but also to resist and persist in the TME and thereby have the chance to

revert it to a more pro-inflammatory milieu optimal for initating potent durable anti-tumor immune reponses. CAR-NK cells equipped with cytokine signaling elements or dominant negative cytokine receptors are examples that hold promise. We are positive that this along with further insights in the basic biology of cytokines and cytokine signaling will help improve NK cell-based cancer immunotherapy.

AUTHOR CONTRIBUTIONS

SG, KW, MC, and SM have together outlined and written the manuscript. All authors contributed to the article and approved the submitted version.

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The Natural Killer–Dendritic Cell Immune Axis in Anti-Cancer Immunity and Immunotherapy

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Natural killer (NK) cells and dendritic cells (DCs) are crucial mediators of productive immune responses to infection and disease. NK cells and a subtype of DCs, the type 1 conventional DCs (cDC1s), are individually important for regulating immune responses to cancer in mice and humans. Recent work has found that NK cells and cDC1s engage in intercellular cross-talk integral to initiating and coordinating adaptive immunity to cancer. This NK cell–cDC1 axis has been linked to increased overall survival and responses to anti-PD-1 immunotherapy in metastatic melanoma patients. Here, we review recent findings on the role of NK cells and cDC1s in protective immune responses to cancer and immunotherapy, as well as current therapies targeting this NK cell–cDC1 axis. Further, we explore the concept that intercellular cross-talk between NK cells and cDC1s may be key for many of the positive prognostic associations seen with NK cells and DCs individually. It is clear that increasing our understanding of the NK cell–cDC1 innate immune cell axis will be critical for the generation of novel therapies that can modulate anti-cancer immunity and increase patient responses to common immunotherapies.

Keywords: cancer immunology, immunotherapy, natural killer cell, dendritic cell, innate immunity

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INTRODUCTION

Natural killer (NK) cells are important innate immune effectors that belong to the family of innate lymphoid cells (ILCs). In humans, NK cells are defined by the expression of CD56 ($CD3^{-}CD56^{+}$), while the expression of NK1.1 and NKp46 ($CD3^{-}NK1.1^{+}NKp46^{+}$) define NK cells in mice. Human NK cells can be divided into two subsets—highly cytotoxic $CD56^{dim}CD16^{high}$ cells located primarily in the peripheral blood, and cytokine-producing $CD56^{high}CD16^{dim}$ cells found predominately in secondary lymphoid organs [rev. in (1, 2)]. Similar functional classes of NK cells are found in mice and defined by the expression of TNF-receptor superfamily member CD27 and the integrin CD11b/Mac-1 [rev. in (1, 2)]. NK cells are heterogeneous and plastic in nature, which leads to variable expression of a number of markers (CD16, natural cytotoxicity receptors, and others) that can be used to further classify NK cells based on their differentiation, tissue location, and microenvironmental cues [rev. in (3, 4)].

NK cell effector functions consist of direct cytotoxicity to infected, transformed, and/or physiologically stressed cells [rev. in (5, 6)] and modulation of immune responses through the

production of cytokines and chemokines [rev. in (2, 7)]. These functions are regulated by an intricate balance of germline-encoded inhibitory and activating receptors [rev. in (2, 4)]. Inhibitory receptors expressed by NK cells include Ly49-type inhibitory receptors (in mice), killer immunoglobulin-like receptors (KIRs; in humans), and the CD94-NKG2A heterodimer (in humans and mice) [rev. in (2)]. Activating receptors expressed by NK cells include the natural cytotoxicity receptors (NCRs) NKp46, NKp44, and NKp30, the lectin-like type 2 transmembrane receptor NKG2D, DNAX accessory molecule 1 (DNAM1/CD226), and adhesion molecule lymphocyte function-associated antigen-1 (LFA-1) [rev. in (2)]. These receptors balance inhibitory signals delivered through the recognition of self MHC-I molecules and activating ligands that are upregulated in response to cellular stress, infection, or transformation [rev. in (2, 8, 9)]. Additionally, NK cell function may be regulated by the immune checkpoint molecules PD-1, CTLA-4, TIGIT, LAG3, and TIM-3 [rev. in (4, 10)].

NK cells are integral to antiviral responses, anti-cancer immunity [rev. in (2, 7, 11, 12)], and have a role in cancer prevention (13). NK cell abundance in the tumor microenvironment (TME) is associated with greater overall survival in patients with melanoma (14–18), hepatocellular carcinoma (19), pulmonary adenocarcinoma (20), renal cell carcinoma (21), gastric cancer (22), breast cancer (23), squamous cell lung cancer (24), non-small cell lung cancer (25), and neuroblastoma (26). The protective immunity provided by NK cells is controlled by their direct cytotoxicity and the production of immunomodulatory cytokines and chemokines that sculpt local and distant immune cell responses [rev. in (2, 4, 7, 11, 12, 27)]. This critical association of NK cells with controlled tumor growth and metastasis highlights their role as dynamic anti-cancer agents [rev. in (4, 7)].

Dendritic cells (DCs) are an innate immune myeloid cell that provide an essential link between the innate and adaptive immune responses. As specialized antigen-presenting cells DCs play a key role in initiating T cell-mediated antigen-specific immunity and tolerance [rev. in (28)]. DCs perform this role by continuously sampling and presenting antigens to T cells *via* major histocompatibility complex (MHC) I and II, a process that is greatly increased upon activation [rev. in (29, 30)]. DC functions are shaped by the integration of environmental cues sensed by pattern recognition (PRRs) and cytokine receptors [rev. in (29, 31)].

Conventional DCs can be classified most simply as cDC1 or cDC2, both of which express CD11c and MHC-II in humans and mice [rev. in (32)]. cDC1s and cDC2s are defined by, and require, distinct transcription factors and cell surface markers, possess differential growth factor requirements, and, critically, undertake distinct functions (29). cDC1s rely on the transcription and growth factors BATF3, IRF8, BCL6, ID2, and FLT3L for development, and can be defined by expression of the chemokine receptor XCR1 and the C-type lectin endocytic receptor CLEC9A [rev. in (29, 32–34)]. Human cDC1s can be further identified by BDCA3 expression, while murine cDC1s can be defined as $CD103^+$ or $CD8\alpha^+$ populations [rev in (29)]. cDC2s, on the other hand, depend on IRF4 and ZEB2 for development and express CD11b and CD172a [rev. in (29, 32)]. Classically, cDC1s are thought to induce robust $CD8^+$ T cell responses, while cDC2s are thought to be more important for $CD4^+$ T cell responses (29). cCD1s and cDC2s both play roles in anti-cancer immunity [rev. in (32) (35)], but we will focus on the protective effects of cDC1s in this review.

The presence of cDC1s in the TME is correlated with improved clinical outcomes in numerous cancers and serves as a strong biomarker for responsiveness to anti-PD-1 immunotherapy in metastatic melanoma patients (16, 17, 26, 32, 36–38). While cDC1s are rare in human and murine tumors, they efficiently cross-present exogenous antigens to $CD8^+$ T cells, are capable of initiating *de novo* cytotoxic $CD8^+$ T cell responses after migrating to the tumor-draining lymph node, and play an integral role in re-priming $CD8^+$ T cells directly in the TME [rev. in (29, 32, 36)] (**Figure 1**). Further, cDC1s can contribute to T helper (Th) 1 cell polarization of naive $CD4^+$ T cells [rev. in (29, 32, 36)]. Augmenting cDC1 frequency in the tumor has resulted in enhanced tumor responses (16, 17, 32, 39). Alternatively, the *in vivo* depletion of cDC1s is associated with failed tumor rejection, immune escape, and an inability to respond to multiple T-cell immunotherapies—such as immune checkpoint blockade and adoptive T cell therapy [rev. in (29, 32)]. Accordingly, cDC1s provide essential roles in anti-cancer immune responses and offer promising immunotherapeutic targets against cancer.

THE NATURAL KILLER–DENDRITIC CELL INNATE IMMUNE AXIS IN CANCER

There is a rich literature surrounding the individual functions of NK cells and DCs in anti-tumor immunity (2, 7, 16, 17). Recent work supports an integral role for NK cells in shaping DC maturation and promoting DC recruitment, retention, and/or survival in the tumor (2, 7, 16, 17). It is well known that NK cells can perform DC editing, a quality control process in which activated NK cells selectively kill immature DCs to ensure successful T cell priming by mature, immunogenic DCs [rev. in (2, 4)]. NK cell editing of DCs established a direct, functional relationship between NK cells and DCs [rev. in (2, 4)].

Emerging evidence indicates that NK cell-cDC1 interactions have a profound effect on anti-cancer immunity (**Figure 1**). NK cells promote the recruitment of cDC1s into the TME through the production of the chemokines CCL5, XCL1, and XCL2 (16). This pathway is abrogated by the presence of tumor-derived prostaglandin E2 (PGE_2), which leads to impaired NK cell function and downregulation of CCR5 and XCR1 receptors on cDC1s (16). *CCL5* expression has further been linked to NK cells and the abundance of cDC1s in neuroblastoma patient samples (26). NK cells also produce FLT3LG, the formative cytokine for cDC1s, in the TME (17) (**Figure 1**). *FLT3LG* expression and NK cell abundance in the tumor are correlated with increased cDC1 levels, better overall survival, and increased responses to anti-PD-1 immunotherapy in metastatic melanoma patients (17). Further, cDC1s and NK cells were positively correlated with

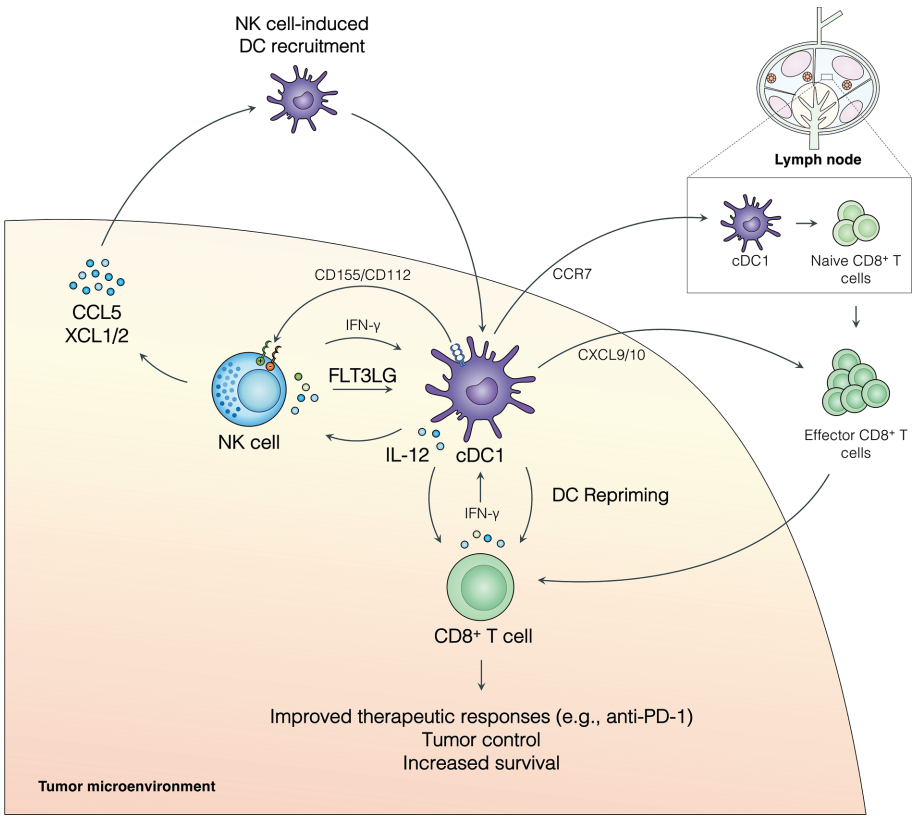

FIGURE 1 | The NK cell-cDC1 axis modulates the TME to boost immune responses to cancer. NK cell production of the chemokines CCL5, XCL1, and XCL2 recruits cDC1s to the tumor. Additionally, FLT3L produced by NK cells increases survival and/or differentiation of cCD1s in the tumor. IFN-γ produced by NK cells enhances cDC1 antigen presentation and maturation and leads to cDC1 production of IL-12, which can increase NK cell activity. cDC1s can further modulate NK cell activity through the expression of CD155 and/or CD112 which can signal through inhibitory receptors (TIGIT and CD96) or activating receptors (CD226/DNAM1) expressed on NK cells. Activated cDC1s in the TME upregulate CCR7 and migrate to the tumor-draining lymph node, where they activate naive $CD8^+$ T cells. Effector $CD8^+$ T cells are recruited to the TME, at least in part, by cDC1-produced CXCL9/10 and, critically, undergo local restimulation by cDC1s. Repriming of $CD8^+$ T cells in the TME increases tumor control, patient survival, and improves responses to anti-PD-1 immunotherapy.

FLT3LG expression, T cell infiltration, increased survival, and the expression of checkpoint molecules (PD-1 and PD-L1) in the tumors of glioblastoma patients (26). NK cell production of FLT3LG may increase cDC1 survival in the TME, but other mechanisms, such as increasing the differentiation of precursor DCs (pre-DCs), remain possible (17). The importance of FLT3L control of cDC1s in the tumor was further demonstrated in a recent murine model of pancreatic ductal adenocarcinoma (PDAC) where FLT3L and anti-CD40 combination therapy restored cDC1 infiltration, improved $CD8^+$ T cell and Th1 control of tumor growth, and boosted responses to external radiation therapy (40, 41). These data suggest that NK cells play an important role in recruiting and retaining cDC1s in the tumor, which subsequently activate protective anti-tumor $CD8^+$ T cell responses (**Figure 1**).

The NK cell-cDC1 innate immune axis is undoubtably a bidirectional relationship with cDC1s playing an important role in regulating NK cells in the tumor. The role of DCs in shaping NK cell responses in cancer has been thoroughly reviewed recently [rev. in (42)]; thus, we will provide a brief description of these mechanisms and how they may affect the NK cell-cDC1 axis. Activated DCs produce IL-12, which stimulates NK cells and anti-tumor T cell immunity [rev. in (32)] (43) (**Figure 1**). Further, IL-12 production by tumor-infiltrating DCs is required for effective anti-PD-1 immunotherapy responses (43). In addition to cytokines, DCs express a number of cell adhesion molecules with immune regulatory functions that can regulate NK cells [rev. in (44–47)] (**Figure 1**). CD155 and CD112 are two such molecules that are upregulated upon DC maturation and activation and can signal through the receptors CD226/DNAM1, TIGIT (T cell immunoreceptor with immunoglobulin and ITIM domains), and CD96 expressed on NK cells [rev. in (44–47)]. CD226 ligation by CD155 or CD112 can induce NK cell activation, while ligation of TIGIT inhibits NK cells (rev. in (44–47)]. Ligation of CD96 acts as an inhibitor of NK cell responses in mice, but the role of CD96 in human NK cells remains less clear (45). A recent study found that a subset of CD112-expressing DCs in the TME of human hepatocellular carcinoma interact with NK cells through CD226 and TIGIT (48). These data suggest that expression of CD155 and CD112 by

DCs can modulate NK cell responses in the tumor. Importantly, NK cell expression of CD226 has been linked to the NK cell-dependent killing of immature and mature DCs expressing CD155 and CD112 (49), suggesting this pathway may play multiple roles in shaping the NK cell-cDC1 axis. The activation of NK cells by DCs (e.g., IL-12, CD226/CD155) may act as a feed-forward loop to increase DC activation through the induction of NK cell production of chemokines and cytokines, subsequently improving anti-tumor immune responses [rev. in (32)] (43). More research is needed to determine how DC-dependent stimulation or inhibition of NK cells may shape production of XCL1, XCL2, CCL5, and FLT3LG in the TME. However, a bidirectional relationship between NK cells and cDC1s in the TME clearly exists and targeting cDC1 factors that influence NK cell activity may be another tool to increase immune responses to cancer.

NK cells and cDC1s make stable and close interactions in the TME of mouse ectopic B78 melanoma tumors (17) and multiplex immunofluorescence imaging of human glioblastoma found a similar close interaction (26). Given these findings, it is intriguing to hypothesize that local concentrations of chemokines and cytokines (e.g., FLT3LG, IFN-γ, or IL-12), or receptor-ligand interactions (e.g., CD226/CD155) that require cell-cell contacts, may control the NK cell-cDC1 axis. The spatial organization of NK cells and cDC1s in the tumor may be an important factor in controlling the NK cell-cDC1 axis (17, 26), but clearly more data is needed to fully define this spatial regulation.

The NK cell-cDC1 axis is integral for controlling immune responses to cancer and is linked to increased patient survival and/or responses to immunotherapies in metastatic melanoma, head and neck squamous cell carcinoma, triple negative breast cancer, and neuroblastoma (16, 17, 26). It is important to note that in certain tumor settings NK cells have been shown to have inhibitory effects on DC functions (50–52) and DCs can have inhibitory effects on NK cell functions [rev. in (44–47)]. These findings suggest that there may be tumor-specific regulation of the NK cell-cDC1 axis and highlight the need for more detailed studies of this innate immune axis across other cancer indications. The data presented here suggest that a better understanding of the mechanisms that influence the bidirectional relationship of NK cells and cDC1s in the tumor could be used to enhance existing therapies or reveal new therapeutic avenues to protect patients from cancer.

NK CELL REGULATION OF cDC1s IN PRE-CLINICAL MODELS OF CANCER

The NK cell-cDC1 axis has been defined within the TME and, as such, these cells are susceptible to tumor-induced immune suppression [rev. in (53–55)]. Conditions in the TME can lead to impairment of antigen presentation, activation of negative costimulatory signals (i.e., immunological checkpoints), and production of immunosuppressive and pro-apoptotic factors [rev. in (53–55)]. Metabolic restrictions within the tumor are also known to inhibit immune responses [rev. in (56)]. Namely, nutrient and oxygen deficiency and increased concentration of metabolic products (e.g., adenosine, lactic acid, retinoic acid) (57–59) can pose significant challenges to infiltrating immune cells [rev. in (32)]. As such, diverse strategies have been explored to improve effector cell responses in cancer. Importantly, the NK cell-cDC1 axis must be studied in the context of these suppressive signals in the TME.

A number of studies have shown that shifting the cytokine milieu or metabolic factors in the TME to modulate NK cells activity can lead to protective immune responses to cancer [rev. in (2, 7)]. Here, we will review recent studies exploring these novel mechanisms and, further pose the question: are the protective responses seen by targeting NK cells, at least partially, due to changes in NK cell regulation of cDC1s in the tumor?

Adenosine 2A Receptor

Adenosine is an immunosuppressive metabolite present at high levels in the TME [rev. in (60–63)]. Adenosine signaling through A2A adenosine receptor (A2AR) on immune cells can dampen anti-tumor immune responses [rev. in (64)]. In a recent study, A2AR signaling was shown to inhibit NK cell maturation in mice at homeostasis and in the tumor (65). Transcriptional profiling of *A2AR*-deficient NK cells revealed decreased expression of the receptor tyrosine kinase *KIT* (*CD117*) and the interleukin-18 (IL-18) receptor *IL18R1* (65). This transcriptional profile is interesting because it is opposite to a population of pro-tumorigenic KIT^+ NK cells found to deplete peripheral pools of DCs (51). These findings suggest that inhibition of A2AR signaling in NK cells may lead to improved anti-tumor activity through a maintenance of DC populations. Further, *A2AR*-deficient NK cells were found to have enhanced maturation, maintain a proliferative advantage over wildtype NK cells, and protect against tumor development in a transplantable BRAF-melanoma tumor model (65). Taken together, these findings demonstrate that pairing A2AR antagonism with NK cell-based immunotherapies may provide a combinatorial strategy to improve therapeutic efficacy (65). Furthermore, these studies suggest that A2AR inhibition may help maintain the NK cell-cDC1 axis. Additional work will provide more insight into the role of the NK cell-cDC1 axis in the anti-tumor protection provided by A2AR antagonism.

Interleukin-18 (IL-18)

Treatment with high doses of IL-18 induce increased inflammation (66) and improved responses to immune checkpoint blockade (67) and CAR-T cells (68). A recent study generated a decoy-resistant IL-18 cytokine (DR-18) that has high binding affinity for the IL-18 receptor, IL-18Rα, but is unable to bind to the decoy receptor, IL-18BP (69). DR-18 treatment protects animals from ectopic models of melanoma (YUMMER1.7) and colorectal cancer (MC38) (69). Further, in β2m-deficient tumors, control of tumor growth by DR-18 requires NK cells, and DR-18 treatment increases the abundance of a cluster of NK cells that produce various effector molecule transcripts (*IFNG*, *PRF1*, *GZMB*), as well as the chemokines *CCL5* and *CCL4*, in the TME (69). CCL5 production by NK cells has previously been linked to increased recruitment of cDC1s to the TME (16). These data suggest that, in certain situations, NK cells upregulate chemokines that could increase cDC1 levels in the TME

in response to DR-18. However, in wildtype tumors, DR-18 acts directly on T cells in the TME and functions independently of cDC1s (69). Thus, it is intriguing to speculate that, depending on the setting, DR-18 treatment may protect against cancer in multiple ways, including by modulating the NK cell-cDC1 innate immune axis. However, it appears that direct stimulation of T cells in the TME may be the primary driver of protection, at least in the tumor models tested thus far.

TIGIT and CD96

TIGIT and CD96 are inhibitory receptors that signal through binding the shared ligand CD155 and CD112 or CD111, respectively (45). The role of TIGIT and CD96 in immune responses to cancer has been thoroughly reviewed elsewhere (45). As such, we will focus our discussion to recent findings regarding the role of TIGIT in regulating NK cell responses to cancer.

$TIGIT^+$ NK cells are found in human hepatocellular carcinoma (70), human colorectal cancer, and a number of murine tumor models, including breast cancer (4T1), melanoma (B16), colon cancer (CT26), and MCA-induced fibrosarcoma (71). Interestingly, genetic or antibody blockade of TIGIT signaling on NK cells was found to increase NK cell function and boost cytotoxic $CD8^+$ T cell responses and protective T cell-mediated memory responses (71). NK cells are required for the protective effects of anti-TIGIT alone or in combination with anti-PD-L1 immunotherapy and, in fact, are partially required for the protective immune responses caused by anti-PD-L1 treatment alone (71). Protective responses induced by anti-TIGIT treatment partially require IFN-γ, and a role for direct NK cell cytotoxicity cannot be ruled out (71). It remains to be seen if these protective effects of NK cells in response to anti-TIGIT therapy function through the modulation of cDC1s in the TME, but, given the strong effects this NK cell-dependent treatment has on $CD8^+$ T cells, it is an intriguing hypothesis.

MODULATION OF THE NK CELL-cDC1 AXIS IN THE CLINIC

NK cell-directed immunotherapies show great promise in the clinic [rev. in (2, 7)]. However, it is unknown if current NK cell-based therapies function through increasing cDC1s in the tumor. Here, we will discuss a recent clinical treatment, the intratumoral electroporation of a plasmid encoding IL-12 (tavokinogene telseplasmid; "tavo") (72–74), and explore its potential role in shaping the NK cell-cDC1 axis.

Interleukin-12 (IL-12), regulates NK cell and T cell responses, promotes Th1 polarization, and is a potent regulator of immune responses to infection and cancer [rev. in (75, 76)]. Systemic treatment with recombinant IL-12 (rIL-12) has shown efficacy in animal models of cancer, but these treatments are associated with modest clinical response and serious adverse events in patients [rev. in (77)]. Alternatively, intratumoral electroporation of tavo (IL-12) was found to be safe in a Phase I clinical trial, demonstrated preliminary efficacy by increasing intratumoral IL-12 and IFN-γ, and led to remission in several patients (74). Two recent Phase II clinical trials of tavo (IL-12) electroporation found that this treatment leads to an increase in NK cell and cDC1-related transcripts in the tumor, an increase in $CD8^+$ T cells in the tumor, and activation of systemic immune responses in treated patients (72, 73). In these studies, it was proposed that intratumoral electroporation of tavo (IL-12) appears to boost NK cell abundance in the tumor, which leads to an increase in abundance of protective cDC1s, increased T cell responses, and, in some patients, durable responses to treatment (72). It was further shown that intratumoral electroporation of tavo (IL-12) can increase immune infiltration in poorly infiltrated metastatic melanoma tumors and subsequently increase patient responses to anti-PD-1 immunotherapy (73).

These studies suggest that targeting the NK cell-cDC1 innate immune axis with the electroporation of IL-12 into the tumor may have efficacy as a single agent and may shape the TME to be more responsive to anti-PD-1 immunotherapy. It is important to note that electroporation of tavo (IL-12) could act directly on T cells to shape immune responses to melanoma, and thus more basic and clinical research is needed to fully elucidate the mechanisms by which this treatment is providing protection to patients. However, these correlative findings are consistent with IL-12 increasing NK cell activity in the TME and subsequently boosting cDC1 abundance and $CD8^+$ T cell responses to the tumor.

CONCLUSION

As highlighted in this review, NK cells and cDC1s have a rich literature demonstrating their important individual roles in supporting protective immune responses to cancer. We propose that at least some of these protective roles are related to the cross-talk between NK cells and cDC1s. We have provided evidence that the NK cell-cDC1 axis is a bidirectional relationship with each cell type shaping the responses of the other. We also highlight pre-clinical and clinical studies that suggest that targeting the NK cell-cDC1 axis may provide novel pathways to increase immune responses to cancer. We propose that the NK cell-cDC1 axis should be considered in future studies exploring the individual association of these cell types in controlling immune responses to cancer. Clearly, the NK cell-cDC1 innate immune axis has important roles in shaping immune responses to cancer and future studies are needed to determine exactly how this axis can be targeted and manipulated as a tool to boost immune responses to cancer.

AUTHOR CONTRIBUTIONS

EP and KB conceived, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Interaction Between MDSC and NK Cells in Solid and Hematological Malignancies: Impact on HSCT

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Myeloid derived suppressor cells (MDSC) are heterogeneous populations that through the release of soluble factors and/or by cell-to-cell interactions suppress both innate and adaptive immune effector cells. In pathological conditions, characterized by the presence of inflammation, a partial block in the differentiation potential of myeloid precursors causes an accumulation of these immunosuppressive cell subsets both in peripheral blood and in tissues. On the contrary, NK cells represent a major player of innate immunity able to counteract tumor growth. The anti-tumor activity of NK cells is primarily related to their cytolytic potential and to the secretion of soluble factors or cytokines that may act on tumors either directly or indirectly upon the recruitment of other cell types. NK cells have been shown to play a fundamental role in haploidentical hemopoietic stem cell transplantation (HSCT), for the therapy of high-risk leukemias. A deeper analysis of MDSC functional effects demonstrated that these cells are capable, through several mechanisms, to reduce the potent GvL activity exerted by NK cells. It is conceivable that, in this transplantation setting, the MDSC-removal or -inactivation may represent a promising strategy to restore the anti-leukemia effect mediated by NK cells. Thus, a better knowledge of the cellular interactions occurring in the tumor microenvironment could promote the development of novel therapeutic strategies for the treatment of solid and hematological malignances.

Keywords: natural killer cells, myeloid-derived suppressor cells, hematopoietic stem cell transplantation, tumor microenvironment, hematological malignancies

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INTRODUCTION

Tumor microenvironment (TME) consists of an assortment of tumor and non-tumor cells (including mesenchymal stromal cells, endothelial cells, regulatory T-cells, and myeloid-derived suppressor cells), as well as soluble components. Tumor associated (TA)-cells may favor neoplastic transformation, tumor growth, and metastasis thus contributing to tumor escape from host immunity. In addition to TA-cells, TME also contains immune cells including innate and adaptive lymphocytes. A growing body of evidences has revealed the existence of a close relationship between tumor and immune components. Many of the interactions between TME and tumor infiltrating (TI) immune cells are already well-known (1). Consistent positive prognostic correlations have been reported for T-cells, especially cytotoxic T-cells, in different tumor types (2). In particular, TI-lymphocyte density can influence prognosis within each tumor, lymph

node, and metastasis (TNM) stage, complementing or even outperforming pathological criteria alone, as shown in colorectal and lung cancers (3–5). In order to develop strategies to overcome immunosuppression and tumor escape it is important to further unravel the cellular interactions occurring in TME. This contribution is focalized on the polymorphonuclear (PMN)-MDSC, an important, strongly immunosuppressive myeloid component, which may greatly impair the anti-tumor defenses in particular those mediated by NK cells.

MDSC in Physiological and Pathological Condition

One important cellular subset present in TME is represented by MDSC. These cells were first identified in 1970 as a heterogeneous group of immune cells with immature features derived from a common myeloid progenitor (CMP) (6). They possess high immunosuppressive and pro-tumorigenic capabilities and actively cooperate with other myeloid regulatory cells as tumor-associated neutrophils (TANs), tumor-associated macrophages (TAMs), and regulatory dendritic cells to favor cancer development (7). MDSC are usually classified in two or three classes in mice and humans, respectively. In mice, MDSC are classified into monocytic (M-MDSC) ($CD11b^+Ly6C^{hi}Ly6G^-$) and PMN-MDSC ($CD11b^+Ly6C^{lo}Ly6G^+$ cells) subsets. In humans, the granulocytic and the monocytic subsets are classified as Lin^-(CD3, CD19, CD56), $CD11b^+CD33^+CD15^+CD66b^+CD14^-HLA\text{-}DR^{low/-}$ and $Lin^-CD11b^+CD33^+CD15^-CD14^+HLA\text{-}DR^{low/-}$cells, respectively. A new subset of immature and early-stage MDSC (e-MDSC) has been found in humans and classified as Lin^- $HLA\text{-}DR^{low/-}$ $CD11b^+$ $CD14^-$ $CD15^-$ $CD33^+$. Recently, the lectin-type oxidized LDL receptor-1 (LOX-1) molecule has been suggested as novel marker to discriminate MDSC from neutrophils (8–10).

In physiological condition, the CMP differentiate into neutrophils or monocytes. In pathological conditions such as cancer or infection the inflammatory milieu (e.g., GM-CSF, TNF-α, VEGF, and PGE2) induces the CMP differentiation into M-MDSC, and subsequently TAM, or PMN-MDSC that can differentiate into TAN. In addition, chronic inflammatory conditions induce release of soluble mediators that are responsible for MDSC accumulation due to their reduced susceptibility to Fas-mediated apoptosis (11). In this setting, MDSC can help to control excessive inflammation, by reducing both innate and adaptive immune responses (12, 13), while their reduction normally occurs following inflammation resolution. Conversely, if MDSC numbers do not decrease, they can be associated to disease progression. Indeed, a continuous inhibitory effect of immune response can interfere with tissue homeostasis, energy metabolism, and dead cell removal.

In cancer, MDSC activity is mainly regulated by three key events: myelopoiesis impairment, MDSC migration to tumor site and subsequent activation. Thus, deregulated myelopoiesis leads to accumulation of immature MDSC in bone marrow that are subsequently recruited to primary and metastatic tumor sites by tumor-released chemokines. Due to their potent immunosuppressive and pro-tumorigenic potential, high levels of MDSC, especially PMN-MDSC, have been observed in high grade cancers and are correlated with poor prognosis, treatment resistance and reduced overall survival in solid cancers (14–18). Breast, ovarian and gastric cancer cells secrete CCL2 that recruits MDSC and sustains tumor growth (19). In addition, $CCR2^+$MDSC can support tumor growth in colon-rectal-cancer (CRC)-bearing mice (20). CXCL1 is another cytokine, highly expressed in CRC that exerts chemoattractant activity on $CXCR2^+$MDSC. Moreover, MDSC also express CCR5 that has been suggested to be involved in MDSC migration to tumor site (21, 22). Regarding MDSC recruitment, it has been demonstrated that the hematopoietic 5-lipoxygenase (5LO), a metabolite of the arachidonic acid implied in colon carcinogenesis, is involved into MDSC migration (23). In addition, MDSC can regulate and induce their recruitment by a positive feedback. Indeed, Reactive Nitrogen Species produced by MDSC lead to chemokine nitration that in turn recruit MDSC (24).

Following recruitment, MDSC are activated through many mechanisms. In particular, PGE2 can induce STAT3 phosphorylation that mediates both MDSC activation (25) and inhibition of their physiological differentiation toward neutrophils or monocytes (26, 27). Histamine can modulate the expression of Arginase-1 (ARG-1) and Nitric oxide synthase (iNOS) in M-MDSCs and PMN-MDSC, respectively (28, 29). Hypoxia can induce the Hypoxia-inducible Factor 1 alpha (HIF-1α) that in turn increases ARG-1 and iNOS activation in MDSC (30).

While many studies on the involvement of MDSC in hematological disorders have been performed, their actual role is still debated. High numbers of PMN-MDSC were reported in chronic myeloid leukemia (CML), possibly playing a role in CML cell immune escape (31–33). The increased numbers of PMN-MDSC, evaluated at diagnosis of CML, return to normal levels after treatment with Imatinib. In acute leukemia, the role of MDSC is not fully defined. Patients with acute myeloid leukemia (AML) display increased numbers of MDSC in PB and BM as compared to patients with acute lymphoblastic leukemia (ALL) and a significant correlation exists with conventional prognostic factors at diagnosis (34). Moreover, a significant decrease of MDSC was observed only in those patients in complete remission after treatment. In pediatric patients, the frequency and the strength of the immunosuppressive function correlated with classical prognostic markers such as MRD and $CD20^+$ blast cell counts and with response to therapy. In addition, patients in remission have been reported to lose MDSC suppressive function further corroborating the effect of these cells in favoring immune evasion mechanisms (35, 36). In diffuse large B-cell lymphoma (37), indolent lymphoma (38), chronic lymphocytic leukemia (39), and Hodgkin lymphoma (40), the frequency of circulating MDSC has been correlated with poor prognosis. Recent studies, in the S100A9 knockout transgenic mice, revealed that MDSC are also involved in the pathogenesis and progression of Multiple myeloma (MM) (41). MDSC isolated from the PB of patients with MM display an inhibitory effect on T-cells which could be abrogated by drugs inhibiting arginase-1 and iNOS

activity (42). Available data would suggest that MDSC represent a sizable subset present in MM patients that may play a role in the pathophysiology of the disease by favoring survival and proliferation of malignant plasma cells as a consequence of their suppressive activity on anti-tumor immune response. Finally, recent evidence suggests that MDSC have also a predominant role in the pathophysiology of Immune Thrombocytopenia and Chronic Idiopathic Neutropenia (43–45).

Immunosuppressive Mechanisms Exerted by MDSC

Several studies described different mechanisms adopted by MDSC to exert their immunomodulatory function either by mechanisms that require cell-to-cell contact or by the release of soluble factors. They can directly inhibit the innate or adaptive immune system or indirectly contribute to tumor progression through regulation of angiogenesis or cell motility. In particular, MDSC-derived Nitric oxide (NO) can suppress proliferation of T-cells by inhibiting the Jak/STAT5 pathway and inducing T-cell apoptosis (46). MDSC-derived NO can also impair T-cell migration by reducing E-selectin expression on endothelial cells (47). In addition, oxygen reactive species (ROS) produced by MDSC can induce apoptosis in T-cells by decreasing expression of the T-cell receptor (TCR) ζ-chain (48, 49). Immunosuppression of T-cell response by MDSC may be accomplished through cleavage of L-selectin on $CD4^+$ and $CD8^+$ T-cells by ADAM metallopeptidase domain 17 (ADAM17) and disintegrin thus impairing T-cell trafficking to tumor sites (50). Moreover, via the ARG-1 or IDO enzymes, MDSC can deprive TME of the amminoacids required by T-cells for proliferation (51–53). Secretion of IL-10 and TGF-β by MDSC represents another mechanism to induce immunosuppression trough Treg induction (54). In lung cancer, IL-10 secreted by M-MDSC has been reported to be in part responsible for Treg induction *in vitro* (55).

Notably, TGF-β and IL-10 can also mediate immunosuppression indirectly, by inducing CD39 and CD73 expression on MDSC that are receptors involved in ATP/ADP hydrolysis and AMP cleavage, respectively, therefore MDSC affect T and NK cell response also by interfering with the adenosine metabolism (56, 57).

Huang B and colleagues showed that IFN-γ secreted by T-cells leads MDSC to release IL-10 and TGF-β that in turn induce Treg (54). In addition to IL-10 and TGF-β, it has been demonstrated that cell-to-cell contact and the CD40 expression on MDSC surface are also required for Treg expansion (58). Contact-dependent mechanism has been demonstrated also in hepatocellular carcinoma where MDSC induce Treg expansion (59). Different factors present in TME (transmembrane TNF-α, TGF-β, lipopolysaccharide, Semaphorin 4D, NKG2D ligands and extracellular vesicles) and hypoxia, can upregulate the secretion of IL-10 by MDSC (60–63). In addition, other factor such as HIF-1α increases the immunosuppressive activity of MDSC by inducing Programmed Cell Death 1 (PD-1) expression and by upregulating the V-domain of Ig suppressor of T-cell activation (VISTA) (64, 65).

Notably, angiogenesis represents another immunosuppressive mechanisms used by MDSC and it is mediated by VEGF upregulation. It has been demonstrated that MDSC, previously activated with VEGF, have a more potent inhibitory activity (66). MDSC can also secrete proangiogenic factors as Metalloproteases (MMP2, MMP8, MMP9, MMP13, and MMP14) that can disrupt the extracellular matrix thus facilitating the extravasation (67).

Another mechanism able to induce immunosuppression is represented by the release of protumorigenic mediators such as S100A8/A9 by MDSC and tumor cells. These factors are capable to induce M2-macrophage polarization and MDSC chemotaxis in TME that results in immunosuppression of effector cells (68, 69).

NK Cells in Tumors

Natural killer (NK) cells belong to the innate lymphoid cell (ILC) family. ILCs have recently been classified into five different subsets: NK cells that represent killer ILC, and ILC1, ILC2 ILC3, and Lymphoid tissue-inducer cells (LTi) that belong to helper-ILC. Unlike NK cells, the other ILC subpopulations were discovered only recently because they are relatively infrequent and are prevalently located in mucosal tissues and secondary lymphoid organs (70).

NK cells are present primarily in the PB, spleen and bone marrow, but they can infiltrate tissues and are also found in the liver, lungs, gut, lymph nodes and uterus (71–73). Two major subsets of PB-NK cells were identified on the basis of the surface density of CD56 antigen ($CD56^{bright}$ and $CD56^{dim}$). $CD56^{dim}$ NK cells are predominant in PB, display a potent cytolytic activity and release cytokines shortly after receptor-mediated signaling. $CD56^{bright}$ predominate in tissues and secondary lymphoid organs, are poorly cytolytic, while they produce cytokines (74, 75).

The anti-tumor activity of NK cells is primarily related to their cytolytic potential and to the secretion of soluble factors or cytokines that may act on tumors either directly or indirectly upon recruitment of other cell types. NK cell cytotoxicity is induced by surface receptors capable of recognizing ligands that are primarily expressed by tumor cells, but not by most normal resting cells (76). These receptors may induce NK cell activation resulting in tumor cell lysis and secretion of cytokines. The major activating NK receptors include Natural cytotoxic receptor (NCR) (i.e., NKp46, NKp44, and NKp30), DNAM-1 and NKG2D. In addition, NK cells, in most instances, do not kill normal cells thanks to a fail-safe mechanism involving inhibitory receptors specific for HLA-class I molecules. These include killer Ig-like receptors (KIRs) that recognize allotypic determinants of HLA-cl I molecules shared by different groups of alleles and CD94/NKG2A that recognizes HLA-E (77).

During cancer progression, the transformed cells display a decrease or even a loss of the surface expression of MHC-I (78) while strongly upregulate or acquire the expression of ligands for activating NK receptors: two events necessary for NK activation and induction of anti-tumor immune cell responses (79). However, the frequent downregulation of activating receptor expression in NK cells may result in decreased activity leading

to increases in tumor expansion and metastases. Indeed, it is well-known that tumor cells may create an immunosuppressive environment through the modulation of inhibitory checkpoints expression on NK cells in order to evade their cytolytic activity and to induce tumor immune escape (79–82).

Some tumors are poorly permeable to NK cells, as the TME may affect their ability to infiltrate the tumor mass. In particular, colorectal carcinoma and melanoma lesions display poor NK cell infiltration (83, 84). On the other hand, NK cell infiltration has been described in other types of tumor and a high number of NK cells in neoplastic tissues has been associated with better survival. For example in breast cancers, tumor-infiltrating (TI) NK cells are used as biomarkers to predict the response to anti-HER2 mAbs therapy (85–87). In the Head and Neck cancers the presence of TI-NK cells correlated with a longer survival (88). Similarly, NK cell infiltration of renal tumors is associated with a good prognosis (85). On the contrary, NK cell infiltration has no impact on clinical outcome in non-small-cell lung cancer (NSCLC) (89, 90).

NK-MDSC Interactions

NK cells may interact with tumor cells and other cells present in TME through three fundamental pathways, i.e., cell-to cell contact, secretion of soluble molecules in the extracellular milieu, and release of extracellular vesicles (EVs) (91). In cancer patients and tumor mice models, an inverse correlation between the presence of MDSC and NK cells exists (92).

In mice, the mechanism of NK cell inhibition exerted by MDSC is mainly related to cell-to-cell contact and it requires TGF-β (93). Membrane-bound TGF-β on MDSC has been shown to induce NK cell anergy thus impairing their cytotoxic capability and reducing NKG2D expression and IFN-γ production (94). Another study reported IL-33 as a novel player in MDSC-NK interaction. Following stress or damage, IL-33 is secreted by endothelial and epithelial cells and recruits both pro-tumorigenic or anti-tumorigenic immune cells (95, 96).

Furthermore, it has been reported that, in the presence of IL-1β, a novel subset of $Ly6C^{neg}$ MDSC with higher inhibitory properties to NK cells expands in mice (97).

In humans, the inhibition of IFN-γ production by NK cells is related to a NKp30-dependent mechanism (98). MDSC can impair NK cell activity also by interfering with NK FcR-mediated cytotoxicity, as shown in cancer patients NK cells displaying reduced antibody-dependent cytotoxicity and production of cytokines (99).

The IFN-γ and other molecules present in the inflammatory microenvironment are able to promote the expansion of MDSC that, in turn, release high amount of IL-10. IL-10 is considered an anti-inflammatory cytokine capable of inhibiting the release of inflammatory cytokines playing an important role in anti-tumor immunoresponse. In particular, IL-10 may induce a pro-tumorigenic microenvironment affecting both NK cell and $CD8^+$cytotoxic T lymphocyte activation and promoting a switch toward type 2 immunoresponse. Targeting either MDSC or IL-10 may favor type1 response and improve the anti-tumor activity of immune cells (100, 101).

Checkpoint blockade immunotherapy targeting the PD-1/PD-L1 inhibitory axis produced remarkable results in the treatment of several types of cancer (102–106). PD-1 is mostly expressed by T-cells, but NK cells with an activated and more responsive phenotype can also express PD-1 (107–110). In TME, tumor cells and their soluble mediators can increase PD-L1 expression on tumor-infiltrating MDSC (111, 112). Thus, PD-L1 expressed by MDSC can suppress NK cell activity while PD-L1 blockade may restore NK and T-cell responses. In different tumor types, increased $PD\text{-}L1^+$MDSC has been observed and, in some instances, a correlation between the percentage of $PD\text{-}L1^+$MDSC and disease stages or clinical outcome has been reported (113). In addition, NO produced by MDSC has a potent inhibitory effect on NK cells by impairing the Fc receptor-mediated killing, the secretion of IFN-γ, TNF-α, and Granzyme B, as detected in MDSC-co-cultured NK cells (99). Furthermore, IDO produced by MDSC can impair development and activation of NK cells by decreasing expression of NKG2D, NCR, DNAM1, and IFN-γ secretion (99, 114). IDO production is regulated by STAT3- induced NF-κB activation. It has been demonstrated that blockage of STAT3 and TGF-β can revert the MDSC-mediated inhibition of NK cell function (115, 116).

On the other hand, STAT5 has an opposite effect to that of STAT3. Indeed, STAT5, induced by Jak3, is responsible for perforin, granzyme and IFN-γ production in IL-2 –activated NK cells (117). NK cells co-cultured with MDSC isolated from spleen of tumor-bearing mice, showed both Jak3-inhibition and reduced STAT5 activation (98).

Another mechanism occurring in NK-MDSC interaction involves the TIGIT-CD155 axis. Thus, analysis of patients with CMV^+ myelodysplastic syndrome revealed the presence of adaptive NK cells with lower TIGIT expression (partially) resistant to MDSC-mediated immunosuppression (118). Recently, in NK cells the IL-1R8 has been suggested as a novel immune check-point that can potentially interact with MDSC cells (119).

MDSC and NK Cross Talk in HSCT

MDSC were originally described as cells able to inhibit T cell activation, proliferation, and function. Other studies provided evidences that MDSC could also interact and interfere with the function of other cells, including NK cells, B cells, NKT cells, and DCs. All these observations are in line with the suppressive effect of MDSC in the context of hematopoietic stem cell transplantation (HSCT). Notably, HSCT from HLA-matched donor, either related or unrelated, is extensively used to cure patients with Acute Leukemia. The HSCT from HLA-haploidentical relatives (haplo-HSCT) gave the opportunity of a prompt transplantation in patients with no HLA-matched donor. Graft-vs.-host disease (GvHD) and post-transplant lymphoproliferative disease (PTLD) are two life-threatening effects of un-manipulated HSCT, due to the presence of T cells and B cells in the graft. In haplo-HSCT, graft of "mega-doses" of highly purified $CD34^+$ HSC has been applied for many years. However, the lack in the graft of different mature lymphoid subsets and of ($CD34^-$) committed hematopoietic progenitors results in a prolonged lymphopenia and delayed

immune reconstitution that causes an increased risk of non-relapse-related mortality (NRM), due primarily to opportunistic infections. Thus, selective depletion of αβ T lymphocytes, and of B cells was used more as a novel method of graft manipulation. This approach allows the infusion in the recipient not only of hematopoietic progenitors but also of high numbers of donor mature NK cells, γδ T-cells and myeloid cells. In particular, NK and γδ T-cells transferred with the graft may contribute to prevent leukemia relapses and severe viral infections and/or reactivation before the establishment of adaptive immune responses thanks to their activity against leukemia blasts remaining in the patient after the conditioning regimen (120). Notably, it has been shown that in αβ T- and B-cell depleted HSCT setting, the contribution of the NK cell alloreactivity to the 5 years' survival probability was partially obscured, possibly by the effect of γδ T-cells (121–123).

Regarding the strategy routinely applied to increase the number of circulating HSC to be infused, donors receive G-CSF for 5 days (**Figure 1**). G-CSF induces a proteolytic microenvironment and inhibits CXCL12 production, thus favoring HSC egress from BM. An adequate number of HSC can be achieved also in "poor mobilizer" donors, who, in addition to G-CSF, receive Plerixafor (PL), a CXCR4 antagonist, which inhibits HSC retention in the BM, favoring their collection in the peripheral blood (PB). The G-CSF mobilization regimen induces an accumulation in PB of PMN-MDSC (124).

We could demonstrate that PMN-MDSC derived from G-CSF mobilized donors did not interfere with the differentiation of donor HSC (124). On the contrary, they could affect the cytotoxic potential of donor-mature NK cells, which are infused into the patients during transplantation, compromising their GvL activity.

PMN-MDSC through the release of IDO metabolites and PGE2 down modulate the expression of intracellular polypeptides involved in the signal transduction and of the major activating NK receptors. In particular, signaling via activating receptor is mediated intracellularly by immunoreceptor tyrosine-based activation motifs (ITAM) and by downstream protein kinases. KARAP/DAP12 and CD3ζ are ITAM-bearing adaptor proteins known to associate with different activating NK receptors. These intracellular molecules involved in signal transduction were down-modulated in NK cells upon interaction with PMN-MDSC.

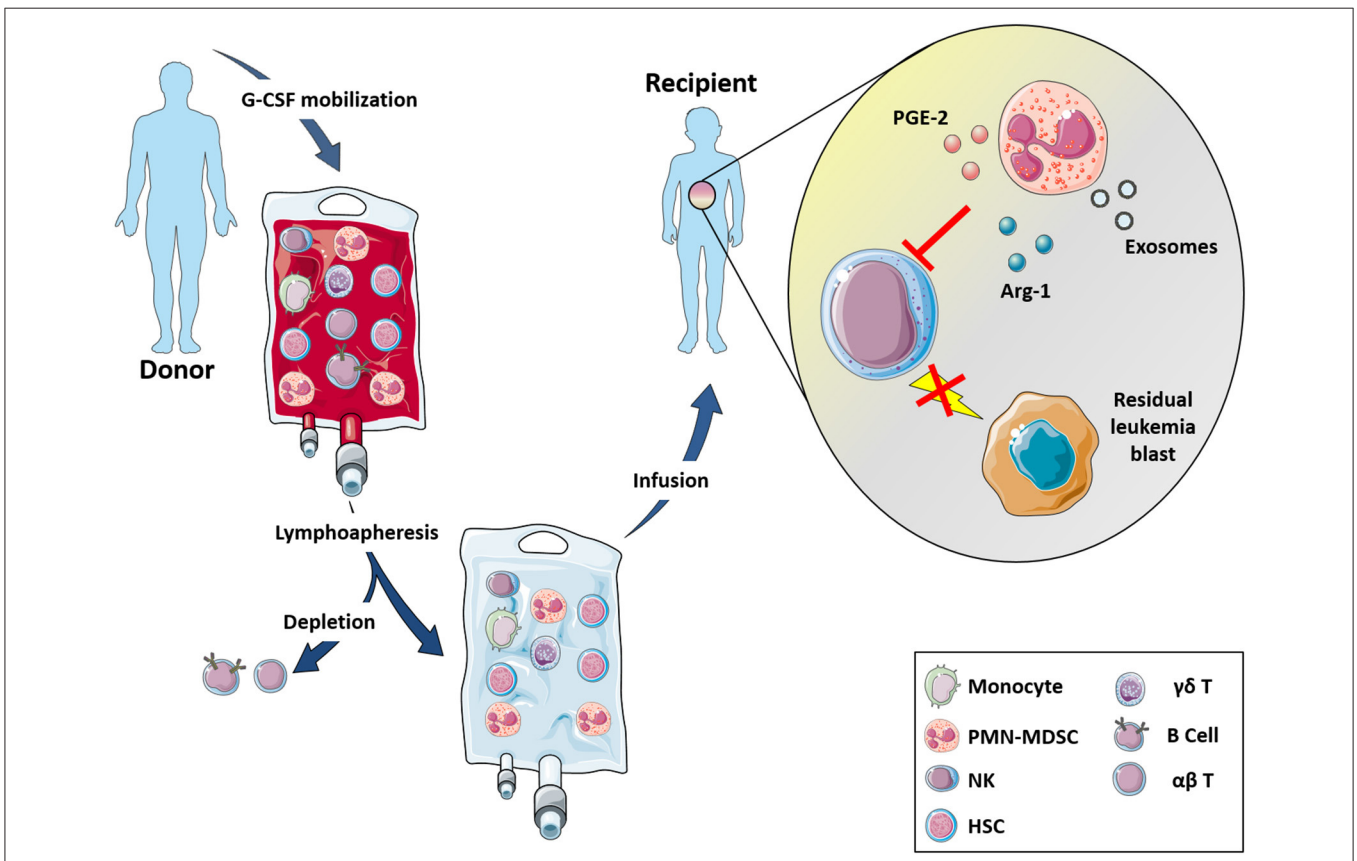

FIGURE 1 | αβ T- and B-depleted hematopoietic stem cell transplantation protocol. Donors undergo lymphoapheresis after 5 days G-CSF stimulation for increasing the number of HSC cells and to induce PMN-MDSC mobilization. Thus, αβ T and B cells are depleted and infusion (enriched in NK cells, γδ T-cells and myeloid cells) is administered to recipient patients. In this transplant setting, the enrichment of PMN-MDSC helps to reduce NK cell cytotoxic effects against the graft by the release of IDO2, PGE2, and exosomes thus suggesting a possible involvement of these myeloid cells into the GvL activity mediated by NK cells.

PMN-MDSC were also shown to affect NK cell degranulation, cytokine release and cytotoxicity. Indeed, in the presence of IDO- and PGE2-inhibitors the NK-cell activity can be recovered suggesting the involvement of IDO catabolites and PGE2 in the inhibition of the NK-mediated killing of leukemia blasts (124). It is known that MDSC may exploit additional immunomodulatory mechanisms including, for example, the release of exosomes. In this context, PMN-MDSC were able to release exosomes that are, in turn, internalized into NK cells and cause an impairment of their cytolytic activity (125) (**Figure 1**). Altogether, these data indicate that PMN-MDSC exert a potent inhibitory effect on anti-tumor NK cell function suggesting their possible involvement in the impairment of GvL activity mediated by NK cells.

Based on the *in vitro* data and on the role of these cells in hematologic malignancies, it is conceivable that MDSC may indeed represent a key immunosuppressive cell type induced in allogeneic HSCT. Further investigation regarding molecular and functional characteristics of MDSC may help to discover new strategies/drugs, to either dampen or enhance MDSC immunosuppressive activity, depending on the therapeutic need in different clinical contexts.

CONCLUDING REMARKS

A deeper comprehension of the mechanisms and relative molecular pathways adopted by MDSC present in TME to impair the anti-tumor function of immune effector cells may allow to identify novel therapeutic strategies capable to disrupt these potent inhibitory mechanisms. Thus, in HSCT the large proportion of PMN-MDSC can counteract the GvL activity mediated by donor-mature NK cells infused in the recipient, particularly in the early post-transplant period. Previous reports revealed that a reduction of the immunosuppressive activity of MDSC could be achieved by inducing their differentiation. It has been reported that the combined administration of ATRA (all-trans-retinoic acid) (126), paclitaxel (ultra-low non-cytotoxic doses) (127), vitamin D (128), and IL-2 (129) is able to induce MDSC differentiation by blocking their immunosuppressive activity and resulting in the recovery of immune response. *In vitro* data showed that chemotherapeutic agents (i.e., gemcitabine or 5-fluorouracil) could be used to selectively deplete MDSC with no toxic effects on other leukocyte populations (130, 131).

A better outstanding of interactions occurring between NK cells and PMN-MDSC, in particular in TME, may offer an interesting clue to further improve the efficacy of immunotherapy. In particular, in αβ T- and B cell-depleted haplo-HSCT setting, removing also PMN-MDSC, could preserve the NK-cell function with a further positive effect on the GvL activity and viral protection, obtaining a better patient's clinical outcome.

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

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REVIEW
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Harnessing the cDC1-NK Cross-Talk in the Tumor Microenvironment to Battle Cancer

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Immunotherapeutic approaches have revolutionized the treatment of several diseases such as cancer. The main goal of immunotherapy for cancer is to modulate the anti-tumor immune responses by favoring the recognition and destruction of tumor cells. Recently, a better understanding of the suppressive effect of the tumor microenvironment (TME) on immune cells, indicates that restoring the suppressive effect of the TME is crucial for an efficient immunotherapy. Natural killer (NK) cells and dendritic cells (DCs) are cell types that are currently administered to cancer patients. NK cells are used because of their ability to kill tumor cells directly *via* cytotoxic granzymes. DCs are employed to enhance anti-tumor T cell responses based on their ability to present antigens and induce tumor-antigen specific $CD8^+$ T cell responses. In preclinical models, a particular DC subset, conventional type 1 DCs (cDC1s) is shown to be specialized in cross-presenting extracellular antigens to $CD8^+$ T cells. This feature makes them a promising DC subset for cancer treatment. Within the TME, cDC1s show a bidirectional cross-talk with NK cells, resulting in a higher cDC1 recruitment, differentiation, and maturation as well as activation and stimulation of NK cells. Consequently, the presence of cDC1s and NK cells within the TME might be of utmost importance for the success of immunotherapy. In this review, we discuss the function of cDC1s and NK cells, their bidirectional cross-talk and potential strategies that could improve cancer immunotherapy.

Keywords: natural killer cells, conventional type 1 DCs, cross-talk, tumor microenvironment, immunotherapy

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INTRODUCTION

Cancer immunotherapy is an approach that aims to activate the immune system to fight cancer. The immune system consists of many different cell types interacting with each other. Natural Killer (NK) cells and dendritic cells (DCs) are two cell types used for immunotherapy that are currently being tested in the clinic.

NK cells are granular innate lymphoid cells that display rapid, contact-dependent cytotoxic activities against viral-infected and cancer cells without prior sensitization. In general, NK cells

rapidly accumulate at sites of inflammation, where they recruit other immune cells *via* cytokine and chemokine secretion. After activation, NK cells induce lysis or apoptosis in mutated cells by releasing granules containing cytotoxic granzymes. Activation occurs in an antigen-independent manner that is regulated by a tight balance of activating and inhibitory germline-encoded surface receptor ligation (1). Activating receptors bind to ligands (e.g., CD155, CD112) upregulated on tumor cells. Inhibitory receptors recognize major histocompatibility complex (MHC) class I, which is expressed by all nucleated cells, and upon binding, suppress NK cell activation. Hence, the highly diverse receptor repertoire on NK cells and the balance of activating and inhibitory receptors determine the magnitude of NK cell-mediated cytotoxicity and allow them to remain tolerant towards healthy cells (1–3). DCs, are a heterogeneous cell population which main function is to initiate an immune response. Immature DCs act as sentinels as they take up antigens, undergo a maturation process, and present these antigens on MHC molecules to naive T cells in lymph nodes. In general, antigen presenting cells present endogenous antigens on MHC class I (MHC-I), and exogenous antigens on MHC class II molecules (MHC-II) and thereby prime and activate $CD4^+$ and $CD8^+$ T cells, respectively (4). However, DCs have the unique capacity to present exogenous antigens on MHC-I molecules to $CD8^+$ T cells, a process known as antigen cross-presentation.

NK cells are exploited as immunotherapeutic tool due to their cytotoxic and immunomodulatory functions and DCs because they are able to antigen-specifically activate T cells. However, both NK cell and DC functions can be restricted by the immunosuppressive tumor microenvironment (TME). In this review, we describe the main features of NK cells and a very rare type of DC, cDC1s, and emphasize the importance of these cell types within the TME. We focus on how to exploit cDC1s and NK cells and their interaction as a potential target to enhance efficacy of cancer immunotherapy.

DENDRITIC CELLS

Both in humans and mice, circulating blood DCs have been classically divided into myeloid or conventional DCs (cDCs), and plasmacytoid DCs (pDCs). The DC subsets are classified by surface marker expression and different functional properties. Human pDCs express CD123, CD303 (BDCA-2) and CD304 (BDCA-4) as distinctive markers and are known for the production of large amounts of type 1 interferon (IFN-I) especially important for strong anti-viral responses (5). Conventional DCs express the common myeloid markers: CD11c, CD11b, CD33, and CD13 and are efficient in antigen presentation and T cell activation (5). They can be subdivided into type 1 conventional DCs (cDC1s) and type 2 cDCs (cDC2s) (6, 7). In humans, cDC1s express CD141 (BDCA-3) and cDC2s express CD1c (BDCA-1) (5). In mice, cDC2s are $CD11b^+$, and cDC1s are characterized by $CD8\alpha^+$ or $CD103^+$ expression (8). Genome-wide association studies of human and mouse cDC1s revealed phenotypic similarities, including expression of nectin-like protein 2 (Necl2), C-type lectin CLEC9a, and the XC chemokine receptor 1 (XCR1) as well as toll-like-receptor 3 (TLR-3) (**Figure 1**) (9–14). Hence, human cDC1s are considered to be the equivalent of the mouse $CD8\alpha^+$ D C subset (10–13, 15, 16) (**Figure 1**).

cDC1s Phenotypic Characterization

Whereas in mice, cDC1s are the most abundant DC subset, human cDC1s are the rarest with approximately 0.03% of PBMCs and lymphoid and non-lymphoid cells are cDC1s (10, 11). This low occurrence of cDC1s resulted in difficulties to characterize them phenotypically. Initially, CD141 (BDCA-3) was described as a distinctive marker for cDC1s. However, CD141 was also found on cDC2s and other myeloid cells like monocytes (17). Therefore, many research groups sought to redefine subset division and attempted to identify conserved

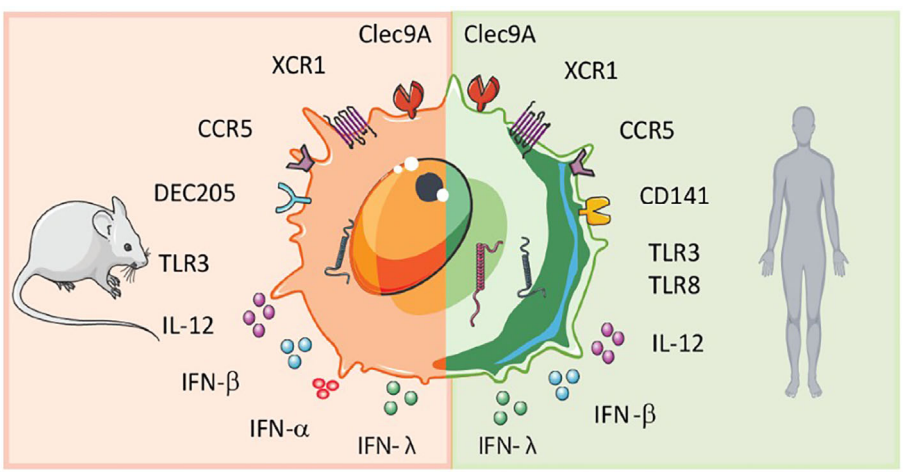

FIGURE 1 | Scheme of murine and human cDC1 features. Human and mouse cDC1s display similarities but also differences in surface receptor expression and cytokine secretion.

and exclusive markers across species. In this context, single-cell RNA-sequencing data recently demonstrated that cDC1s indeed form a single, separate cluster with CLEC9a as a specific marker for human cDC1s (18). CLEC9a is a receptor for necrotic cell-derived antigens (19). Yet, also CLEC9a is not exclusively expressed on cDC1s (13, 20, 21). The chemokine receptor XCR1 was established to be a specific marker for cDC1s. It is exclusively expressed on both human and mouse cDC1s (12, 13, 22–24). XCR1 mediates chemotaxis of cDC1s towards $CD8^+$ T cells and NK cells, because they are the main producers of the ligand of XCR1, XC chemokine ligand 1 (XCL1), the ligand of XCR1 (12, 23). Besides exclusive expression of XCR1, cDC1s have a higher expression of the TLR-3 compared to other DCs subsets. TLR-3 signaling triggers IRF3/7, leading to IFN-ß secretion, thereby providing cDC1s with the enhanced capability to initiate T helper 1 responses (10, 11). As a result of the expression of CLEC9a and XCR1, cDC1s can take up necrotic cell-derived antigens and can migrate towards $CD8^+$ T cells.

cDC1s Activate T Cells *via* Cross-Presentation

Cross-presentation of extracellular antigens is essential to activate $CD8^+$ T cells specific for antigens derived from tumor cells (25). In mice, cDC1s are the only subset that cross-present antigens. Depletion of cDC1s, for example using *Batf3* knockouts, results in loss of cross-presentation. Mice lacking cDC1s have less tumor-specific $CD8^+$ T cells hence an impaired anti-tumor response (24, 26–29). Of note, depletion of cDC1s leads, besides to diminished cross-presentation also to loss of other functions mediated by cDC1s like attracting $CD8^+$ T cells *via* XCL1-XCR1 interactions as well as less IL-12 and IFN-β since it is produced by cDC1s upon TLR-3 triggering. These processes also enhance anti-tumor responses and are less efficient upon depletion of cDC1s (6, 24).

In line with data obtained from mouse experiments, human cDC1s are shown to be highly efficient at antigen cross-presentation (10–13). However, in humans, cDC1s are not the only subset capable to cross-present. Plasmacytoid DCs, cDC2s and monocyte-derived DCs are also able to cross-present (30–34). However, data show that cDC1s are the most competent DC subset to cross-present antigens, especially of necrotic cells after uptake *via* CLEC9A (27, 35). The efficiency of cross-presentation of cDC1 is enhanced after triggering of TLR3 (11). Hence, human cDC1s are also highly specialized to cross-present antigens from necrotic tumor cells and to initiate a tumor antigen specific $CD8^+$ T cell response (36, 37).

NATURAL KILLER CELLS

Human blood NK cells are a heterogeneous immune cell population classified by surface receptor expression of CD56 into two main functionally different subsets. $CD56^{high}$ NK cells secrete high levels of IFN-γ and other cytokines important for immunoregulatory functions, but produce low levels of perforin (38). Furthermore, $CD56^{high}$ NK cells express high levels of natural killer group 2A (NKG2A) and C-C chemokine receptor type 7 (CCR7) and low levels of Fcγ receptor IIIa (CD16) and killer cell immunoglobulin-like receptors (KIRs) (38, 39). In contrast, $CD56^{low}$ NK cells display a high expression of KIR and CD16 receptor. The receptor CD16 enables $CD56^{low}$ NK cells to mediate antibody-dependent cellular cytotoxicity (ADCCs). This CD16 expression together with the secretion of perforin and granzyme B provides $CD56^{low}$ NK cells with efficient killing abilities (38).

In general, NK cells display either killer (CD56lowNK cells) or helper (CD56high) functions against virus-infected or tumor cells due to activating and inhibitory receptor signaling and cytokine secretion (**Figure 2**). Inhibitory KIR and NKG2A receptors recognize MHC-I molecules. In the case of absent, changed, or mismatched MHC-I expression, the inhibitory receptor signal is lost, and NK cells get activated. The diversity of inhibitory receptors expressed on NK cells allows to recognize the high polymorphism of the *MHC class I* genes resulting in NK cell tolerance for healthy cells (3). Virus-infected or tumor cells often show gradually or complete loss of MHC-I expression to escape cytotoxic T cell recognition, simultaneously leading to a lack of inhibitory signal and NK cell activation (38, 40). Besides NK cell activation by loss of inhibitory signals, ligands overexpressed on mutated cells engaging with activating receptors such as natural cytotoxicity receptors (NKp30, NKp44, and NKp46) and NKG2D also lead to NK cell activation (41). Exposure of NK

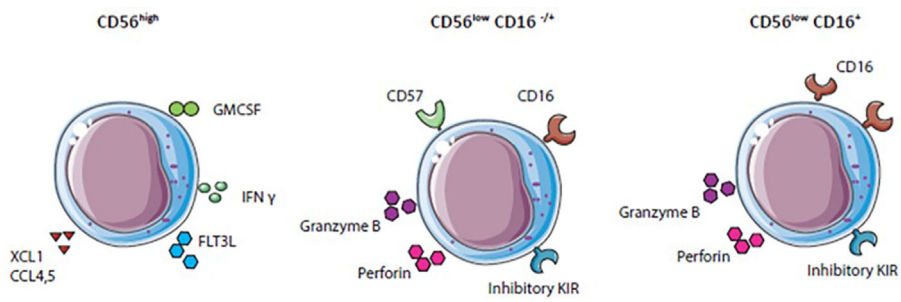

FIGURE 2 | Natural killer (NK) cell subset differentation: NK cell subsets express variouus activating and inhibitory receptors and secrete different cytokines.

cells to cytokines such as IL-2 or IL-15 enhances activating receptor upregulation and promotes survival and proliferation (42–44). Activated NK cells can kill by forming a synapse with target cells and releasing perforin and cytotoxic granules. Perforin penetrates the membrane of target cells and granules containing cytotoxic granzymes enter target cells to provoke programmed cell death (45, 46). Another killing mechanism of NK cells is *via* ADCC. CD16 on NK cells recognize IgG antibody-coated tumor cells upon which granules are released by NK cells and target cells are killed (47). Some anti-tumor therapies based on neutralizing antibodies such as Rituximab or Trastuzumab demonstrate that the clinical benefit is partly mediated by ADCC (47).

Immunomodulatory functions, mainly ascribed to $CD56^{high}$ NK cells are the secretion of different cytokines, chemokines, and growth factors, for example, IFN-γ, TNF-α, fms-like tyrosine kinase 3 ligand (FLT3L), Chemokine (C-C motif) ligand 3 (CCL3), CCL4, CCL5, granulocyte-macrophage colony-stimulating factor (GM-CSF) and XCL1 (41). These factors attract other immune cells like DCs, induce Th1 polarization, and CTL responses against target cells (41, 48). NK cells are essential for anti-tumor immune responses especially for eradication of MHC-I negative tumors. Different strategies to boost NK cell activity, particularly in the suppressive TME, are currently investigated.

TUMOR MICROENVIRONMENT AND IMMUNE ESCAPE MECHANISMS

Escaping immune surveillance is one of the key hallmarks of cancer (49). Different suppressive mechanisms are employed by cancer cells to bypass immune system attacks. These mechanisms include the upregulation of co-inhibitory ligands (such as programmed death-ligand 1, PD-L1), down-regulation or loss of MHC-I, or secretion of immunosuppressive factors like IL-10, VEGF, and TGF-β (50–54).

The composition of the TME differs between cancer type and patient and plays an essential role in the tumor immune escape. This highly heterogeneous TME is composed of tumor cells, blood vessels, tumor-infiltrating lymphocytes, and other immune cells, fibroblasts, endothelial cells, signaling molecules (cytokines, chemokines), and the extracellular matrix (55). Tumors can be divided into "hot" and "cold" depending on the presence or absence of effector immune cells within the tumor. The occurrence of effector immune cells, for example T cells which directly can eliminate tumor cells, impacts patient outcome (55). In some cancer types, such as epithelial ovarian cancer and colorectal cancer T cell infiltration, specifically in the tumor has been associated with positive clinical effects (56–58). Moreover, pre-existing $CD8^+$ T cells within the tumor are crucial for tumor regression upon PD-1 checkpoint inhibition (59). In contrast to $CD8^+$ T cells, regulatory T cells, myeloid-derived-suppressor cells, or tumor-associated macrophages inhibit the immune response and are therefore associated with tumor growth and a poor clinical outcome (60, 61).

NK Cells and DC Within the TME

Next to the presence of $CD8^+$ T cells, the occurrence and active state of NK cells in the tumor are related to a positive prognostic outcome (62–64). In some types of cancer such as head and neck or prostate cancer, a high ratio of tumor infiltrating NK cells is associated with a positive clinical outcome for patients, whereas in non-small-cell lung cancer, the presence of NK cells within the TME has no clinical impact due to down-regulation of activating receptors on the infiltrated NK cells (63, 65). NK cells isolated from prostate cancer are mainly $CD56^{high}$ and display reduced cytotoxicity because of an increased inhibitory and decreased activating receptor expression pattern. In non- small cell lung carcinoma CTLA-4 expression is upregulated on NK cells in the TME compared with healthy tissue NK cells and negatively effects DC maturation (66). This unfavorable activating and inhibitory receptor expression pattern have been implicated as cause for impaired killing activity of NK cells in the TME (67, 68).

NK cells isolated from tumors differ phenotypically and transcriptomically from NK cells isolated from blood (69). These variations can arise from several cellular and soluble factors present within the TME, which influence phenotype, functionality, and migration characteristics of infiltrated NK cells (63, 66, 70). Soluble factors influencing NK cell cytotoxicity and infiltration into the TME for instance TGF-β and Prostaglandin E2 (PGE2) are secreted by immunosuppressive cells such as regulatory T cells, tumor-associated macrophages, myeloid-derived-suppressor cells (68, 71–73). TGF-β suppresses NK cell cytotoxicity by inhibiting IFN-γ secretion, down-regulating activating receptor expression, and the adapter molecule DAP12 (74–76). PGE2 hampers NK cell cytotoxicity by decreasing IFN-γ secretion, thereby facilitating cancer progression (77, 78). Other factors within the TME, like hypoxia, high expression of checkpoint receptor ligands, and chronic activation of activating receptors, can cause functional exhaustion of NK cells (79–81). Exhausted NK cells show a dampened inflammatory cytokine secretion pattern, a reduced activity due to decreased activating receptor expression and an upregulation of inhibitory receptors and checkpoint receptors such as PD1, TIM3, CD69, and LAG3 (63, 68, 82, 83).

Immunosuppressive factors within the TME also strongly influence the function of DCs within this microenvironment (**Figure 3**). Tumor-infiltrated DCs are less efficient in antigen presentation and cytokine production upon TLR stimulation than peripheral blood DCs (84, 85). Factors in the TME, including IL-10, IL-6, TGF-ß, and PGE2, modulate DCs, for example, inhibition of DC maturation. Hence, tumor-infiltrated DCs often display an immature phenotype with a low expression of co-stimulatory markers: CD80, CD86, and CD40, while co-inhibitory markers like PD-L1 and TIM3 are upregulated. Consequently, DCs within the TME are often associated with immunosuppressive and impaired functions (86–88). Notably, the infiltration of pDCs is associated with poor prognosis in breast- and ovarian cancer (89, 90). In contrast, cDC1s in the TME have been linked with favorable patient survival (91). Several reports indicate that cDC1s in tumors are associated

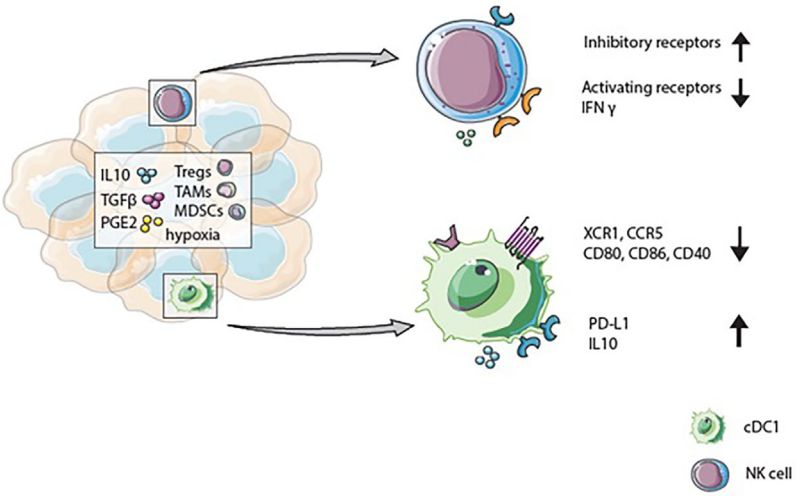

FIGURE 3 | The tumor microenvironment influences cDC1 and natural killer (NK) cell phenotype and function. Immunosupressive factors like IL-10, TGF-β, and PGE2 can be secreted by tumor- and immune cells such as regulatory T cells (Tregs), tumor-associated macrophages (TAMs), or myeloid-derived-suppressor cells (MDSCs) present in the tumor microenvironment (TME). These factors, and hypoxia, can upregulate inhibitory receptors and decrease activating receptors on NK cells. Together with diminished IFN-γ secretion, the changed receptor expression results in reduced cytotoxicity of NK cells. Due to immunosuppressive factors in the TME cDC1s express low levels of XCR1 and CCR5 and display an immature phenotype with reduced CD80, CD86, and CD40 expression. Whereas checkpoint receptors and anti-inflammatory cytokine expression are upregulated.

with a higher number of $CD8^+$ T cells within the TME, better prognosis of cancer patients, and immunotherapeutic success (36, 92–95). In addition to the presence of $CD8^+$ T cells, the occurrence of cDC1s in the TME also correlates with high numbers of NK cells and better overall survival of melanoma patients (14, 94, 96). Positive effects of cDC1s within the TME seem to be partly mediated *via* their IL-12 secretion which is essential for the induction of T-helper 1 (Th1) responses and $CD8^+$ T cell activation, both of which are crucial for a long-lasting anti-tumor response (97). Moreover, IL-12 effects IFN-γ production by NK cells. The lack of IL-12 secreting cDC1s in mice leads to reduced IFN-γ production by NK cells and growth of metastases (98). In human breast cancer, IL-12 expression correlates with cDC1s and cytotoxic effector molecules like *IFNG* (99). Nevertheless, positive effects of the presence of cDC1s can be negatively affected by different factors in the TME. For example, in mice, tumor-infiltrating cDC1s show increased PD-L1 expression upon antigen uptake and IFN-γ stimulation. High PD-L1 expression protects cDC1s from killing by $CD8^+$ T cells. However, within the TME, this protective high PD-L1 expression on cDC1s can lead to a diminished anti-tumor immune response (100). Macrophage-derived IL-10 inhibits IL-12 production by cDC1s during chemotherapy, resulting in a lower $CD8^+$ T cell cytotoxicity and, tumor progression in a mammary carcinoma mice model (99). A factor that affects the presence of cDC1s within the tumor is cyclooxygenases (COX), an enzyme crucial for PGE2 production. In mice, increased COX levels lead to diminished cDC1 numbers within melanoma tumors and a dampened activity by suppressed IL-12 production. COX inhibition, together with anti-PD-1 treatment, enhanced eradication of tumors. Changed immune cell infiltration due to COX expression is further confirmed in various tumor types in mice and in human tumors; COX levels correlate negatively with immune cell infiltration (101). Even though, cDC1s in the TME positively affect patient outcome different factors in the TME suppress their functional properties.

Migration Patterns of NK Cells and cDC1s

The migration of immune cells towards lymph nodes and solid tumors is essential for proper immune activation and cancer cell elimination (55, 102). In general, the attraction and migration of immune cells occur through chemokine production and the interaction with their receptors (55). DC migration to lymph nodes is mediated by CCL19 and CCL21, the ligands for CCR7, where they present antigens to T cells and induce an antigen-specific immune response (103). The expression of CCR7 on cDC1s is crucial for migration and therefore important for antigen trafficking to lymph nodes. The lack of CCR7 expression in mice results in reduced numbers of cDC1s in the draining lymph node and increased tumor growth compared to wild type mice. In melanoma patients, cDC1s in the TME express high levels of CCR7, which predict T cell infiltration and improves clinical outcome, underscoring the importance of CCR7 expressing cDC1s for antigen trafficking and T cell priming (104).

After antigen specific activation of T cells in the lymph nodes these effector cells should migrate to the tumor to be able to kill tumor cells. Differences in chemokine and cytokine expression between hot and cold tumors impact the infiltration of effector cells (55, 105, 106). Hot tumors express high gene- and protein levels of chemokines CXC ligand (CXCL)9, CXCL10, and CXCL11 (106). All these three chemokines are secreted by DCs

and bind to CXCR3 expressed on activated T- and NK cells. The high presence of CXCL9, CXCL10, and CXCL11 in the TME is associated with favorable clinical outcome in some cancers in humans (107). Interestingly, cDC1s are the primary source of those chemokines, underscoring the importance of cDC1s to attract effector cells towards the TME (95). For T cell chemotaxis into the tumor, the CXCR3/CXCL9,10,11 axis seems crucial (108, 109). For NK cells the role of CXCR3 for tumor infiltration is less straight forward. CXCR3 expression differs per NK cell subset, which influences their migration pattern. $CD56^{high}$ NK cells express CCR7 and CXCR3, $CD56^{low}$ NK cells express CXCR1 and CXCR2 (107, 110, 111). In breast and lung cancer, CXCL9 and CXCL10 mediate directed migration of $CD56^{high}$ NK cells, but not of cytotoxic $CD56^{low}$ NK cells (112). Moreover, suppressive factors present in the TME can influence chemokine production leading to altered NK cell migration. TGF-β favors $CD56^{high}$ and dampens $CD56^{low}$ NK cell recruitment. TGF-β decreases chemokine secretion of CXCL2, CX_3CL1, and CXCL1 which attract $CD56^{low}$ and increases chemokine production, which recruit $CD56^{high}$ (CXCL9, CXCL10, CXCL11, and CCL5) (107, 110, 113). Even though factors in the TME can influence migration patterns of effector cells, the secretion of chemokines by cDC1s seem to be crucial for the recruitment of T cells and NK cells.

CROSS-TALK CDC1S AND NK CELLS

The cross-talk between NK cells and cDC1s is bidirectional and NK cells as well as T cells can recruit cDC1s into the tumor *via* chemokine secretion and thereby promote anti-tumor immunity (14). In both preclinical and clinical settings, cDC1s are found enriched in TMEs with a specific chemokine profile. This profile includes XCL1, mainly secreted by tumor resident $CD56^{low}$ NK cells, and CCL4 and CCL5 mostly produced by $CD56^{low}$ and $CD56^{high}$ NK cells and $CD8^+$ T cells (14). cDC1s express the receptors for those chemokines, namely XCR1 and CCR5 (14, 23). Indicating the importance of NK and $CD8^+$ T cells for intratumoral migration of cDC1s due to XCL1, CCL5, and CCL4 (14). NK cells stimulated with IL-18 and IFN-α attract immature DC based on CCR5 expression, and stimulate DCs to increase CXCL9, CXCL10, and CCL5 production, promoting the attraction of effectors cells (114). *In vitro*, XCR1 and CCR5 expression on cDC1s is downregulated by PGE2, diminishing responsiveness to XCL1 and CCL5. In addition, PGE2 inhibits XCL1 and CCL5 secretion by NK cells, underlining the role of PGE2 as an immunosuppressive mediator to interfere with cDC1s migration to the tumor (14). In human tumors, cDC1 and NK cell gene signatures correlate with CCL5 and XCL1 gene expression and with $CD8^+$ T cell infiltration. Further, NK cell and cDC1 gene signatures in the TME correlate positively with patient survival (14).

The cross-talk between NK cells and cDC1s influences the migration pattern of both cell types in addition to multiple other mechanisms by which DCs and NK cells interact (**Figure 4**). One mutual process is the maturation of DCs initiated by NK cells. Mature DCs release cytokines (IL-2, IL-12, or IL-18), which provoke NK cells to produce IFN-γ, TNF-α, or GM-CSF. These cytokines promote DC maturation (115, 116). Besides cytokine secretion, NK cells mature DCs *via* the ligation of CD40/CD40L

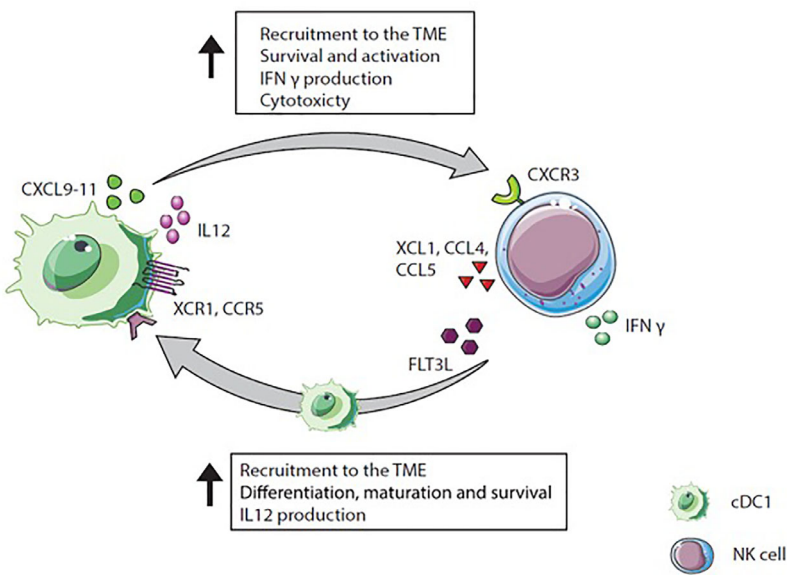

FIGURE 4 | Natural killer (NK) cell and cDC1 cross-talk. NK cells secrete XCL1, CCL4, and CCL5 which attract XCR1 and CCR5 expressing cDC1s. Moreover, NK cells produce FLT3L, a differentiation factor for precursor DCs to cDC1s recruit NK cells *via* CXCL9-11 secretion into the TME. In a positive feedback loop, IL-12 produced by cDC1s activates IFN-γ extraction by NK cells, which again increases IL-12 secretion by cDC1s.

(117). Upon CD40/CD40L ligation, the membrane bound IL-15 expression on DCs is upregulated promoting proliferation of NK cells (118, 119). The expression of CD40L on NK cells is regulated by IL-12 and IFN-γ and might determine cytotoxicity of NK cells (117, 120). Indeed, in a mouse tumor model, IL-12 and IFN-γ inhibition resulted in the down-regulation of CD40L expression on NK cells and diminished NK cell cytotoxicity (120).

Besides maturation factors, NK cells produce the differentiation factor FLT3L, which stimulates cDC1 survival, differentiation, and recruitment and thereby positively influence cDC1s in the TME (94, 121). Interestingly, intratumoral NK cells in humans and mice are a major source of FLT3L, which maintains DC viability within the TME (94). The depletion of NK cells in mice reduced the frequency of cDC1s within the tumor, indicating that FLT3L production by NK cells is required for stable cDC1 numbers within the TME. In humans, genes encoding for *FLT3LG* within the tumor are linked to NK cell presence. In melanoma patients, the presence of cDC1s and NK cells within the tumor correlate with increased survival and responsiveness to anti-PD1 treatment. This underscores the hypothesis that NK cells producing FLT3L are responsible for the abundance of cDC1s within the tumor resulting in improved patient survival (94).

Taken together, chemokines such as XCL1, CCL4, and CCL5 secreted by NK cells and activated T cells within the TME recruit cDC1s into the tumor where they efficiently process antigens and then migrate to lymph nodes while they undergo maturation. In the draining lymph nodes, cDC1s prime naïve $CD8^+$ T cells *via* cross-presentation. The cytotoxic T cells expand and are attracted to the tumor site, guided by local cDC1s secreting CXCL9 and CXCL10 (**Figure 5**). Thus, NK cells, cDC1s and their interaction are crucial for anti-tumor activity and, therefore, are promising targets to improve cancer immunotherapy outcome (6, 14, 37, 122).

MANIPULATION OF NK-CDC1 CROSS TALK

Targeting cDC1s and XCR1-XCL1 Axis

The presence and interaction of cDC1s and NK cells within the TME are associated with activation and increased cytotoxicity of NK cells, cDC1s infiltration and maturation, and better prognosis for cancer patients (14, 93, 95). Hence, interfering with the XCR1-XCL1 axis could be an approach to increasecDC1s numbers and thereby also increase numbers of T cells and NK cells within the tumor (**Figure 6**). Activated intratumoral $CD8^+$ T cells and NK cells produce XCL1, attracting XCR1 expressing cDC1s. The intratumoral injection of XCL1 can increase the migration of cDC1s towards the TME (91). In mice, administration of XCL1 linked with antigen (e.g., OVA) targeting cDC1s results in an antigen-specific T cell response and reduced tumor growth (123–125). Nonetheless, there is skepticism about using XCL1 to attract cDC1s because of its unstable structure and relatively weak chemotactic activity (126, 127). For this reason, Kazuhiko *et al.* engineered a stabilized, more potent agonist form of murine XCL1. Upon injection in mice, this potent version of XCL1 showed increased recruitment of $XCR1^+$ DCs, compared to wild-type XCL1 (126).

Besides targeting XCL1 to increase $CD8^+$ T cell and NK cell numbers within the TME, direct administration of cDC1s into the TME could also be an approach. In mice, the application of cDC1s loaded with tumor-cell derived antigens enhanced tumor T cell infiltration and reduced tumor cell growth (128). DC therapy in the clinic shifts the application of monocyte-derived DC towards the application of blood-derived cDC2s and the combination of cDC2s and pDCs (129). Even though cDC1s are associated with improved survival in different types of cancer, the application of cDC1s is not yet assessed due to their low frequency in human blood (14, 93, 104, 129). Proposed

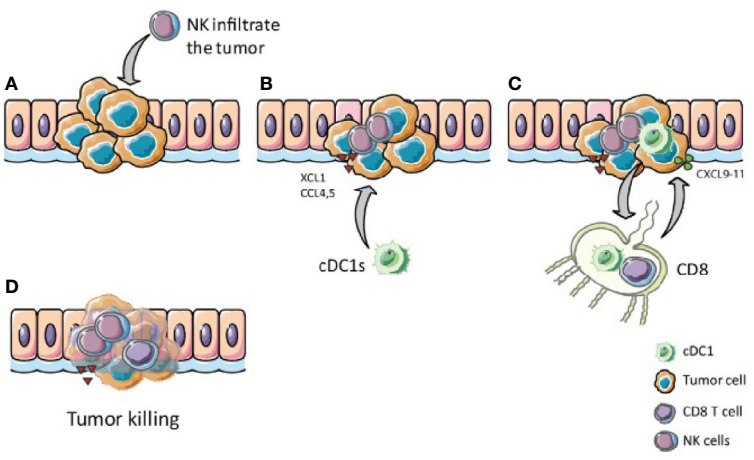

FIGURE 5 | The XCR1 – XCL1 axis plays a role in tumor clearance. **(A)** NK and T cells, which infiltrate the tumor, produce XCL1 upon stimulation. **(B)** XCL1 attracts XCR1 expressing cDC1s to the tumor **(C)** cDC1s internalize, process, and cross-present tumor antigens to CD8+ T cells in the lymph nodes, thereby activate CD8+ T cells, which migrate to the tumor attracted by CXCL9-11 secreted by local cDC1s. **(D)** Activated CD8+ T cells kill tumor cells antigen-specifically

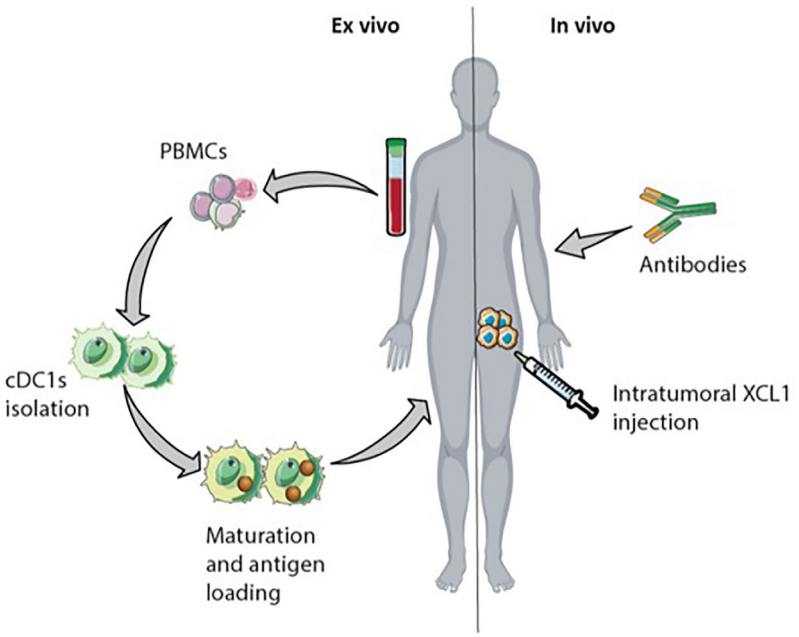

FIGURE 6 | Possible immunotherapeutic approach targeting natural killer (NK) cells and cDC1s. Interfering with the XCR1-XCL1 axis in the tumor could increase antigen-specific CD8+ T cell infiltration. The intranodal reinfusion of cDC1s from patients after isolation them from PBMCs, maturation, and antigen-loading could be an *ex vivo* approach. *In vivo*, intratumoral XCL1 injection could recruit cDC1s into the tumor. Injected or attracted cross-presenting cDC1s in the tumor enhance CD8+ T cells activation. Monoclonal antibodies initiate antibody-dependent cellular cytotoxicity (ADCC) by NK cells leading to increased cross-presentation of tumor-cell derived antigens by cDC1s.

alternatives to overcome the low abundance of cDC1s are the *ex vivo* generation of cDC1s from progenitor cells or expanding cDC1 progenitors *in vivo* (94, 130, 131). *In vitro* stimulation of human hematopoietic progenitors with Notch signaling and FLT3L induces cDC differentiation and yields phenotypical cDC1s with cross-presenting abilities (132). In mice, injection of FLT3L and intratumoral TLR3 stimulus poly IC, leads to expansion of cDC1 progenitor cells in the bone marrow and promotes cDC1s accumulation within the TME (131).

Monoclonal Antibody Treatment

Checkpoint blockade therapies targeting CTLA-4 and PD-1/PD-L1 are successfully used as cancer immunotherapy (133). PD-1 and CTLA-4 expression on NK cells is upregulated in several types of cancer and is associated with reduced cytotoxicity and cytokine secretion (66, 82, 134, 135). Especially in low MHC-I expressing tumors, the effect of mAbs blocking PD-1/PDL-1 might partly be facilitated by NK cells (136). Antibodies targeting PD-1 or CD40 stimulate IFN-γ secretion, which drives IL-12 production by cDC1s that licenses cytotoxic T cell responses in both mice and cancer patients (137). Mice lacking cDC1s display no specific T cell response upon mAbs targeting of PD-1, indicating that the effectiveness of anti-PD1 mAbs depends on the presence of cDC1s within the tumor (94, 131, 138). Moreover, the expression of PD-L1 on DC seems to influence the efficacy of anti-PD-L1 mAb treatment as mice lacking PD-L1 expression on DCs show no response to anti-PD-L1 mAb treatment (100). Based on pre-clinical data, it is proposed that therapies recruiting and activating NK cells within the TME could increase cDC1s within the tumor, benefitting responsiveness to checkpoint inhibitors (94). In line with that, enhanced NK cell activity in mice resulted in increased anti-PD-1 and anti-CTLA4 responses and a better control of tumor growth (139). In melanoma patients, anti-PD-1 treatment response correlates with the presence of cDC1s and NK cells within the TME (94).

Other mAbs target tumor-associated antigens like Her2/neu which are overexpressed on malignant cells compared to healthy cells (140). Monoclonal Ab administration induces NK cell-mediated ADCC (47). NK cells activated *via* ADCC release soluble factors like cytotoxic granules and cytokines (IFN-γ and TNF-α). The uptake of cytotoxic granules initiates tumor cell apoptosis causing the release of tumor-cell derived antigens, which can be taken up and presented by DCs (47, 141). Simultaneously, IFN-γ and TNF-α secreted by NK cells activate cDC1s to cross-present antigens leading to an antigen-specific $CD8^+$ T cell activation (47, 142). Moreover, mAb-activated NK cells can enhance NK cell-cDC1 cross-talk by activating cDC1s to secrete IL-12 which amplifies NK cell activation (141).

In some tumor types, inhibitory receptors such as KIRs and NKG2A are upregulated on NK cells (3, 143, 144). Therefore, mAb against these inhibitory receptors could be targets for blocking immune inhibition and thereby increase the cytotoxic

potential of NK cells and anti-tumor immunity (145). mAbs targeting KIRs, NKGA2, and other inhibitory receptors are currently investigated in clinical trials and are until now considered safe with limited side effects (3, 145).

CONCLUSION

Both cDC1s and NK cells are important for a proper anti-tumor immune response. cDC1s due to their specialization in cross-presenting tumor antigens and initiating an antigen-specific T cell response. NK cells because of cytotoxic and immunomodulatory functions. Even though both cell types can be affected by immunosuppressive factors in the TME, the NK-cDC1 cross-talk within this microenvironment establishes the cooperative nature of protective immunity against tumors. cDC1s, as producer of IL-12, CXCR9, CXCR10, and CCL5, attract NK cells to the TME, initiate cytokine production and boost NK cell cytotoxicity. At the same time, NK cells enhance cDC1 accumulation in the TME *via* XCL1 and CCL4, CCL5 secretion, and favor differentiation of cDC precursors to cDC1s. NK cells further stimulate DC maturation *via* cytokine secretion and CD40 ligation. Moreover, NK cell-mediated lysis of target cell releases cell debris including tumor-antigens, processed by cDC1s and cross-presented to $CD8^+$ T cells. Thus, NK cell-cDC1 cross-talk is a promising target for immunotherapy to improve the clinical outcome of cancer patients.

AUTHOR CONTRIBUTIONS

JB wrote the manuscript and designed the figures. TZ and RS helped with the literature and wrote the manuscript. GS, IV, and GF-G designed and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Platelet-Mediated Protection of Cancer Cells From Immune Surveillance – Possible Implications for Cancer Immunotherapy

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The growing insights in the complex interactions between metastatic cancer-cells and platelets have revealed that platelet tumor cell interactions in the blood stream are an important factor supporting tumor metastasis. An increased coagulability of platelets facilitates the vascular evasion and establishment of solid tumor metastasis. Furthermore, platelets can support an immunosuppressive tumor microenvironment or shield tumor cells directly from engagement of cytotoxic lymphocytes as e.g., natural killer (NK) cells. Platelets are both in the tumor microenvironment and systemically the quantitatively most important source of TGF-β, which is a key cytokine for immunosuppression in the tumor microenvironment. If similar platelet-tumor interactions are of physiological relevance in hematological malignancies remains less well-studied. This might be important, as T- and NK cell mediated graft vs. leukemia effects (GvL) are well-documented and malignant hematological cells have a high exposure to platelets compared to solid tumors. As NK cell-based immunotherapies gain increasing attention as a therapeutic option for patients suffering from hematological and other malignancies, we review the known interactions between platelets and NK cells in the solid tumor setting and discuss how these could also apply to hematological cancers. We furthermore explore the possible implications for NK cell therapy in patients with solid tumors and patients who depend on frequent platelet transfusions. As platelets have a protective and supportive effect on cancer cells, the impact of platelet transfusion on immunotherapy and the combination of immunotherapy with platelet inhibitors needs to be evaluated.

Keywords: NK cells, platelets, immunosuppressive, tumor microenvironment, antitumor immunity, metastasis, immunotherapy, cytotoxicity

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INTRODUCTION

Natural killer (NK) cells represent the largest fraction of innate lymphocytes, accounting for 10–15% of all peripheral lymphocytes in humans (1, 2), The physiological importance of NK cells is commonly ascribed to their capability to form early responses against viral infections and malignant cells (3). The main effector functions of NK cells encompass elimination of cells identified as targets, along with the secretion of proinflammatory cytokines, which can attract further immune cells and thereby promote the formation of an adaptive immune response (4). NK cell activation is regulated by the integration

of signals from an array of different germline-encoded activating and inhibitory receptors (5). Activating receptors on NK cells bind many stress-induced ligands as for example MICA and MICB, which are recognized by NKG2D on NK cells (6). The main inhibitory receptors are the killer cell immunoglobulin like receptors (KIR) which bind to HLA class I and NKG2A which binds to HLA-E, thereby allowing NK cells to kill virally infected or transformed cells that escape T cell-mediated immunosurveillance by down-regulation of HLA (3, 7, 8). This unique capability of immediate cytotoxicity toward malignant and virally infected cells makes NK cells attractive for antitumor therapy approaches (9). Especially since a clinically relevant antitumor effect has been described before, when a NK cell-mediated graft vs. leukemia effect (GvL) was found after haploidentical hematopoietic stem cell transplantation (HSCT), in case of HLA mismatch when NK cell tolerance is broken (10, 11). Currently, different anti-tumor therapies rely on tumor cell lysis through cytotoxic lymphocytes, mainly NK- and T cells. This includes beside NK- and T-cell-mediated GvL after HSCT (10, 12) the therapies inhibiting immune checkpoints (13). Moreover, the success of monoclonal antibody therapies including e.g., Rituximab depends on NK cell-mediated antibody dependent cytotoxicity (ADCC) as a main effector function (14). Lastly, there are many studies evaluating the therapeutic value of expanded NK cells or therapies with engineered NK cells or T cells (15–18).

Platelets have a well-established and important role in hemostasis and wound healing, but it has become clear that they also function as immune cells (19). In the context of cancer, they have been shown to support various steps of tumor expansion, including local growth, migration in and out of the blood stream and metastasis establishment. During several of those steps platelets are important for evasion of the immune system (20). The protection from immunosurveillance can be the result of a direct or indirect inhibition of tumor cell engagement by cytotoxic lymphocytes. Here, we review the different ways how platelets can support cancer cells to avoid or disarm lymphocyte cytotoxicity focusing on NK cells. We furthermore explore the possible implications of NK cell platelet-interactions on NK cell therapy and platelet transfusions.

PLATELET-MEDIATED IMMUNE ESCAPE MECHANISMS IN THE TUMOR ENVIRONMENT

High platelet counts were identified as a risk factor associated with adverse outcome in numerous different tumor entities including lung cancer, breast cancer, ovarian cancer, gastric cancer, pancreas carcinoma, hepato-cellular carcinoma, colon carcinoma, renal cell carcinoma or glioblastoma to name just a few (21–28). Based on these robust data, and studies showing that low lymphocyte counts correlate with shorter survival time (29), the ratio between lymphocytes and platelets has been investigated and identified as a predictive marker for the disease outcome with low platelet-lymphocyte ratios (PLR) favoring a beneficial course of the disease (30–32). Of note, a meta-analysis including 1,340 cancer patients that were treated with an immune checkpoint inhibitor, showed a clear advantage of patients with a low PLR (33). In sum, these observational studies gave rise to the question if platelet count and PLR are mere surrogate markers indicating strong systemic inflammatory response, reflecting advanced progression, or if clinically relevant interactions between platelets and lymphocytes can influence the disease outcome by themselves. Notably, these two explanations are not mutually exclusive. The understanding of how platelets can interfere with the function of different lymphocyte subsets has significantly grown and different mechanisms were uncovered. It was shown that platelets protect tumor cells from different cytotoxic lymphocytes including NK cells and effector T cells. The protective mechanisms from lysis by NK cells can be divided in those resulting from direct interaction including cell contact and cytokine interaction and those involving further cell types (34).

When a single cell or micro metastasis, consisting of a few cells enters the blood stream, it is at the same time leaving the immunosuppressive, protective environment of the tumor. It becomes vulnerable and is more exposed to potential recognition and elimination by the immune system. Metastatic tumor cells that enter the blood can activate platelets by tissue factor (TF)-mediated thrombin generation and the release of ADP or Thromboxane A2 (TXA2) (35, 36). The activated platelets can attach to the cancer cells via integrins, fibrin, and P-selectin, forming a layer of platelets, hiding the malignant cell from cellular components of the immune system (37) (**Figure 1**, left). This "cloaking" of cancer cells with platelets protects them from NK cell-mediated lysis as it was first described in mouse models of metastatic cancers (38, 39). Initially, it was hypothesized that the platelets would simply physically shield the cancer cells from direct interaction with the NK cells. More recent research on solid tumor-derived cancer cells showed that there are several more specific mechanisms by which the adherent platelets inhibit activation of NK cells. Adherent platelets can transfer their ligands for inhibitory NK cell receptors to the cancer cell surface, namely HLA class I (40), glucocorticoid-induced TNF-related protein (GITR) ligand (41), and the receptor activator of NFκB (RANK) ligand (42) (**Figure 1**, right). While KIRs, the receptors for HLA class I, are constitutively expressed on the majority of circulating NK cells, the latter two inhibitory receptors are only up-regulated under certain circumstances. GITR is expressed at low levels in resting NK cells from healthy donors but is up-regulated after activation through IL-2 or IL-15 (43). RANK expression is also absent on resting NK cells from healthy donors but is found on NK cells from patients with AML (44), breast cancer, and colon cancer (42). A recent report suggested that platelet-derived PD-L1 could protect PD-L1-negative solid tumors from elimination by T cells (45), a mechanism that extends also to NK cells (46). In addition, adherent platelets can promote the shedding of the NKG2D ligands MICA and MICB from the cancer cell surface through ADAM10/17-mediated cleavage (47, 48) (**Figure 1**, right). It has also been shown that platelet-coated tumor cells have less detectable CD112 and CD155 on their surface, the ligands to the activating NK cell receptor DNAM-1

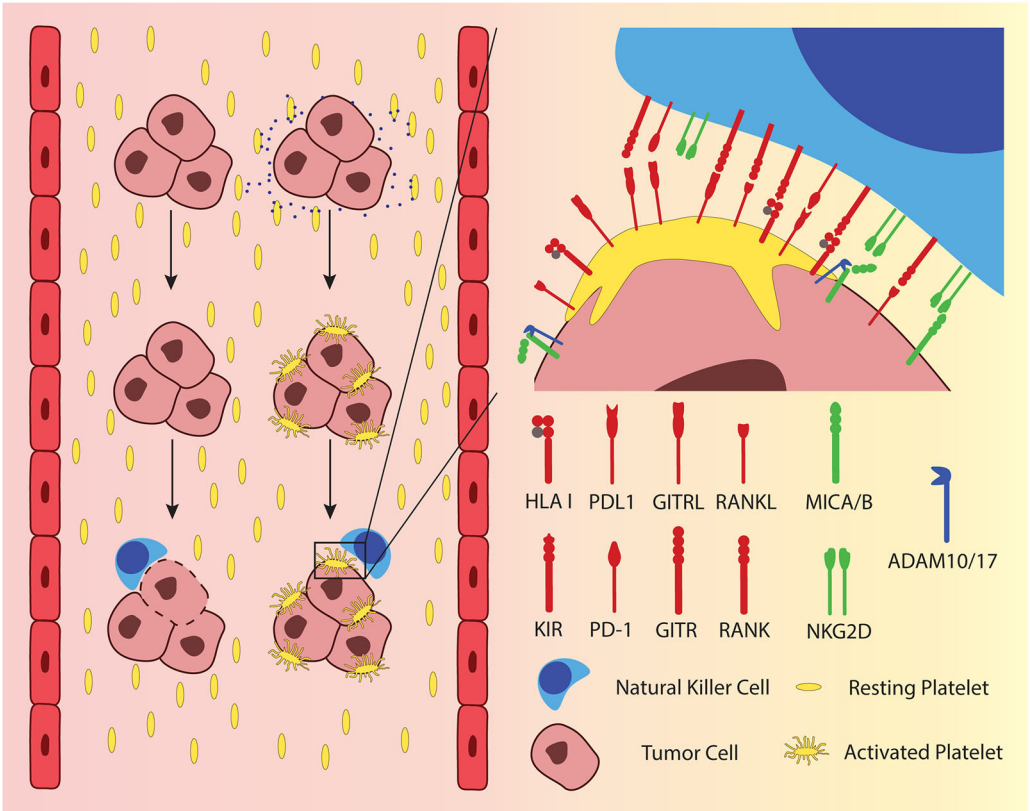

FIGURE 1 | Metastasizing tumor cells exploit platelets as physical and immunological shielding from NK cells in peripheral blood. Platelets are activated upon encountering tumor cells, which release adenosine diphosphate (ADP) and tissue factor (TF), depicted as blue dots. Activated platelets adhere to the tumor cells surface, providing a physical and an immunological shield by presenting ligands to inhibitory NK cell receptors. Moreover, activated platelets exchange surface receptors with tumor cells. Several ligands to inhibitory NK cell receptors have been shown to be passed over from platelets to tumor cells, including HLA class I, glucocorticoid-induced TNF-related protein (GITR) ligand, receptor activator of NFκB (RANK) ligand and PD-L1. The figure furthermore delineates the shedding of MICA and MICB, which are ligands to the activating NK cell receptor NKG2D.

(48). If the mechanism of this reduction involves the interaction of platelet DNAM-1 with its ligands on the cancer cells was not investigated.

The "cloaking" of cancer cells by platelets has mostly been studied in the context of solid tumor metastasis. However, it is very likely to play a role in hematological malignancies, too. Platelets attach to leukocytes in the circulation of healthy donors (49) and have been shown to adhere to the erythroleukemia cell line K562 (48), as well as primary acute myelogenous leukemia (AML) cells *in vitro* (50). Shedding of NKG2D ligands was observed when platelets attached to K562 cells, similar to cell lines derived from solid tumors (48). Hematological malignancies are often accompanied by thrombocytopenia or platelet dysfunction, a fact that makes platelets appear less likely to play a significant role in the immune evasion of these cancer types, which can explain why the interactions of platelets with hematological cancer cells have been less well-studied. However, in a small study, platelets were found attached to circulating AML blast in three out of eight patients (50) and a study with cryopreserved material from over 1,000 AML patients found platelets adherent to AML blasts in about one third of the cases (51). It appears therefore plausible that leukemia cells may benefit from adhering platelets in a similar way as metastasizing cells from solid tumors do. In consequence, those leukemia cells might be more difficult to target by host immunity, monoclonal antibodies or cellular immunotherapies.

Beside the direct contact-dependent tumor cloaking, platelets can support tumor growth and metastasis through the secretion of various factors (**Figure 2**). The granules released by platelets upon activation contain amongst others several factors of the coagulation cascade, growth factors and cytokines including TGF-β (52). Platelets are, in fact, the main source of TGF-β in the human body, both systemically and also specifically in the tumor microenvironment (53–56). TGF-β figures among the most extensively investigated immunosuppressive cytokines in the tumor microenvironment and it has been demonstrated to exert deleterious effects by affecting different lymphocytes. TGF-β inhibits the differentiation of T cells into cytotoxic T cells and raises the number of regulatory T cells (Tregs) (57–59). Tregs in turn can inhibit effector T cells and NK cells. TGF-β also exerts direct impact on NK cells: It has been shown that TGF-β

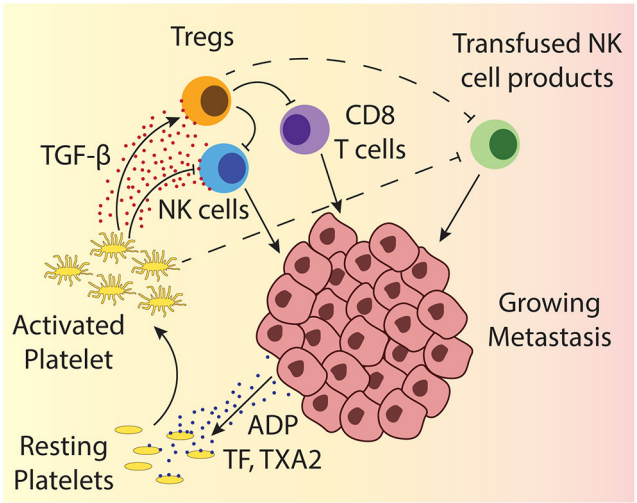

FIGURE 2 | Depicted is a growing metastasis, highlighting the interactions between platelets and different lymphocytes. Tumor cells can activate platelets through release of ADP and TF. ADP directly activates platelets through engagement of the ADP receptor, while TF activates platelets together with other coagulation factors. Platelet activation results in the release of the dense granules and α-granules that contain amongst others more ADP, different clotting factors, growth factors, and TGF-β, which can impact different tumor infiltrating lymphocytes. The impact of TGF-β on T cells and NK cells is indicated by solid line arrows and the suspected impact on transfused cellular therapy products is shown by dashed line arrows.

impairs the lytic activity as well as IFN-γ production (60, 61). The mechanisms how TGF-β reduces NK cell activity encompass downregulation of activating receptors on NK cells as e.g., NKp30 or NKG2D, resulting in decreased capability to kill target cells with the respective ligands (55, 62) and interference with IFN-γ transcription (63). Importantly and beside the fact that platelets are the main source of TGF-β it has been specifically proven that platelet-derived TGF-β can impair cytokine production and degranulation of NK cells (55, 64). Taken together, the TGF-β provided by platelets can restrain cellular immune responses in solid tumors, as well as in the bone marrow microenvironment of hematological malignancies and thus support the survival of cancer cells.

DISCUSSION

Beside the physiological role of NK cells in the control of transformed or virally infected cells, they are also a cornerstone of monoclonal antibody therapies as a mediator of ADCC (14, 65). In addition, NK cells exert, beside T cells, graft vs. tumor effects in allogeneic HSCT settings (10, 15) and various cellular therapy approaches based on *ex vivo* activated or expanded NK cells are pursued (18). In all these settings, it is a common goal to maximize cytotoxicity to achieve tumor eradication. While it remains so far elusive if and to which extent the killing capacity of cellular therapy products are reduced by platelets in the tumor microenvironment, we extrapolate that the infused cellular therapies are likewise inhibited by platelets. Therefore, it is necessary to assess the impact of tumor PLT interactions on cytotoxicity of cellular therapy products. Similar considerations apply to further immunotherapies, that rely on tumor cell lysis mediated by other cytotoxic lymphocytes, especially the inhibition of checkpoints for T cell activation e.g., by anti-CTLA4, anti-PD1 or anti-PDL1 antibodies, as well as therapies with engineered T cells. An increasing number of different immunotherapies which rely on NK cell and other lymphocyte-mediated effector functions find already broad application or have the perspective of doing so (18, 66). With a growing understanding of the adverse effects of tumor cell-platelet interactions and the consequences on various lymphocytes, new questions arise. Starting from practical questions; a large portion of patients with hematological malignancies e.g., receive frequent infusions of platelet products (67). Regarding the immunosuppressive potential of platelets described above, an optimization of the transfusion management might be worth considering if the patient is in need of both platelets and cellular therapy at the same time, especially as platelet storage increases the available TGF-β in the transfusion unit (64). Beyond this, it is highly desirable to find a generally applicable approach to prevent the adverse interactions between platelets and NK cells. This may also be beneficial for cancer patients regardless if they receive immunotherapy. While it was previously not the scope to target interactions between platelets and NK cells specifically, different approaches were evaluated either aiming to reduce the number of platelets in the tumor microenvironment or to prevent the activation of platelets or their interaction with tumor cells: It was shown that a specific inhibition of tumor-associated platelets by directing the platelet inhibitor ticagrelor to tumor-associated platelets, using a tumor homing liposomal nanoparticle strongly reduced lung metastases in a mammary carcinoma mouse model (68). Another recent study investigated the possibility of targeting cancer cell TF expression with nanoparticle-mediated delivery of siRNA to the site of metastasis in a breast cancer mouse model. This led to a tumor specific silencing of tissue factor, which in turn resulted in reduced platelet adhesion and ultimately in lower numbers of lung metastases (69). Similar observations were made studying the impact of systemic ticagrelor treatment in a breast cancer mouse model (70). However, while NK cell-mediated killing of K562 *in vitro* was inhibited in the presence of platelets, no difference was observed if the platelets were pre-treated with ticagrelor or not (71). A very recent study in mice showed reduced metastasis and a reversal of TGF-β mediated immunosuppression upon delivery of NO-releasing nanoparticles. The NO released, inhibited platelet activation and therefore TGF-β release specifically in the tumor microenvironment (72). A limitation that all approaches targeting the platelet activation share, is that the desired interruption of tumor platelet interaction with tumor cells comes with the risk of reduced coagulation function of platelets. Inhibiting platelet activation is therefore likely unsuitable for thrombocytopenic patients but might be a promising therapy for patients with normal thrombocyte counts, especially when the delivery of the platelet inhibitor can be targeted to the tumor

microenvironment and unspecific coagulation inhibition can be further reduced.

CONCLUSION

As platelets can protect cancer cells from cytotoxic lymphocytes by various mechanisms, it is very suggestive that they have an impact on the efficiency of cancer immunotherapies. So far, most studies have investigated either immunotherapy or inhibition of platelet activation, but not the combination of both. Future research will show if targeting the protective interaction of platelets with tumor cells can improve the efficiency of NK cell therapy in solid tumors where results so far were less promising than in immunotherapy of hematological malignancies. On the other hand, in hematological malignancies that are often accompanied by thrombocytopenia, it is less likely that inhibition of platelet activation would be beneficial for the patients. Here, however, the effects of platelets on NK cell cytotoxicity should be considered in the scheduling of platelet transfusions and treatment.

AUTHOR CONTRIBUTIONS

LS, PH, and SM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Therapeutic Approaches Targeting the Natural Killer-Myeloid Cell Axis in the Tumor Microenvironment

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Immunotherapy has transformed cancer treatment by promoting durable clinical responses in a proportion of patients; however, treatment still fails in many patients. Innate immune cells play a key role in the response to immunotherapy. Crosstalk between innate and adaptive immune systems drives T-cell activation but also limits immunotherapy response, as myeloid cells are commonly associated with resistance. Hence, innate cells have both negative and positive effects within the tumor microenvironment (TME), and despite investment in early clinical trials targeting innate cells, they have seen limited success. Suppressive myeloid cells facilitate metastasis and immunotherapy resistance through TME remodeling and inhibition of adaptive immune cells. Natural killer (NK) cells, in contrast, secrete inflammatory cytokines and directly kill transformed cells, playing a key immunosurveillance role in early tumor development. Myeloid and NK cells show reciprocal crosstalk, influencing myeloid cell functional status or antigen presentation and NK effector function, respectively. Crosstalk between myeloid cells and the NK immune network in the TME is especially important in the context of therapeutic intervention. Here we discuss how myeloid and NK cell interactions shape anti-tumor responses by influencing an immunosuppressive TME and how this may influence outcomes of treatment strategies involving drugs that target myeloid and NK cells.

Keywords: immunotherapy, cancer immunotherapy, myeloid cell, NK cell, tumor microenvironment

INTRODUCTION

Immunotherapy has transformed cancer treatment by harnessing the immune system to target solid and hematological cancers (1), achieving durable responses across multiple tumor types (2). However, only approximately 20% of patients have a durable response, and intrinsic or acquired resistance is often observed in the clinic (3, 4). Therefore, novel combination approaches are needed to expand

the therapeutic benefit of these drugs. Currently, several anticancer therapies employing multiple drug modalities and combinatorial approaches are being tested clinically (5), but few have been found to enhance benefit in combination with checkpoint inhibitors.

Because the balance of immunosuppressive versus immunostimulatory cells varies among individual tumors, a major goal of these approaches going forward is to understand immune contexture and segmentation. Most solid tumors include a variety of immunosuppressive cells, such as regulatory T cells, polymorphonuclear (PMN) myeloid-derived suppressor cells (MDSCs), mononuclear MDSCs (M-MDSCs), tumor-associated macrophages (TAMs) [as defined by Bronte et al., (6)] and neutrophils that can suppress effector $CD8^+$ T cells and NK cells (7).

Two cell types of the innate immune system that shape the tumor microenvironment (TME) and can initiate anti-tumor immune responses are natural killer (NK) cells and cells of the myeloid lineage, including immunosuppressive PMN-MDSCs, M-MDSCs, and immune-activating macrophages, dendritic cells, and neutrophils. Although the individual roles of these cell types in the anti-tumor immune response have been extensively studied [for review, see Neophytou et al., (8)] the ways in which interactions between these cell types affect immune responses is only just emerging. Consideration of the interplay between NK cells and suppressive myeloid cells could give new insight into the effects of therapies combining PD-1/PD-L1 and CTLA-4 blockade in the clinic and may also have an impact in early-stage cancers and hematological diseases.

The most advanced therapies are those that modulate myeloid cells, depleting or inhibiting recruitment or promoting reprogramming to activate or de-repress tumoricidal mechanisms (9), but these modalities have been disappointing in the clinic (**Table 1**). These trials include inhibitors of CSF1R, CCR2, CXCR2, CXCR4, and most recently, PI3Kγ (10, 11). Although many of these modalities have been tested clinically, few have passed beyond phase 2 studies, due to either lack of efficacy or associated adverse effects. It is important to gain insight into the mechanism of action and biomarker changes associated with efficacy in order to refine therapeutic strategies for myeloid-targeting agents and to identify patients who could benefit from these therapies as monotherapies or in combination with immune checkpoint blockade.

n contrast, only a few drugs targeting NK biology to reverse NK tumor immune tolerance have been progressed to clinical trials (**Table 1**). These therapies include the anti–KIR2DL-1, -2, and -3 antibody IPH2102/BMS-986015 (lirilumab), the anti-NKG2A antibody IPH2201 (monalizumab), and the anti-CD16 innate cell engager AFM13.

To date, the concept of modulating NK-myeloid cell interactions to relieve tumor immunosuppression is underexplored. However, further consideration of NK-myeloid cell interactions in the TME and periphery may provide insights into both innate and adaptive immune anti-tumor responses. Here we discuss possible mechanisms that can attenuate or enhance a productive immune response through innate cell–mediated responses and the consequence for activation of effector cell types in the TME.

NK CELL BIOLOGY AND THE TME

NK cells are large, granular lymphocytes that can kill target cells without previously encountering an antigen. NK cells also produce proinflammatory cytokines like interferon-alpha (IFN-α), tumor necrosis factor-alpha (TNFα), and granulocyte macrophage–colony-stimulating factor (GM-CSF), as well as chemokines such as CCL1, CCL3, CCL4, CCL5, CCL22, and CXCL8. Their activity is regulated by a balance of signals from activating and inhibitory receptors (12). Most of the inhibitory receptors bind to major histocompatibility complex (MHC) class I–like proteins, which enable NK cells to detect the downregulation of MHC class I molecules on target cells. Activating receptors, on the other hand, bind a variety of molecules, some of which are derived from pathogens such as CMV protein pp65, which is recognized by NKp30 (13), or are induced by cell stress, transformation, or infection (e.g., MICA/B and ULBP1-6, the ligands of NKG2D) (14). NK cells not only play an important role as a first line of defense against viral, bacterial, and fungal infections (15, 16), but are also important in tumor immuno-editing (17), tumor development (18), and control of metastasis (19–21).

Under nonpathological conditions, NK cells and myeloid cell subtypes crosstalk through multiple mechanisms. NK cells interact with macrophages and dendritic cells through both soluble factors, such as IL-12, IL-15, IL-27, and IL-18, and cell-to-cell contact (22–24). These interactions can induce maturation of NK cells, cytotoxicity, and cytokine release. Reciprocally, NK cell–derived cytokines can drive stimulation of macrophages. Pathogen-induced upregulation of ligands for activating NK-cell receptors can result in the elimination of monocytes and macrophages by NK cells (25), as well as the killing of immature but not mature dendritic cells *in vitro* (26), a process thought to limit the generation of potentially tolerogenic dendritic cells.

MYELOID CELLS IN THE TME INFLUENCE NK FUNCTION

Tumor-derived myeloid cells are plastic and heterogeneous and have both positive and negative roles in anti-tumor immunity. There are two main subsets of suppressive myeloid cells in tumors, PMN-MDSCs and M-MDSCs (6, 27, 28). Monocytes, M-MDSCs, and TAMs are abundant in solid tumors (29) and are associated with poor prognosis (30, 31). M-MDSCs support tumor progression through both immune-mediated mechanisms and mechanisms not directly associated with immune suppression (32). Macrophages and monocytic MDSCs isolated from mouse murine and human solid tumors can directly suppress T-cell responses (29, 33) and NK-cell cytotoxicity (34). M-MDSCs are implicated in the recruitment of T regulatory cells and inhibition of T-cell cytotoxicity and have been shown to inhibit NK cell function *in vitro* and *in vivo* (35). Normally, neutrophils respond to tissue damage and defend against pathogens (36), but in the TME, tumor-associated neutrophils or PMN-MDSCs express various cytokines, including CCL2 and CCL17, depending on their immunosuppressive or immune-

TABLE 1 | Myeloid and NK target therapies tested in clinical studies.

Target	Mechanism of Action	Modality	Drugs/ Company	Dose regimen	Current clinical status	Combinations	Indications	Clinical trial number
CCR2	CCR2 is expressed by monocytes and macrophages and interacts with CCL2 to mediate chemotaxis of monocytes and TAMs, promoting tumor progression	Small molecule	PF-04136309 (Pfizer)	Continuous	Discontinued post-phase 1b/2	Folfirinox	Pancreatic ductal adenocarcinoma	NCT01413022
						Nab-paclitaxel		NCT02732938
CSF1R	CSF1 receptor (CSF1R)-mediated signaling is crucial for the differentiation, recruitment, and survival of the mononuclear phagocyte system and macrophages	Small molecule	Pexidartinib (Turalio) (PLX7486), Daiichi Sankyo	Continuous	Phase 2/ approved	Monotherapy	Tenosynovial giant cell tumor	NCT01804530
		Small molecule	JNJ-40346527 (J&J)	21-day cycle or PO BID for 4–5 weeks	Discontinued after phase 1b/2	Monotherapy	Relapsed or refractory Hodgkin lymphoma	NCT01572519
							Relapsed or refractory AML	NCT03557970
						Surgery	Advanced Prostate Cancer	NCT03177460
		Small molecule	ARRY-382 (Array/Pfizer)	21-day treatment cycles	Phase1b	Keytruda (anti–PD-1 antibody)	Relapsed or refractory Hodgkin lymphoma, AML	NCT02880371 NCT01316822
		Small molecule	BLZ945 (Novartis)		Phase I (ongoing)	PDR001 (anti–PD-1)	Advanced solid tumors	NCT02829723, NCT02404441
		Antibody	RG7155/ emactuzumab (Roche)	IV Q3W	Phase 2	Atezolizumab (anti–PD-L1 mAb)	Advanced solid tumors	NCT02323191
						Selicrelumab (anti-CD40)		NCT02760797
						Paclitaxel and bevacizumab	Platinum-resistant ovarian cancer	NCT02923739
		Antibody	AMG 820-mAb (Amgen)	IV weekly	Phase 1/2	Pembrolizumab (anti–PD-1 mAb)	Advanced solid tumors	NCT02713529, NCT01444404
		Antibody (human mAb)	LY3022855 (Lilly)	IV Q4W	Phase 1	Durvalumab (anti–PD-L1 mAb) or tremelimumab (anti–CTLA-4 mAb) GVAX	Advanced solid tumors	NCT02718911
							Pancreatic cancer	NCT03153410
CXCR2/IL8 axis	CXCR2 plays a critical role in the regulation of neutrophil homeostasis and recruitment to the tumor	Small molecule	AZD5069 (AstraZeneca)	Continuous + PD-L1	Phase1/2	Durvalumab (anti–PD-L1 mAb)	Head & neck/ pancreatic cancer	NCT02499328, NCT02583477
						Enzalutamide	mCRPC	NCT03177187
		Antibody	HuMax-IL8/ BMS-986253 (BMS)	IV Q2W	Phase1/2	Nivolumab + degarelix	Hormone-sensitive prostate cancer	NCT03689699
						Nivolumab	HCC	NCT04050462
							metastatic or unresectable solid tumors	NCT03400332
							NSCLC/HCC	NCT04123379
		Small molecule	Navarixin/MK-7123 (Merck)	IV infusion on day 1 of each 3-week cycle	Phase 2	Pembrolizumab	Advanced/ metastatic solid tumors	NCT03473925
					Phase 1	Pembrolizumab		NCT03161431

(Continued)

TABLE 1 | Continued

Target	Mechanism of Action	Modality	Drugs/ Company	Dose regimen	Current clinical status	Combinations	Indications	Clinical trial number
		Small molecule	SX-682 (Syntrix Pharmaceuticals)	SX-682 monotherapy for 21 days, then 90 days with pembro			Metastatic melanoma	
			Reparixin (IL-8) (Dompe)		Phase 2; discontinued	Paclitaxel	$HER2^-$ breast cancer	NCT02001974 NCT02370238
		Small molecule						NCT01861054
PI3Kγ	PI3Kγ signaling promotes macrophage pro-inflammatory profile and anti-tumor activity	Small molecule	Eganelisib (IPI-549)	Continuous	Phase 2	Nivolumab	Advanced urothelial carcinoma	NCT03980041 UC
						Tecentriq and abraxane (TNBC)/ bevacizumab (RCC)	TNBC and RCC	NCT03961698 RCC
						AB928 (A2ARi)/ pegylated liposomal doxorubicin (PLD)/ nanoparticle albumin-bound paclitaxel (NP)	TNBC and ovarian cancer	
								NCT03719326 TNBC/OV
								NCT03719326 TNBC/GC
CCL2	CCL2 chemokine interacts with CCR2 in monocytes and macrophages, impairing migration	Antibody (human mAb)	Carlumab (CNTO888)	IV Q2W	Phase 2	Monotherapy Chemotherapy (SoC)	MCRP advanced solid tumors	NCT00992186 NCT01204996
CD47/CD47-SIRPα	Promotes the adaptive immune response and enhances the phagocytosis of tumor cells by macrophages	Antibody (hu mAb)	Magrolimab (Hu5F9-G4)/ Gilead Sciences	IV every 3 cycles	Phase 3	Azacitidine	MDS AML DLBCL FL	NCT03248479
		Antibody (hu mAb)	CC-90002/ Celgene	IV infusion on a 28-day cycle	Phase 2	Rituximab	Advanced solid and hematologic cancers	DOI: 10.1056/ NEJMoa1807315 NCT02367196
NK2GA	NKG2A/CD94 are inhibitory receptors expressed on T and NK cells. Inhibition of interaction with HLA-E relieves inhibitory signals and leads to cell activation and cytotoxicity	Antibody (hu mAb)	Monalizumab	IV	Phase 1/2 Phase 3	Durvalumab (MEDI4736) Ibrutinib	advanced solid tumors Relapsed, refractory or previously untreated CLL	NCT02671435
						Durvalumab	Advanced NSCLC (resistance CPI)	NCT02557516
						Durvalumab	NSCLC	NCT03833440
						Durvalumab	Resectable NSCLC	NCT03822351
						Cetuximab	Metastatic HNSCC	NCT03794544
								NCT02643550
						Cetuximab	Recurrent or metastatic HNSCC	NCT04590963
CD30xCD16a	AFM13 is a bispecific, tetravalent chimeric antibody designed for the treatment of	Affimed	AFM13	Weekly IV	Phase 2	Pembrolizumab	Relapsed or refractory classical	NCT02665650

(Continued)

TABLE 1 | Continued

Target	Mechanism of Action	Modality	Drugs/ Company	Dose regimen	Current clinical status	Combinations	Indications	Clinical trial number
	CD30-expressing malignancies. AFM13 recruits NK and macrophage cells *via* binding to CD16A as immune effector cells. https://dx.doi.org/10.1182%2Fblood-2014-12-614636			Weekly IV	Phase 1/2 approved (orphan drug designation)		Hodgkin lymphoma Peripheral T-cell lymphoma	NCT04101331
EGFRxCD16A	AFM24 NK-cell–engaging bispecific antibodies to target EGFR-expressing tumor cells irrespective of their mutational status.	Bispecific engager	Affimed (AFM24)	Weekly IV	Phase 1		Advanced solid cancers	NCT04259450
BCMAxCD16a	Bispecific antibody (IgG-scFv) targeting B-cell maturation antigen and CD16a (FcγRIIIA) being developed for treatment of multiple myeloma	Bispecific engager	Roche (RO7297089)	Weekly IV	Phase 1		Multiple myeloma	NCT04434469
HER2 x NKG2D x CD16A	HER2 trispecific NK cell engager; binds to HER2 on tumor cells and simultaneously binds to NK cells	Trispecific engager	Dragonfly Therapeutics (DF1001)		Phase 1/2	Pembrolizumab	Advanced solid tumors	NCT04143711
KIR2DL-1, -2, -3	Inhibits major inhibitory receptors on NK cells	Humanized mAb	Innate Pharma/ BMS (IPH2102/ BMS-986015/ lirilumab)	4 cycles Q4W IV	Phase 1/2	Ipilimumab or nivolumab	Advanced solid tumors	NCT01750580 NCT01714739
CD16/IL-15/ CD33	Trispecific scFv recombinant fusion protein conjugate composed of heavy and light chains of anti-CD16 and anti-CD33 antibodies and human IL-15	Trispecific engager	GT Biopharma (GTB-3550)	3x weekly IV	Phase 1/2		High-risk heme malignancies	NCT03214666

AML, acute myeloid leukemia; BID, twice daily; CLL, chronic lymphocytic leukemia; CPI, checkpoint inhibitor; EGFR, epithelial growth factor receptor; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; IV, intravenous; mAb, monoclonal antibody; m-CRPC, metastatic castration-resistant prostate cancer; MDS, myelodysplastic syndrome; NP, nonpegylated; NSCLC, non–small-cell lung carcinoma; PLD, pegylated liposomal doxorubicin; PO, orally; PTCL, peripheral T-cell lymphoma; Q2W, Q3W, Q4W, every 2, 3, 4 weeks; RCC, renal cell carcinoma; scFv, single-chain variable fragment; SoC, standard of care; TNBC, triple-negative breast cancer.

activating state, and can degranulate to release various types of bioactive molecules (37, 38). The formation of neutrophil extracellular traps can convert dormant cancer cells, drive aggressive lung metastases in mice (39), and accelerate hepatocellular cancer (40) in patients and in mouse models (41).

NK CELLS IN THE TME

The TME not only shapes the adaptive immune response but also has profound effects on NK cells, which in many tumors are functionally distinct. Anti-tumor NK effector mechanisms such as cytotoxicity and secretion of pro-inflammatory cytokines are impaired due to low expression of effector molecules perforin and granzyme in patients with lung adenocarcinoma (42), downmodulation of activating receptors NKG2D or NKp30 in gastric cancer (43), and upregulation of inhibitory receptors like NKG2A in cervical cancer (44).

NK cells in tumors also acquire pro-angiogenic and pro-tumor functions, including the secretion of vascular endothelial growth factor (VEGF) (45), angiogenin, and MMP9 (46, 47). Indeed, NK cells play an important role in the menstrual cycle and establishing the placenta (48). The induction of some of these phenotypic features have been attributed to immune-modulatory molecules present in the TME, such as indoleamine-pyrrole 2,3-dioxygenase and tumor growth factor-beta (TGF-β), which can be secreted by MDSCs (49). NK cells in which STAT5 has been silenced express VEGF-A at a level sufficient to promote the growth of murine syngeneic tumors (50). NK cells with a pro-angiogenic phenotype have been identified in non–small-cell lung cancer (47) and colorectal cancer (51). However, it is not clear whether there is a meaningful or broad contribution of these potentially pro-angiogenic NK cells to drive tumor progression or whether they represent the primary angiogenic drive. In one study, genetic inactivation of VEGF in myeloid cells prevented tumor growth and chemotherapy-induced cachexia in B16 and LLC mouse tumor models (52). This study also suggested that increased levels of circulating chemerin by the tumor endothelium improved NK-cell recruitment to the tumor site, suggesting that an indirect mechanism of targeting myeloid cells affects NK recruitment and function. It would be important to understand whether pharmacological interventions would have a similar effect and whether this is a dominant mechanism.

Some of the factors that are known to contribute to functional impairment of NK cells, such as hypoxia, are tumor intrinsic, whereas others are secreted by tumor-associated cells, in particular MDSCs and TAMs. In mouse models, one such mechanism is induction of NK-cell scavenger receptor expression, which is involved in lipid metabolism. Uptake of MDSC-derived factors leads to lipid accumulation and functional impairment (53).

NK cells have also been implicated in anti-tumor immune responses after checkpoint blockade. PD-1 is expressed on about 25% of NK cells in some healthy donors, usually at low levels (54), but has been found to be expressed at substantial levels in patients with ovarian cancer (54); digestive cancers, including esophageal, liver, colorectal, and gastric cancers (55); multiple myeloma (56); Kaposi sarcoma (57); and renal cell carcinoma (58). However, infiltrating NK cells in non–small-cell lung cancer do not express PD-1 (59), although a recent systematic study using multiple methods to detect PD-1 protein and mRNA concluded that NK cells showed only minimal expression of PD-1 in primary human tumor samples of round-cell sarcoma and colorectal cancer, as well as in multiple mouse tumor models (60). Despite these findings, several studies have reported upregulation of PD-1 expression on NK cells in various mouse models (61, 62), and although blockade of PD-1/PD-L1 interaction has been shown to enhance activity of NK cells *in vitro* and in animal models (63), this is suggested to be mediated mainly through expression of PD-L1 by NK cells (64). Moreover, it is not clear how NK cells contribute to anti-tumor responses in patients. NK cells have also been implicated in playing a role in response to treatment with agonistic anti-CD137/4-1BB antibodies. CD137 is upregulated by Fc receptor cross-linking on NK cells (65) and in patients after treatment with monoclonal antibodies (66). CD137 ligation contributes to activation *in vitro* (67) and in humanized mice (68) but reports that enhances antibody-dependent cell cytotoxicity have been retracted (69, 70).

HUMORAL VS. CELL-CELL INTERACTION CROSSTALK BETWEEN NK AND MYELOID CELLS

In the TME, cell-cell interactions and humoral responses build an anti-tumor immune response; therefore, it is important to consider how different therapeutic approaches can affect these interactions. The role of myeloid cells in the TME has been extensively studied, and a number of therapeutics have been developed to target these cells. Both neutrophil/PMN-MDSC and macrophage/M-MDSC–like myeloid cells can influence both T-cell and NK-cell activation and play both positive and negative roles in tumor growth and metastatic progression.

During infection, macrophages can modulate NK function either through direct cell-to-cell contact or through secretion of soluble mediators such as IL-18, IL-12, and TGF-β (25) (**Figure 1A**). $CD56^{bright}$ NK cells accumulate in inflammatory lesions in the presence of IL-12, IL-15, and IL-18 and engage with $CD14^{+}$ monocytes in a reciprocal activation loop, amplifying the inflammatory response by increasing TNFα production by monocytes and IFNγ by NK cells (71). *In vitro*, appropriately activated myeloid cells can also facilitate activation of NK cells *via* cell-cell interactions, enhancing CD69 expression and secretion of IFNγ in co-cultures (71, 72). In contrast, monocytes and macrophages isolated from hepatocellular carcinoma patient samples (34) and gastric cancer (73) tumors can induce NK-cell dysfunction *via* direct cell-cell interaction and indirectly, e.g., through soluble TGF-β signaling. In other studies, macrophages and monocytes isolated from hepatocellular carcinoma samples expressed high levels of CD48, driving NK-cell dysfunction. This effect was attenuated by blocking the NK-cell CD48 receptor 2B4 (34). Macrophage or M-MDSC secreted factors can have direct and indirect effects on myeloid and NK-cell crosstalk. Soluble TGFβ modulates NK-cell function *via* activating receptors NKG2D and CD16 antibody-dependent cell-mediated cytotoxicity in tumors by impairing cytotoxicity potential *in vivo* and in co-culture experiments with acute myeloid leukemia and colon cancer models (74, 75). Conversely, IL-15 plays a role in maintaining NK activation to suppress tumor escape and metastasis (76). Other secreted factors may act indirectly; these include tumor-derived prostaglandin-E2, which induces MDSCs and inhibits NK-cell function in melanoma samples (77). Restoring NK-cell function by co-targeting immunosuppressive myeloid cells may be an important therapeutic strategy to prevent tumor immune escape (**Figure 1A**).

Pro-inflammatory macrophages, such as IL-12–secreting macrophages (9, 78), that promote NK function in infection and mouse tumor models highlight the importance of understanding the difference between specific myeloid phenotypes and their influence on NK activation and function (79). Some myeloid-targeting therapies rely on cell depletion mechanisms, whereas others attempt to block recruitment or to reprogram these cells into a pro-inflammatory anti-tumor state (9). This is an important consideration when developing therapies, given the high plasticity of myeloid cell types and multiple cell interactions, including activating and suppressive impacts on T- and NK-cell effector functions.

Most studies have focused on the effects of myeloid-cell inhibitors (e.g., CSF1R inhibitors) on primary tumors, but not much information is available in the context of metastasis. CSF1R inhibition reprograms the TME to increase responses to chemotherapy and checkpoint inhibitors and to decrease metastatic spread (80). In some studies, inhibition of CSF1R depleted tumor-associated macrophages but unexpectedly promoted metastasis in 4T1 orthotopic syngeneic models. In one report, CSF1R inhibition reduced the number of NK cells due to a decrease in IL-15, a T-cell and NK-cell survival factor secreted by myeloid cells (22). Moreover, dosing exogenous IL-15 during CSF1Ri treatment restored NK-cell numbers and metastasis control. Genetic ablation of IL-15 in mice and in Th2-polarized CD4 T cells has been found to promote the formation of M2 macrophages that are thought to contribute to

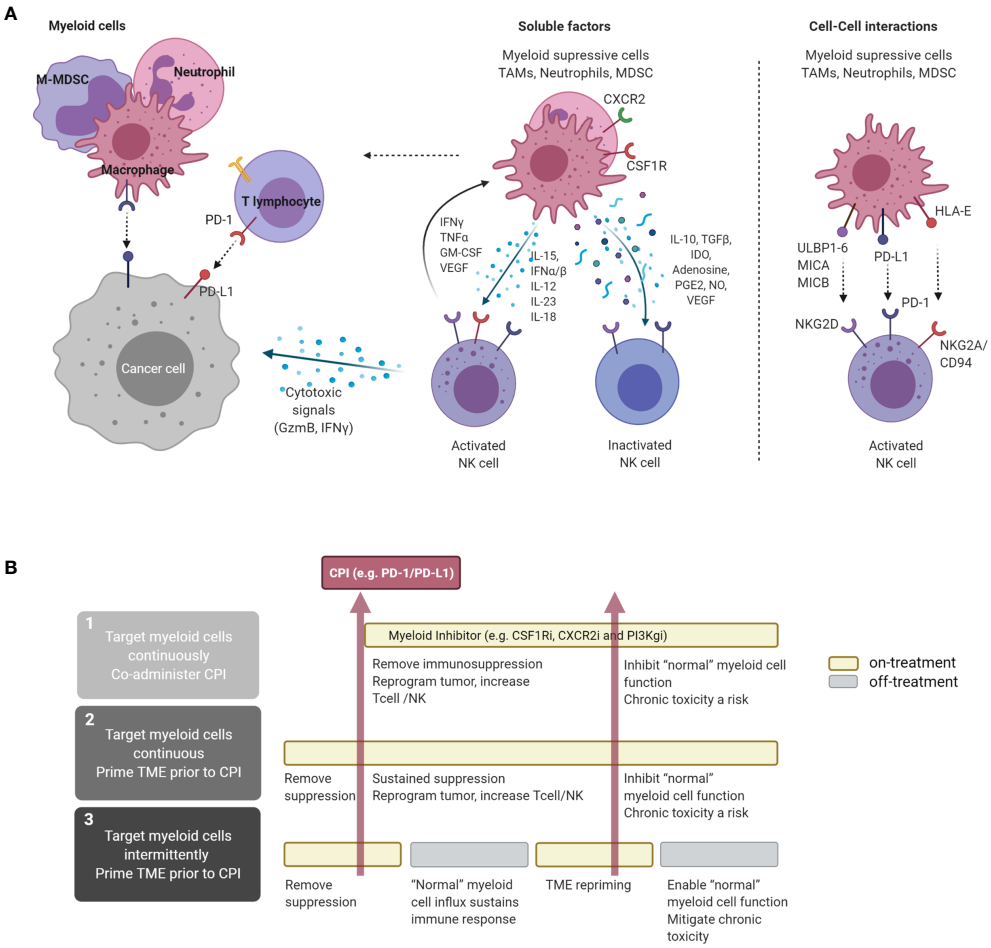

FIGURE 1 | Direct and indirect interaction of NK and myeloid cells in the TME and therapeutic concepts. **(A)** Interaction of NK and myeloid cells in the TME. NK cells can directly target tumor cells *via* cytolytic granules, independent of antigen recognition. Macrophages with immunostimulatory properties can independently induce tumor-cell killing through antigen presentation and production of pro-inflammatory cytokines. In the TME, myeloid cells, including TAMs, M-MDSCs, neutrophils, and PMN-MDSCs, can secrete a variety of soluble factors that inhibit NK activation and therefore suppress NK-mediated cytotoxicity. Cytokines secreted by NK cells (e.g., IFNγ, TNFα, and GM-CSF) can stimulate macrophages, driving a pro-inflammatory activated state. These two cell types can also interact at the receptor level, where myeloid cell-surface ligand and NK receptors interact, attenuating downstream signaling, e.g., NKG2D. **(B)** Proposed therapeutic approaches targeting myeloid-cell subsets in the TME and proposed alternative treatment sequences that can be explored to maximize immune-mediated anti-tumor response. CPI, checkpoint inhibitor. Figure created with BioRender.com.

metastasis formation (81). NK cells may control the seeding of circulating tumor cells due to crosstalk with myeloid cells, a process that is affected when tumors are treated with depleting CSF1R antibodies (82). It is interesting to contrast this finding with the observation that neutrophils or PMN-MDSCs promoted metastasis in this model (83). Other therapeutic approaches can influence this axis. Blocking CD39 activity in myeloid cells has been shown to improve control of metastases *via* NK-cell effector function (84). CD39 expression by myeloid cells, but not NK cells, was required for efficacy, suggesting that blockade of CD39 on myeloid cells limits the impact of eATP in driving intratumor myeloid pyroptosis or the release of IL-18, both of which have been shown to stimulate NK-cell effector function (84).

CCL2 (MCP1), which interacts with CCR2, is an alternative mechanism that influences macrophage-related myeloid recruitment to tumors and subsequent tumor progression (85). Inhibition of CCL2 has been shown to limit early metastatic processes in breast cancer; however, after cessation of therapy, increased metastatic spread is observed due to enhanced recruitment of monocytes to micrometastatic lesions in breast (86, 87) and lung (88, 89) metastasis mouse tumor models. Importantly, it has been suggested that combined inhibition of CCL2 and IL-6, a cytokine expressed by myeloid cells, reduced metastasis and improved survival in prostate cancer (90). Targeting CCR2 also reduces tumor progression associated with an influx of T cells in preclinical glioma (91) and pancreatic models (92).

Although macrophages can contribute to anti-tumor immunity, both monocyte-derived MDSCs and TAMs can also promote cancer initiation, stimulate angiogenesis, and suppress anti-tumor immunity during malignant progression. Pro-inflammatory, or "anti-tumor", macrophages contribute to an anti-tumor response by producing pro-inflammatory cues such as IFNγ and IL-12 secretion or by acquiring an antimicrobial and tumoricidal phenotype (93, 94). Therefore, "reprogramming" macrophages into an anti-tumor and proinflammatory state is an attractive strategy to tip the balance on tumor immunity. Targeting STAT3 (95) or PI3Kγ signaling has been shown preclinically to change the TME in tumors by remodeling suppressive macrophages into proinflammatory macrophages. Selective targeting of PI3Kγ signaling in combination with checkpoint blockade is thought to promote reprogramming of macrophages into a pro-inflammatory state, leading to cytotoxic T-cell–mediated anti-tumor response in preclinical mouse models (96, 97). The combination of PI3Kγ with PD-1 blockade is currently under investigation in clinical trials and recently received FDA Fast Track designation in urothelial cancers (ClinicalTrials.gov NCT03980041).

In normal physiological processes, neutrophil depletion impairs NK-cell maturation, function, and homeostasis (98). The role of neutrophils and PMN-MDSCs in cancer has been extensively studied, and these cells play an important role in facilitating tumor progression. In various tumor models, targeting or depleting neutrophils or PMN-MDSCs reduces metastasis in both autochthonous models of pancreas (99), colon cancer (100), breast (101), and metastatic syngeneic models (83, 102). This metastatic process may be through γδT-cell–orchestrated suppression of CD8 T cells by modified neutrophils (101). However, there is evidence that immunosurveillance of metastatic 4T1 cells by NK cells is inhibited by interaction with $CD11B^{+}/Ly6G^{+}$ neutrophils (most likely PMN-MDSCs), increasing residence time for metastatic tumor cells arriving at the lung and enabling extravasation and establishment of the metastatic niche (83). Soluble factors such as IL-17, granulocyte-CSF (G-CSF) (101), and TGFβ signaling (100, 103) play pivotal roles in establishing this suppressive network. The crosstalk between neutrophils and PMN-MDSCs is not a one-way process. In MCA205-Luc2 tumors, depletion of NK cells with antibodies or CXCR3 blockade has been shown to promote tumor growth due to reduced IFNγ and upregulation of IL-17A and VEGF-A, modifying the TME and recruitment of suppressive neutrophils of PMN-MDSCs (104).

Therapeutic targeting of CXCR2 (or IL-8) inhibits neutrophil-granulocytic myeloid cells or PMN-MDSCs, leading to suppression of metastasis in mouse models of pancreatic cancer (99) and colorectal cancer (100), as well as in metastatic syngeneic models 4T1 and B16F10 (105). In preclinical efficacy studies, CXCR2 inhibition resulted in an influx of T cells (99, 100, 105, 106); however, the impact on the broader immune environment, including NK biology, has not been explored. Although CXCR2 blockade inhibits recruitment of granulocytic myeloid cells to the tumor, it may also inhibit NK recruitment. CXCR1 and CXCR2 are highly expressed by cytotoxic $CD56^{dim}$ NK cells (107), and increasing CXCR2 expression on NK cells promotes recruitment to tumors that overexpress CXCR2 ligands (108). Importantly, the CXCR2 ligand CXCL8 is secreted within the TME of melanoma-infiltrated lymph nodes and may play a role in the efficient recruitment of highly cytotoxic NK cells (109). Because it has been suggested that chronic combined inhibition of both CXCR2 and CSF1R can increase the efficacy of checkpoint inhibition in syngeneic models (110), understanding the potential impact of comprehensive myeloid suppressor cell inhibition on NK-cell activity should be considered.

Taken together, these studies of different myeloid lineages exemplify how depletion of specific subsets of myeloid cells can affect different features of the TME, modulating innate effector-cell activity and promoting tumor progression and metastasis. However, because myeloid cells, and particularly macrophages, play an important role in assisting NK- and T-cell activation, it is important to target the right population of cells. Moreover, given that these are essential cell types, translation to a clinical setting may be limited by tolerability, as observed in studies targeting the CSFR1 axis with antibodies or small molecules (80, 111, 112), which resulted in increased liver enzymes and induction of periorbital edema. Less toxicity was observed when the alternate macrophage regulating receptor CCR2 was targeted (113–115).

Nontargeted therapies, such as chemotherapy, can also deplete myeloid cells from tumor. Paclitaxel-carboplatin treatment was shown to alter circulating and intratumoral myeloid cell populations and to promote anti-tumor responses when combined with vaccination in HPV-16–positive tumors in mice (98). In a phase 2 trial in patients with extensive small-cell lung cancer, it was reported that ipilimumab treatment beginning with the third cycle of paclitaxel-carboplatin treatment produced better clinical outcomes than giving the drugs during cycles 1 to 4 (116). An understanding of the pivotal points in these complex signaling and transcriptional networks that program the myeloid cell phenotypes is essential to guide more effective therapeutic approaches.

PERSPECTIVE: IMPACT OF DOSE AND SCHEDULE IN MYELOID TARGET THERAPIES AND CHECKPOINT INHIBITORS

Translation to the clinic of preclinical concepts, which were largely developed using fast-growing subcutaneous *in vivo* models, presents a challenge. Subcutaneous models are limited because they do not reflect the variations observed in the tissue of residence, and the speed of cell growth in these models does not enable elucidation of the longer-term consequences of the treatment strategy. As shown in **Table 1**, most clinical studies have taken a standard approach in which the myeloid therapy is co-administered with the checkpoint inhibitor or chemotherapy and then dosing is maintained chronically (80, 111–113, 115). This approach has a number of drawbacks. Myeloid cells exhibit both positive and negative effects on the TME, as described

above. Accumulation of myeloid cells in the tumor (macrophage-like and neutrophil-like cells) clearly defines a resistance phenotype, and depletion of macrophages and neutrophils can remodel the TME. In addition to preventing the suppressive crosstalk to immune cell types, including T cells and NK cells, removal of these cells results in remodeling of the stroma and, in some cases, reprogramming of the tumor cell compartment. These changes make the tumor more susceptible to appropriate recruitment of activated immune cells. Hence, it is likely that pretreatment with a myeloid modulation agent prior to treatment with immunotherapy or even chemotherapy would "prime" the TME by reversing the resistant features in the tumor, facilitating more effective stimulation of the immune system. However, once the immune response is progressing, then more "normal" myeloid cells could be required to sustain that response, especially in situations where there is less effective immune recognition of the tumor. Paradoxically, chronic suppression of the myeloid cells may result in attenuation of the immune response in certain situations, mitigating the advantages gained from targeting the suppressive cells. Therefore, therapies that deplete myeloid cells or prevent recruitment to the TME may be more effective with intermittent or sequenced dosing, using the myeloid therapy for a short time prior to treatment to "prime" the TME, but then stopping dosing after introduction of the checkpoint inhibitor to allow the more normal immune response to progress (**Figure 1B**). These types of intermittent approaches could also mitigate clinical toxicity.

DISCUSSION

The development of cancer immunotherapies, specifically immune checkpoint blockade, has shifted the treatment of cancer by promoting complete and durable responses (117, 118). Immunotherapies focus on enhancing the activities of T cells; however, the complexity of the TME limits the response. The pivotal role of tumor myeloid cells, particularly macrophages, in conditioning the TME and regulating the broader response to host immune response and therapy is broadly appreciated. Unfortunately, the development of targeted therapeutics has only just started to teach us about the complexity of this cross-regulation, particularly in the context of different tumor mutational backgrounds and TMEs, as well as the broader systemic immune response. To enhance success, it is worth considering the positive influence of myeloid cells on the other components of the immune system, such as NK cells, and their role in sustaining persistent T-cell responses. Although myeloid therapies have largely been combined with checkpoint inhibitors and, to a lesser extent, chemotherapeutics, little consideration has been given to combinations with therapies targeting other functional nodes, such as NK cells or stimulators of innate immunity. As we seek to improve responses in patients earlier in disease progression, at the point of metastatic spread, such alternative strategies could become important.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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NK Cells and PMN-MDSCs in the Graft From G-CSF Mobilized Haploidentical Donors Display Distinct Gene Expression Profiles From Those of the Non-Mobilized Counterpart

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A recent approach of hematopoietic stem cell (HSC) transplantation from haploidentical donors "mobilized" with G-CSF is based on the selective depletion of $\alpha\beta$ T and B lymphocytes from the graft. Through this approach, the patient receives both HSC and mature donor-derived effector cells (including NK cells), which exert both anti-leukemia activity and protection against infections. We previously showed that donor HSC mobilization with G-CSF results in accumulation in the graft of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), capable of inhibiting *in vitro* the anti-leukemia activity of allogeneic NK cells. Here, we performed a detailed gene expression analysis on NK cells and PMN-MDSCs both derived from mobilized graft. Cytotoxicity assays and real time PCR arrays were performed in NK cells. Microarray technology followed by bioinformatics analysis was used for gene expression profiling in PMN-MDSCs. Results indicate that NK cells from the graft have a lower cytolytic activity as compared to those from non-mobilized samples. Further, mobilized PMN-MDSCs displayed a peculiar transcriptional profile distinguishing them from non-mobilized ones. Differential expression of pro-proliferative and immune-modulatory genes was detected in mobilized PMN-MDSCs. These data strengthen the concept that G-CSF-mobilized PMN-MDSCs present in the graft display unique molecular characteristics, in line with the strong inhibitory effect on donor NK cells.

Keywords: myeloid-derived suppressor cells, NK cells, hematopoietic stem cell transplantation (HSCT), leukemia, microarray gene expression analysis

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a life-saving treatment for children with both malignant and non-malignant severe disorders. However, a suitable HLA-compatible donor is not always available (1, 2). Thus, different HSCT strategies have been developed to offer an allograft to these patients with no alternative therapeutic options. The first approach was based on the infusion of megadoses of highly purified $CD34^+$ precursors (3, 4). However, the complete lymphoid cell depletion caused a prolonged lymphopenia and a delayed immune reconstitution, resulting into an increased risk of leukemia relapses and opportunistic infections, especially in the early period after HSCT (5). In an attempt to overcome these severe complications, a novel method of graft manipulation based on the specific depletion of $\alpha\beta$ T lymphocytes (responsible of graft-*versus*-host disease; GvHD) and B cells (in order to prevent Epstein–Barr virus-related post-transplant lymphoproliferative disorders), has been more recently introduced (6, 7). Through this new approach, the patient receives not only HSCs but also high numbers of cells of the innate immunity (including mature NK cells, $\gamma\delta$ T lymphocytes and different myeloid cells), some of which may rapidly exert anti-leukemia activity (graft-*versus*-leukemia; GvL) and early protection against infections. In particular, infused mature donor NK cells can promptly exert a GvL effect (8–12).

To obtain high numbers of HSC in the peripheral blood (PB), donors were treated with G-CSF for 5 days. G-CSF induces HSC mobilization from the bone marrow (BM). However, this treatment also induces an increase of circulating myeloid cells, including myeloid-derived suppressor cells (MDSCs) (13–15).

MDSCs represent an intrinsic myeloid compartment derived from a common myeloid precursor present in the BM (16). Human MDSCs are classified in two major subsets based on their surface markers' expression, namely monocytic MDSCs (Mo-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) (17). Mo-MDSCs are $CD45^+Lin^-$(CD3/CD19/CD56)$HLA\text{-}DR^{-/low}CD33^+CD11b^+CD14^+CD66b^-$ (or $CD15^-$) while PMN-MDSCs are $CD45^+Lin^-$ (CD3/CD19/CD56)$HLA\text{-}DR^{-/low}CD33^+CD11b^+CD14^-CD66b^+$ (or $CD15^+$) (18). Low numbers of Mo- and PMN-MDSC cells are present in PB of healthy individuals. However, a substantial accumulation of these cell subsets both in PB and in tissues was detected in different pathological conditions (including acute/chronic viral and bacterial infections), characterized by an inflammatory response (19–22). As a consequence of inflammation, a partial block in the differentiation of myeloid precursors occurs contributing to their accumulation. Importantly, MDSCs have also been detected in the microenvironment of various tumors (22, 23).

Emerging evidence indicates that MDSCs can interact and regulate the function of other immune cells, including NK cells. Several studies described different mechanisms adopted by MDSCs to exert their immunomodulatory function either by mechanisms that require cell-to-cell contact and/or by the release of soluble factors (24). In addition, a large number of data indicate that some genes are critical for MDSC function and/or represent useful markers to identify PMN-MDSCs. Thus, lectin-type oxidized LDL receptor 1 (LOX1) is undetectable in neutrophils while it is expressed by PMN-MDSC with potent immunosuppressive activity (18). Arginase 1 (ARG1) and nitric oxide synthase 2 (NOS2) are enzymes involved in L-arginine metabolism and are expressed in activated MDSCs. ARG1 promotes depletion of L-arginine, whereas NOS2 stimulates nitric oxide synthesis. In an inflammatory/tumor microenvironment, both enzymes cause suppression of immune cell function (25). Another characteristic of MDSCs associated with their suppressive capability is the production of interleukin 10 (IL-10) and tumor growth factor β (TGFβ), both acting as potent immunosuppressive cytokines on T and NK cell function (26). Furthermore, two important genes in MDSC biology are the S100 calcium-binding proteins S100A8 and S100A9. These proteins are constitutively expressed by myeloid cells including MDSCs, and they are down-regulated during normal differentiation of myeloid precursors. Some studies indicate that tumor-derived factors promote sustained up-regulation of S100A9 in myeloid precursors inducing accumulation of MDSCs (27).

In a previous study, we showed that, in HSCT, donor HSC mobilization with G-CSF induces the accumulation of PMN-MDSCs in PB and, consequently, in the graft and that these cells are capable of inhibiting *in vitro* the anti-leukemia cytolytic activity of donor-derived mature NK cells. Their potent immunosuppressive activity was exerted both by cell-to-cell contact mechanism and by their ability to release prostaglandin E2 (PGE2), indoleamine-pyrole 2, 3-dioxygenase (IDO) metabolites, and exosomes with immunosuppressive activity (28).

In the present study, we show that NK cells isolated from "mobilized" donors display a reduced cytolytic activity paralleled by a decreased expression of genes involved in this function as compared to non-mobilized NK cells. Thus, NK cell suppression detected in mobilized donors and the striking increase of PMN-MDSCs in the graft prompted us to explore more deeply the molecular features of these PMN-MDSCs. In particular, we evaluated the global pattern of gene expression in PMN-MDSCs present in the donor both before and after G-CSF-induced mobilization. Bioinformatics-based approaches were used to better investigate the role of these cells in HSCT. In mobilized PMN-MDSCs, we found de-regulation of some immune-modulatory genes and a striking activation of genes involved in the cell cycle program. These results show that mobilized PMN-MDSCs are characterized by a peculiar transcriptional profile compatible with an intense proliferative activity. Moreover, these data suggest of a further PMN-MDSC expansion in the patient after transplantation. Thus, removal or inactivation of this immunosuppressive cell subset could represent a promising strategy to restore/improve the NK-mediated GvL activity.

MATERIALS AND METHODS

G-CSF Mobilized Donors, Samples, and Ethical Statements

Healthy donors were enrolled at Bambino Gesù Children's Hospital, Rome, Italy. G-CSF mobilized donors received

subcutaneous administration of G-CSF for five days (until apheresis) at the dose of $10^{-}{}_{12}$ μg/kg/day. Peripheral blood mononuclear cells (PBMCs) were obtained from G-CSF mobilized healthy donors (n = 12) and non-mobilized healthy donors (HD; n = 9). PBMCs were obtained after density gradient centrifugation (Ficoll-Lympholyte, Cederlane) as described before. Both G-CSF mobilized donors and HD gave their informed consent to participate in this study, which was approved by the Bambino Gesù Children's Hospital (Rome, Italy) ethics committees (Prot. n. 1724/2018) and was conducted in accordance with the tenets of the Declaration of Helsinki.

Cell Isolation and Antibodies

NK cells and PMN-MDSCs were isolated from PBMCs of mobilized and non-mobilized donors using NK isolation kit and $CD66b^{+}$ microbeads (purity >98%, data not shown) following manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). Before starting any experiment, we determined the purity of isolated cells by flow cytometry using anti-CD3-APC, anti-CD19-ECD, and CD56-PC7 (Beckman Coulter, Brea, CA) for NK cells. PMN-MDSCs were labeled with anti-CD3-AF700, anti-CD19-AF700, anti-CD11b-FITC, anti-CD33-PC7, anti-HLA-DR-PE, anti-CD14-ECD, anti-CD45-KrOr, and anti-CD66b-APC desiccated in the Duraclone custom design platform (Beckman Coulter, Brea, CA) adding anti-CD56-BV650 (BioLegend, San Diego, CA) and following manufacturer's instruction. After the staining procedures, cells were acquired at Cytoflex LX and analyzed with Cytexpert software (v2.4, Beckman Coulter, Brea, CA). Freshly isolated NK cells were immediately used for functional studies and gene expression evaluation.

Functional Assays

Cell cytotoxicity assays were performed using as target NALM-18 cell line (childhood B-cell acute lymphoblastic leukemia) or K562 cell line (erythroleukemia) and as effector cells (mobilized or not) NK cells at different Effector/Target (E/T) cell ratios. Killed cells were evaluated after 4 hours. At the end of the co-culture, the assay was stopped by chilling cells on ice, and Propidium Iodide (PI) was added to each sample immediately before acquisition in order to identify the percentage of target cell lysis, as previously described (29). For each set of experiment, all the acquisitions (5,000 target cells/sample) were performed within 20 min. Statistical analysis was performed using GraphPad Prism.

PMN-MDSC apoptosis was examined using Annexin V-FITC apoptosis detection kit (BD biosciences, Franklin Lakes, NJ, USA). PMN-MDSCs derived from non- and post-mobilized donors were separated and cultured in 24-well plates at a density of 1×10^{6} cells/well. Analysis of cell viability and apoptotic status was evaluated at different time points (from 1 to 6 days). PMN-MDSCs were incubated with 5 μl of Annexin V-FITC and 2 μl of PI at room temperature for 15 min. The PMN-MDSC apoptotic rate was measured using a flow cytometer (Cytoflex LX, Beckman Coulter, Brea, CA, USA). Chemotaxis of NK cells was measured by migration through a polycarbonate filter with an 8.0 μm pore size in 24-well trans-well chambers (Sarstedt, Nümbrecht, Germany). The assay medium consisted of Roswell Park Memorial Institute (RPMI) supplemented with 10% fetal bovine serum (FBS) derived from purified PMN-MDSCs non-mobilized and post-mobilization after 24 h of culture. 500 μl of assay medium was added to the lower chamber in the presence or not of CCL4 blocking antibodies (1.2 μg/ml; R&D Systems, Minneapolis, MN, USA). 500 μl of assay medium was used as a control for spontaneous migration. Then, 1×10^{5} NK cells were seeded to the upper chamber. After 1 hour of incubation at 37°C, NK cells that migrated to the bottom chamber were harvested and counted by flow cytometry (absolute count).

RNA Extraction and Real Time PCR Analysis

Total RNA extraction from purified NK cells and PMN-MDSCs was performed with miRNeasy mini kit, combined with on-column DNase I treatment, following the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). RNA concentration and purity were evaluated by spectrophotometric analysis (Nanodrop 2000; Thermo Fisher Scientific, Wilmington, DE). For gene expression analysis in NK cells and PMN-MDSCs, total RNA was reverse transcribed with random primers using Super Script IV first-strand synthesis system following manufacturer's instructions (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). Real Time PCR on NK cells was performed by 384-wells TaqMan array microfluidic cards with a custom configuration for the detection of selected genes implicated in NK cell biology (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). Briefly, 200 ng of cDNAs for each sample was mixed with an isovolume of TaqMan Advanced Master Mix 2× and loaded in 384-wells cards (100 ng/channel). Real time PCR on PMN-MDSCs was performed in 20 μl of total volume with TaqMan™ Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, U.S.A). The following TaqMan™ Gene Expression assays were used: MS4A4A (Hs00254780_m1), CLEC7A (Hs01902549_s1), CD177 (Hs00360669_m1), CCL4 (Hs99999148_m1), 18S (Hs99999901_s1). Reactions were carried out on a QuantStudio 12 Flex instrument using thermal PCR cycling conditions suggested by the manufacturer (Applied Biosystems, Foster City, CA, U.S.A). Data analysis was performed on Thermo Fisher Cloud with Design and Analysis New qPCR application (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). PCR array data were analyzed with relative threshold algorithm and normalized using the mean of ACTB and HPRT expression, used as reference genes. Real time PCR on PMN-MDSC samples was analyzed with baseline threshold algorithm and $2^{-\Delta Ct}$ method. Data were normalized with 18S housekeeping gene.

Microarray Analysis

The samples of non-mobilized (n = 5) and mobilized (n = 8) PMN-MDSCs from which we obtained enough amount of high quality total RNA were used as start material for the microarray gene expression analysis. Biotin labeled RNA was generated using the GeneChip 3′ IVT Plus labeling kit (Thermo Fisher

Scientific, Wilmington, DE) according to the manufacturer's instructions. The Biotin labeled RNA was subsequently hybridized to a HT U133 plus 16-array plate (Thermo Fisher Scientific, Wilmington, DE). The hybridization, wash, staining, and scanning procedures were done in a GeneTitan™ Instrument according to the manufacturer's protocol (cat#: 00-0373; Thermo Fisher Scientific, Wilmington, DE). For labeling, hybridization, washing, staining, and scanning procedures we took advantage of Eurofins Genomics microarray service (Eurofins Genomics; Ebersberg, Germany).

The cel file output was used as input in the Partek Genomics Suite Software (Partek, St. Louis, MO) for generation of Robust Multi-Array Average (RMA) normalized data.

Bioinformatics and Statistical Analysis

Data in **Figures 1**, **2A** were expressed as mean ± standard deviation (SD) or ± standard error of the mean (SEM) as indicated. For RNA yield evaluation, statistical significance was calculated using paired Student's t-test. A p value ≤0.05 was considered statistically significant. DEGs between non-mobilized and mobilized PMN-MDSC groups were calculated by ANOVA one-way test. Lists of DEGs were filtered applying fold change (FC) >2 or <-2 (FC>10 or <-10 in **Figure 3A**) and a p-value ≤0.05 with Benjamini–Hochberg (1995) False Discovery Rate (FDR) correction criteria. PCA, ANOVA test, unsupervised hierarchical clustering in **Figure 3A**, and box plots in **Supplementary Figures 2A, B**, were generated by Partek Genomics Suite (Partek, St. Louis, MO). Enrichment of GO BP terms was performed by GOrilla tool (30) applying a p-value threshold of 10-3, calculated according to the mHG or HG model. Graphical summarization of enriched GO BPs in a semantic treemap was performed by REVIGO allowing medium similarity and applying the simRel score (31). Two-way Anova was used for **Figure 4A**, Mann–Whitney test was used for **Figures 3B**, **4B, C**.

RESULTS

NK Cells Present in the Donor Graft Exhibit a Reduced Cytolytic Activity

As previously shown, the HSC mobilization regimen induces accumulation of PMN-MDSCs in the PB of G-CSF-mobilized donors. Moreover, these cells sharply inhibited the effector function of autologous and allogeneic NK cells *in vitro* (28). Based on these results, we verified whether the cytolytic activity of NK cells, isolated from the PB of mobilized donors, was affected by the concomitant presence of high numbers of PMN-MDSCs. Fresh NK cells were isolated from apheresis (post-mobilization) or from PB of unrelated HD, and their ability to kill NALM-18 tumor target cells was assessed. As shown in **Figure 1A**, NK cells isolated from mobilized donors showed a lower cytolytic activity as compared to HD NK cells. As previously demonstrated, PMN-MDSCs are capable, after 48 h of co-culture, to strongly impair the cytolytic activity of NK cells. In order to clearly demonstrate the impact of PMN-MDSCs on NK cell function, we further removed PMN-MDSCs after 48 h from NK/MDSC co-cultures and tested the cytolytic activity of these NK cells after additional 24 h of culture (referred as NK/MDSC 24 h alone; **Supplementary Figure 1**). The inhibitory effect of PMN-MDSCs was reversible, since the cytolytic activity of NK cells could be restored by removing PMN-MDSCs from co-cultures (**Supplementary Figure 1**).

NK Cells in the Donor Graft Display an Altered Expression of Genes Controlling Immune Effector Functions

To support the functional data, we further investigated whether NK cells of mobilized donors were modified in their gene expression profiles. Thus, we analyzed by PCR array the

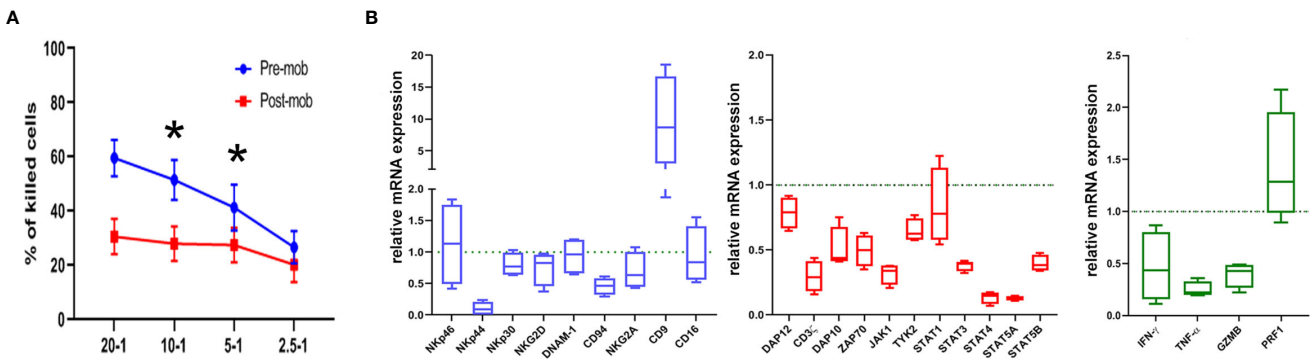

FIGURE 1 | Cytolytic activity and expression of functional genes are altered in NK cells from mobilized grafts. **(A)** Freshly isolated NK cells derived from donors who did (*post-mob*; in red) or did not (*pre-mob*; in blue) undergo mobilization with G-CSF were purified, and their cytolytic activity was assessed using NALM-18 as target cells. Percentages of killed cells ± SEM at different Effector/Target (E/T) ratios are shown. Statistical analysis was performed using Mann–Whitney test (n = 5). *p ≤ 0.05; where not indicated, data were not statistically significant. **(B)** Box and whisker plots representing the expression of the indicated panels of genes (*blue*: cell surface markers; *red*: transduction signal molecules; *green*: effector molecules) measured by real time PCR array in NK cells freshly isolated from apheresis of mobilized donors (n = 4). Values are expressed as fold change with respect to their expression in NK cells from PB of non-mobilized donors, used as control (n = 4).

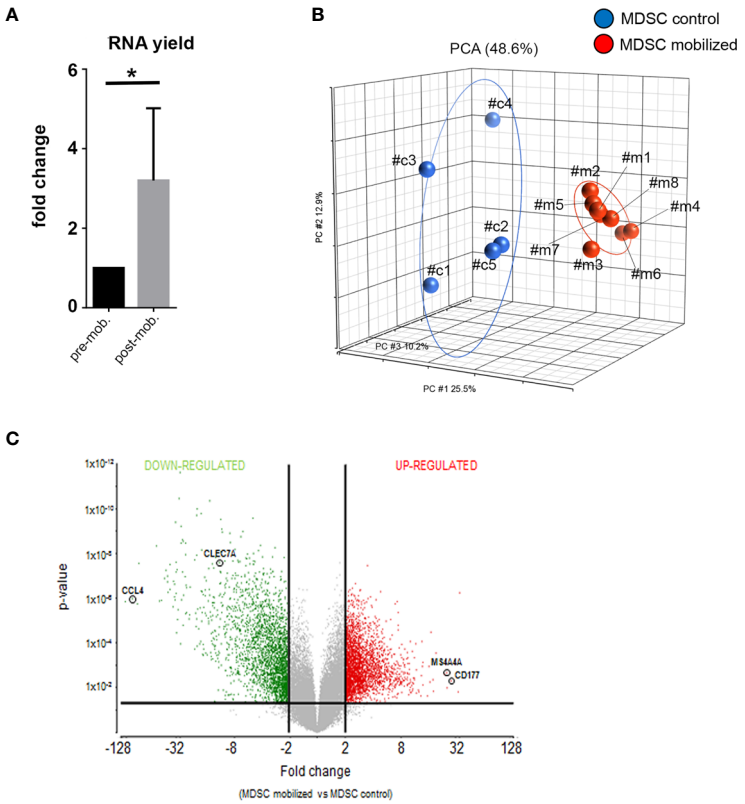

FIGURE 2 | Global gene expression analysis reveal distinct transcriptional profiles in mobilized PMN-MDSCs. **(A)** Total RNA yield obtained from freshly-isolated PB PMN-MDSCs of donors before (pre-mob) and after mobilization (mob). Data are expressed as fold change with respect to non-mobilized samples. Bars indicate SD. *p-value ≤ 0.05 (Student's t-test). **(B)** Principal Component Analysis (PCA) summarization of microarray data for non-mobilized (MDSC control; blue circles; n = 5) and mobilized (MDSC mobilized; red circles; n = 8) PMN-MDSCs. **(C)** Volcano plot representing DEGs between mobilized and non-mobilized PMN-MDSCs upon ANOVA of microarray data. Vertical lines indicate FC≤-2 and ≥2, and horizontal line indicates p-value ≤ 0.05, representing cut-off lines applied to filter significant DEGs. Probeset significantly up-regulated and down-regulated are highlighted in red or green, respectively. The positions of CCL4, CLEC7A, MS4A4A, and CD177 genes in the volcano plot are highlighted.

expression of a wide panel of genes regulating cell function in NK cells purified from mobilized and non-mobilized donors. In particular, we evaluated genes considered important in the control of immune effector function of NK cells. As shown in **Figure 1B**, in comparison to NK cells from HD, "mobilized" NK cells revealed a decrease in the expression of some genes encoding for surface receptors involved in anti-tumor activity including NKp44 and CD94, or signal transduction molecules CD3ζ, ZAP70, DAP10, DAP12, or STAT family members, or effector molecules such as IFN-γ, TNF-α and GZMB. Interestingly, in NK cells from mobilized donors, we also found a higher expression of the CD9-encoding gene (**Figure 1B**). CD9 up-regulation was reported in NK cells converted into a pro-angiogenic, non-cytotoxic state upon exposure to TGFβ that, in turn, can be released by PMN-MDSCs as well (32, 33). Taken together, these results suggest that the effector function and the activation status of NK cells in the graft may be influenced by the presence of PMN-MDSCs.

PMN-MDSCs in the Donor Graft Are Characterized by Distinct Gene Expression Profiles

Next, we verified whether the mobilization regimen induced changes in the molecular properties of PMN-MDSCs present in mobilized donors. First, we compared the frequency of PMN-MDSCs in G-CSF mobilized and non-mobilized donors. In G-CSF mobilized donors PMN-MDSCs were ≈20% while they were <5% non-mobilized donors (28). Their percentages increased to ≈40% in the collection bag (due to their enrichment related to the B- and $\alpha\beta$ T-cell depletion; data not shown). Notably, RNA yield from freshly isolated PMN-MDSC in the donor graft was significantly higher than that of the same cells isolated from the same pre-mobilized donors (~three-fold) (**Figure 2A**). The increased RNA content suggested an activated metabolic state in PMN-MDSCs of mobilized grafts. Thus, we investigated whether these cells

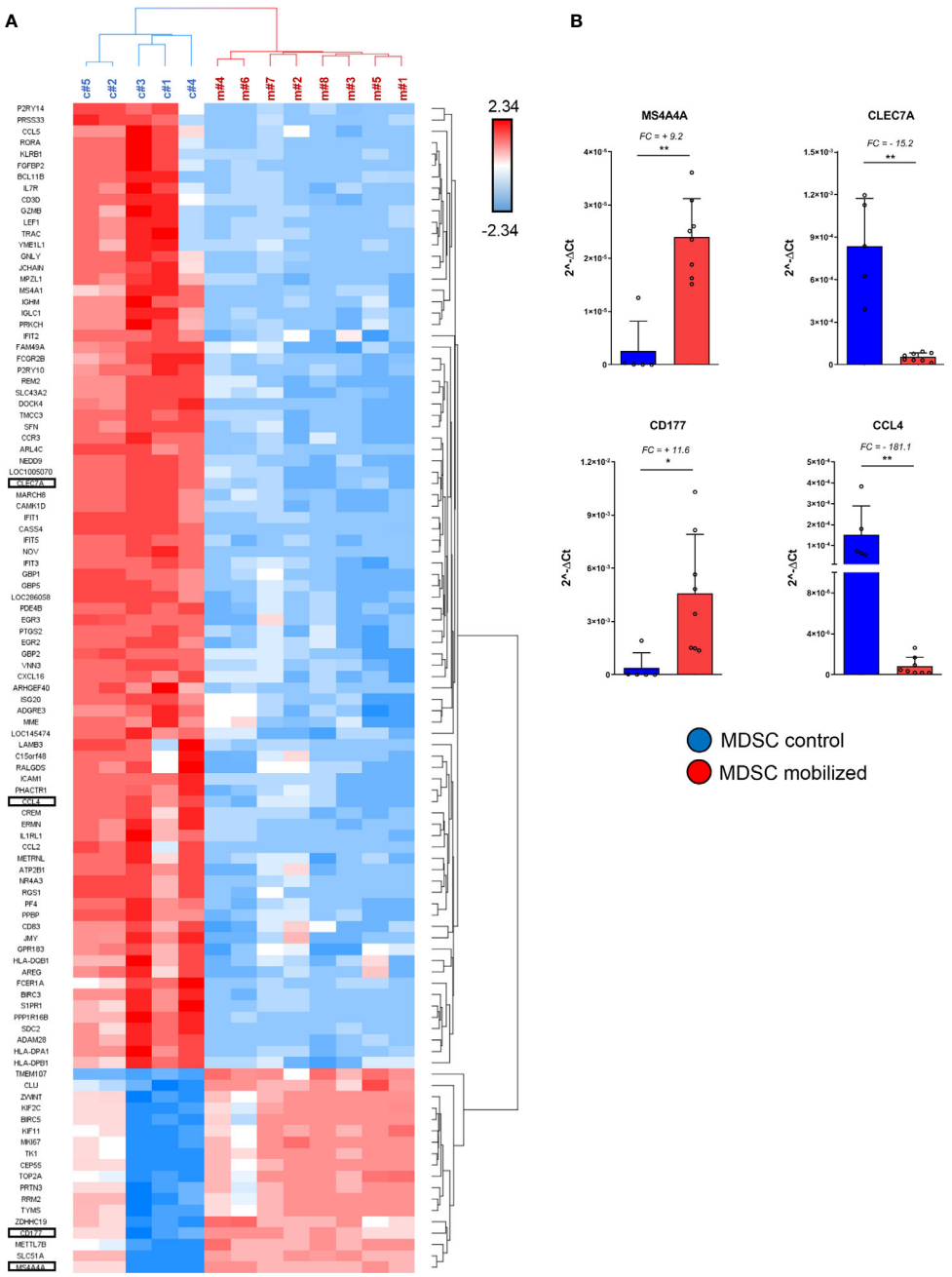

FIGURE 3 | Specific immuno-modulatory genes are differentially expressed in mobilized PMN-MDSCs. **(A)** Heat-map of the top up-regulated and down-regulated genes in PMN-MDSCs, based on the filter FC ≥10 or ≤-10; FDR adjusted p-value ≤ 0.05. Red squares indicate up-regulated genes; blue squares down-regulated genes in mobilized PMN-MDSCs. Each row represents a gene; each column represents individual samples analyzed by microarray (control: c#1–5; mobilized: m#1–8). Specific genes selected for further analysis are highlighted. **(B)** Real time PCR analysis on the samples used for microarray for the indicated genes. Values were calculated with ΔCt method. 18S was used as endogenous control. Bars indicate SD. *p-value ≤ 0.05; **p-value ≤0.01 (Mann–Whitney test).

displayed transcriptional features different from non-mobilized PB PMN-MDSCs. To this purpose, we performed a global gene expression profiling by microarray to compare eight samples of PMN-MDSCs isolated from PB of mobilized donors with a control group represented by five PMN-MDSC samples derived from non-mobilized HD. In the microarray, three out five of the control samples were derived, before mobilization, from the same donors analyzed in the mobilized group (samples *c#1*, *c#,2* and *c#3 versus m#1*, *m#2*, and *m#3* respectively; **Figure 2B**). We used a three-dimensional Principal Component

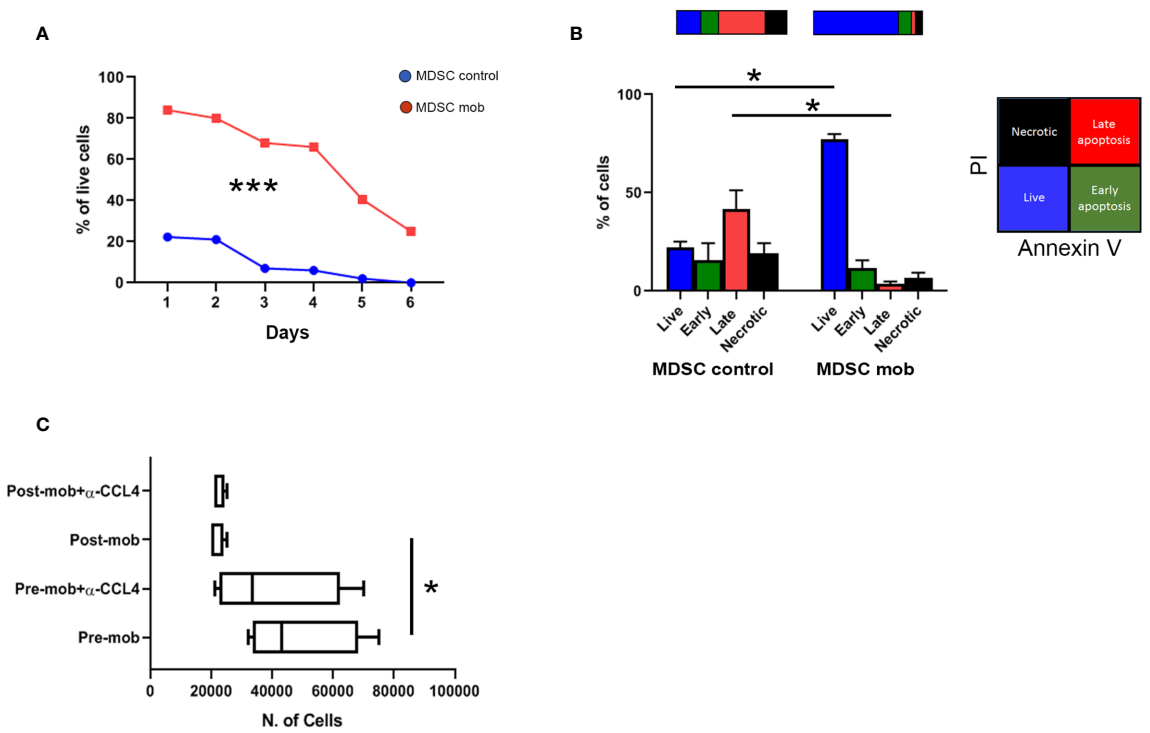

FIGURE 4 | Prolonged survival and altered chemo-attraction of mobilized PMN-MDSCs. **(A)** Percentages of PMN-MDSC live cells (PI^-) along 6 days of non-mobilized (PMN-MDSCs ctrl, red square line) and mobilized (PMN-MDSCs mob, blue dots line) donors. ***p-value < 0.001 (two-way ANOVA test, n = 3). **(B)** Percentages of live (Annexin V^- and PI^-, blue), early apoptotic (Annexin V^+ and PI^-, green), late apoptotic (Annexin$^+$ and PI^+, red), and necrotic (Annexin V^- and PI^+, black) control (left) and mobilized (right) PMN-MDSCs measured by flow cytometry after 24 h of culture. *p-value ≤ 0.05; (Mann–Whitney test, n = 4). **(C)** Numbers of migrated NK cells after 1 h of culturing in conditioned medium derived from pre- and post-mobilized PMN-MDSC, in the presence or not of anti-CCL4 blocking antibody. *p-value ≤ 0.05; (Mann–Whitney test, n = 4).

Analysis (PCA) plot to summarize the microarray data. This exploratory analysis showed that all the mobilized PMN-MDSCs samples finely clustered together and were clearly distinguished from non-mobilized PMN-MDSCs samples, indicating that, indeed, the two groups are characterized by distinct transcriptional profiles (**Figure 2B**).

Specific Pro-Proliferative and Immune-Modulatory Genes Are Differentially Expressed in PMN-MDSCs of Mobilized Donors

We further investigated the differentially expressed genes (DEGs) in mobilized or non-mobilized PMN-MDSCs. To this end, we performed an Analysis of Variance (ANOVA) test, imposing a p-value ≤0.05 and a fold change (FC) ≥|2|. We found 4,625 differentially expressed probe sets displaying robust and statistically significant variations between mobilized and non-mobilized PMN-MDSCs. Among them, 2,589 probe sets were up-regulated and 2,036 down-regulated in mobilized *versus* non-mobilized PMN-MDSCs (**Figure 2C** and **Supplementary Table 1**). Hierarchical clustering based on these significantly deregulated genes also confirmed a clear discrimination between mobilized and non-mobilized PMN-MDSC samples (data not shown). It should be noted that some important genes characterizing the PMN-MDSCs subset such as LOX1, S100A9, GAS6, TGFβ1 and IL-10 were expressed in both groups with no significantly different expression level, suggesting that mobilized PMN-MDSCs retain some features of typical PMN-MDSCs (**Supplementary Figure 2A**). Remarkably, in mobilized PMN-MDSCs we detected a differential expression of some relevant genes associated with cell proliferation and immunosuppressive function. In particular, MS4A4A (↑), MKI67 (↑), ARG1 (↑), CEACAM1 (↑), FKB5 (↑) genes were up-regulated whereas CCL4 (↓), IL7R (↓) were down-regulated in mobilized PMN-MDSCs (**Supplementary Table 1** and **Figure 3A**).

A heat-map showing the most modulated genes in mobilized PMN-MDSCs is represented in **Figure 3A**. In this analysis, we filtered the list to DEGs with FC ≥ |10| to narrow down to the most deregulated genes. One of the most up-regulated genes in mobilized PMN-MDSCs was CD177 (FC = +27.4) (**Supplementary Figure 2B**), a surface membrane glycoprotein expressed in neutrophils and neutrophil precursors (34). Consistent with the wide up-regulation of other genes involved in MDSC proliferation and activation, this gene is involved in neutrophil proliferation and was found to be up-regulated in

human MDSCs upon inflammation (35, 36). Furthermore, the tetraspan molecule MS4A4A was one of the most up-regulated genes in mobilized MDSCs (FC = +24). It has recently been reported that MS4A4A expression is restricted to human mononuclear phagocytes, where MS4A4A co-localizes with the β-glucan receptor dectin-1 (CLEC7A), mediates NK cell activation and exert a control of metastases in murine models (37). Our data indicate that Dectin-1/CLEC7A is down-regulated (FC= −11) in mobilized PMN-MDSCs. Since Dectin-1 signaling is crucial for mediating cell-to-cell contact and subsequent NK cell activation, the up-regulation of MS4A4A might have a different role besides inducing NK cell activation *via* MS4A4A-CLEC7A. A sharp increase of MS4A4A expression, usually associated with cells of the monocytic lineage, suggests that PMN-MDSCs may acquire unique molecular characteristics upon G-CSF induced mobilization. Conversely, one of the most down-regulated genes was CCL4, encoding for an inflammatory chemokine functioning as chemoattractant for different leukocytes, including NK cells (**Supplementary Figure 2B**). The above mentioned genes were selected for PCR validation on the same samples used in the microarray. Real time PCR analysis confirmed a striking differential expression of all the genes tested, confirming the reliability of the microarray approach (**Figure 3B**).

PMN-MDSCs of Mobilized Donors Display Prolonged Survival and Altered Chemo-Attraction Properties on NK Cells

Transcriptome data indicated that mobilized PMN-MDSCs are characterized by an activated phenotype. Thus, we asked whether their distinct gene expression profile was associated with an altered cell survival. To this purpose, mobilized and non-mobilized PMN-MDSCs were cultured *in vitro*, and cell viability was evaluated for 6 days. Whereas non-mobilized PMN-MDSCs underwent rapid cell death upon *in vitro* culturing, mobilized PMN-MDSCs displayed a significantly prolonged survival (**Figure 4A**). Of note, after 24 h of *in vitro* culture, the large majority of mobilized PMN-MDSCs were represented by live cells, whereas their non-mobilized counterpart already showed an extensive fraction of apoptotic cells (**Figure 4B**).

Transcriptional profiles also suggested that mobilized PMN-MDSCs could have different immune properties as compared to the non-mobilized ones. Based on down-regulation of several chemokines including CCL4 (see **Figure 3A** and **Supplementary Table 1**), we tested whether mobilized PMN-MDSCs could exhibit different chemo-attractive properties on NK cells. Supernatants of non-mobilized and mobilized PMN-MDSC cultures displayed significant differences in the capability to attract NK cells in trans-well chambers system. In particular, supernatants of mobilized PMN-MDSCs exhibited a reduced ability to attract NK cells as compared to non-mobilized ones (**Figure 4C**). The use of a blocking antibody against CCL4 chemokine caused a slight decrease of NK cell migration in non-mobilized supernatants, indicating that CCL4 chemokine is involved, at least in part, in this process (**Figure 4C**).

Biological Processes Associated With Proliferation and Myeloid Activation Are Enriched in Mobilized PMN-MDSCs

In order to deepen the biological significance of DEGs identified in PMN-MDSCs of mobilized donors, we performed an enrichment analysis of biological processes (BPs) from Gene Ontology (GO) database. We separately analyzed the list of up-regulated (FC ≥2; p value ≤0.05) and down-regulated (FC ≤-2; p value ≤0.05) genes in mobilized MDSCs to find emerging BPs in these groups. A list of BPs significantly over-represented is shown in **Figure 5**, whereas the complete list of enriched BPs in up-regulated and down-regulated genes is reported in **Supplementary Tables 2** and **3**. This analysis clearly highlighted a striking predominance of BP associated with cell proliferation in the up-regulated genes in G-CSF mobilized PMN-MDSCs, including cell cycle process (GO:0022402), chromosome organization (GO:0051276) and segregation (GO:0007059). Another important group of BPs regards the immune defense compartment, in particular, the myeloid cell activation (GO:0002274) and other processes specifically associated with granulocytes, such as neutrophil degranulation (GO:0043312), activation (GO:0042119) and extravasation (GO:0072672) (**Figure 5A**). Conversely, down-regulated genes in mobilized PMN-MDSCs were enriched in some BPs associated with macromolecule metabolism, *e.g.* transcription DNA-templated (GO:0006351) and nucleic acid metabolic process (GO:0090304). Other significantly enriched BPs were associated to covalent chromatin modification (GO:0016569) and histone modification (GO:0016570) (**Figure 5B**). A summarization of the GO BPs significantly enriched in up-regulated and down-regulated gene lists was performed by REVIGO (31) to graphically represent in a semantic tree-map all the BPs identified (**Figure 5**). Collectively, these data suggest that the mobilization regimen with G-CSF induced a transcriptional program promoting cell proliferation and activation of myeloid cells.

DISCUSSION

In the present paper, we first show that NK cells present in the graft after G-CSF mobilization regimen display a lower cytolytic activity as compared to NK cells from HD. We then performed a detailed gene expression analysis on both NK cells and PMN-MDSCs derived from apheresis after mobilization with G-CSF. Notably, PMN-MDSC and NK cells represent two important cellular components of the $\alpha\beta$ T cell- and B cell-depleted graft. Since, upon G-CSF-induced mobilization and $\alpha\beta$ T and B cell-depletion of the graft, PMN-MDSCs are present in high percentages, it was conceivable that they could exert a strong negative impact on NK cell effector function. Notably, mature donor-derived NK cells were shown to contribute to both GvL activity and control of infections at early stages after transplantation, particularly in the haplo-HSCT setting using pure $CD34^+$ HSC. However, we found that NK cells present in mobilized graft in $\alpha\beta$ T and B cell-depleted HSCT setting show a reduced anti-leukemia activity as compared to non-mobilized NK cells. This functional evidence was further supported by

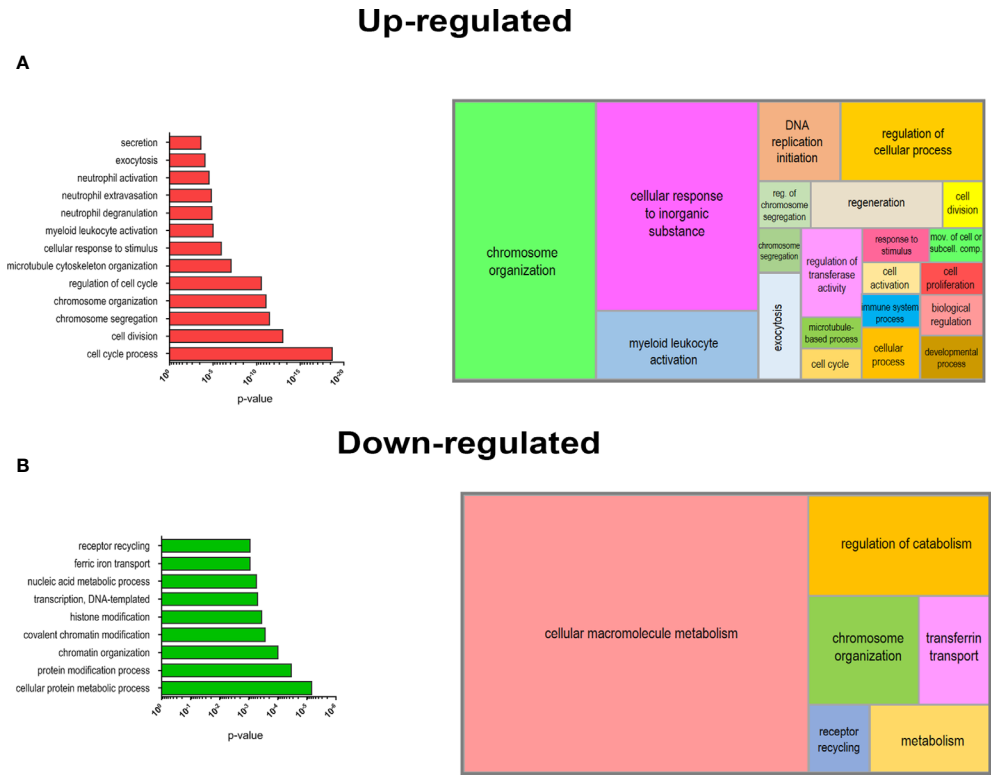

FIGURE 5 | Biological processes (BPs) enrichment in DEGs of mobilized PMN-MDSCs. Selected list of BPs from the GO database enriched for up-regulated **(A)** o r down-regulated **(B)** genes of mobilized PMN-MDSCs (left panels). Histograms represent –log10 p-value for each enriched BP calculated by GOrilla tool (see *Materials and Methods*). Graphical summarization of the GO BPs significantly enriched in up-regulated and down-regulated gene lists is shown with a semantic clustering performed by REVIGO tool (right panels).

transcriptional data, revealing that some molecules involved in NK cell-mediated anti-tumor activity were down-regulated in mobilized samples. Although the reduced cytotoxic activity of NK cells *in vivo* upon G-CSF regimen could reflect also MDSC-independent mechanisms, the high proportion of PMN-MDSCs in the graft raises the intriguing possibility that G-CSF could inhibit donor NK cell cytotoxicity through PMN-MDSC activation and expansion. Indeed, we show that the inhibition of NK cell cytolytic activity *in vitro* is due to the presence of PMN-MDSCs and that their removal results in the restoration of NK cell function. Our data are supported by a previous study in which we showed that PMN-MDSCs present in high concentrations in the $\alpha\beta$ T and B cell-depleted transplants exert a sharp inhibitory effect *in vitro* on both allogeneic and autologous NK cell function (28).

G-CSF-induced mobilization causes the release in the PB and the expansion of a broad spectrum of hematopoietic cell precursors including PMN-MDSCs. An activated state of these cells was suggested by the increase in the total RNA amount per cell in mobilized donors. Thus, we asked whether PMN-MDSCs in the graft possess peculiar molecular characteristics or whether they are similar to PMN-MDSCs isolated from non-mobilized donors. Interestingly, our comprehensive gene expression profiling by microarray revealed that PMN-MDSCs in the graft showed a peculiar transcriptional profile distinguishing them from non-mobilized PMN-MDSCs. All the mobilized samples finely clustered together and were clearly distinct from non-mobilized ones. Notably, three out of five of the control samples were derived, before mobilization, from the same donors analyzed in the mobilized group (samples *c#1*, *c#2*, and *c#3 versus m#1*, *m#2*, and *m#3* respectively; **Figures 2B** and **3A**), thus further supporting that the variability between the two groups was indeed related to the mobilization regimen. The most prominent difference in mobilized PMN-MDSCs resides in the strong up-regulation of genes promoting DNA replication, cell cycle, and cell division such as the marker of cell proliferation Ki-67 (MKI67), topoisomerase II alpha (TOP2A), cyclin B (CCNB2) or kinesin family member 2 C (KIF2C). Transcriptional data would clearly support the scenario of a rapidly expanding PMN-MDSC population in PB of mobilized donors, in agreement with their high concentration in the graft. Both the high number of PMN-MDSCs in the graft and their possible further expansion after transplantation offer a reasonable explanation for the impaired cytolytic activity of NK cells.

Although there was a pronounced difference in the transcriptome, several key genes associated with PMN-MDSCs such as LOX1, NOS1, GAS6, TGFβ1, IL10, and S100A9 were not significantly de-regulated in the two compared groups.

This finding indicates that PMN-MDSCs isolated from mobilized or non-mobilized donors share a number of similar features and display some common functional properties. On the other hand, the expression of some genes with important immune-regulatory functions was sharply deregulated in mobilized PMN-MDSCs. Among them, we found a strong up-regulation of the tetraspanin MS4A4A. MS4A4A was reported to be a fundamental molecule in tumor-infiltrating macrophages as well as in dectin-1-dependent activation of NK cells in cancer (37). Up-regulation of MS4A4A was reported to be restricted to cells of the macrophage-lineage (37). Of note, we show that such up-regulation also occurs in PMN-MDSCs upon G-CSF induced mobilization. In addition, we found Dectin-1 (CLEC-7A) was significantly down-regulated in these cells. Since Dectin-1 is critical for NK cell-mediated killing of tumor cells (38), it is possible that MS4A4A may not be functional in mobilized PMN-MDSCs. Interestingly, an increased expression of MS4A4A was also observed in circulating MDSCs after surgical sepsis (35), suggesting that pro-inflammatory stimuli could up-regulate this molecule in MDSCs. Further studies are clearly needed to clarify the functional significance of MS4A4A up-regulation in these cells.

We also observed a striking increase of several other genes encoding for transmembrane proteins, suggesting that the membrane surface composition of mobilized PMN-MDSCs may be significantly different from that of their non-mobilized counterpart. Among these genes, the one encoding the transmembrane protein 107 (TMEM107) is the most up-regulated in mobilized PMN-MDSCs. This molecule plays a critical role in ciliated cells, while to date, their potential functional role in blood cells remains to be defined. CD177 is another of the most up-regulated genes in mobilized PMN-MDSCs. CD177 has been associated with subpopulations of immature developing human neutrophils and it might be involved in their migration (34). Altered migration properties of these cells were also suggested by the up-regulation of other genes such as integrin alpha 9 (ITGA9). Of note, genes regulating neutrophil extravasation were up-regulated in mobilized PMN-MDSCs. Thus, the migration properties and the ability to extravasate may greatly differ in these cells as compared to other mature neutrophil populations.

Regarding the group of down-regulated genes, enrichment analysis underlines an emerging and strong over-representation of genes involved in transcriptional regulation and chromatin remodeling, further supporting the occurrence of a wide alteration of the metabolic state in mobilized MDSCs as well as of their prolonged survival, possibly associated with a trained immunity process. Metabolic rewiring could be explained as the response of PMN-MDSCs to the environment upon G-CSF stimulus, leading to their activation and expansion. As described above, these changes could also affect immune properties of PMN-MDSCs in the graft modulating their capability to attract NK cells. In this regard, it is also worth mentioning the sharp down-regulation occurring in the CCL4 gene expression. This chemokine is produced by neutrophils and is an important chemo-attractant for NK cells and other immune cells.

A deeper comprehension of the molecular characteristics and functional effects of PMN-MDSCs could highlight the impact of these cells in the HSCT.

Circulating and tumor-infiltrating PMN-MDSCs have a short *in vivo* lifespan, whereas during inflammation the half-life of PMN-MDSCs may be significantly prolonged (39). The persistence of mobilized PMN-MDSCs in transplanted recipient patients and their possible durable effects on NK cells is an interesting, unanswered, question. The evidence that mobilized PMN-MDSCs display an increased survival *in vitro* as compared to non-mobilized PMN-MDSCs suggests they may persist for longer time intervals *in vivo*. However, further investigation is needed to directly address this question.

For example, removal or inactivation of PMN-MDSCs could represent a promising strategy to restore/improve the NK-mediated GvL activity and to control viral infections. On the other hand, PMN-MDSCs could also exert a control on GvHD due to their potent immunosuppressive effect on T cells.

The immunologic and bioinformatics-based analyses of PMN-MDSC cell function in response to donor treatment with G-CSF may be useful for better understanding their interaction with donor effector cells involved in GvL, while a better comprehension of the molecular mechanisms involved in the function of PMN-MDSCs may allow for exploiting or inactivating these cells not only in HSCT but also in other pathological conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE159069.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bambino Gesù Children's Hospital (Rome, Italy) ethics committees (protocol number 1724/2018). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AP designed and performed the research, interpreted the data, and wrote the article. FB performed the experiment and analyzed the data. GLP, PM, and MA provided samples and discussed the data. LQ and LM interpreted the data and revised the manuscript. PV and NT designed the research, interpreted the data, and wrote the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.657329/full#supplementary-material

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REVIEW
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Natural Killer Cell Interactions With Myeloid Derived Suppressor Cells in the Tumor Microenvironment and Implications for Cancer Immunotherapy

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The tumor microenvironment (TME) is a complex and heterogeneous environment composed of cancer cells, tumor stroma, a mixture of tissue-resident and infiltrating immune cells, secreted factors, and extracellular matrix proteins. Natural killer (NK) cells play a vital role in fighting tumors, but chronic stimulation and immunosuppression in the TME lead to NK cell exhaustion and limited antitumor functions. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of myeloid cells with potent immunosuppressive activity that gradually accumulate in tumor tissues. MDSCs interact with innate and adaptive immune cells and play a crucial role in negatively regulating the immune response to tumors. This review discusses MDSC-mediated NK cell regulation within the TME, focusing on critical cellular and molecular interactions. We review current strategies that target MDSC-mediated immunosuppression to enhance NK cell cytotoxic antitumor activity. We also speculate on how NK cell-based antitumor immunotherapy could be improved.

Keywords: natural killer cells, myeloid derived suppressor cells, tumor mircroenvironment, natural killer cell immunotherapy, cancer

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INTRODUCTION

Tumorigenesis is a complex and dynamic process involving three stages: initiation, progression, and metastasis (1). Besides blood and lymphatic vessels, composed in part of vascular endothelial cells, the major constituents of the tumor microenvironment (TME) are a heterogeneous population of cancer cells, fibroblasts, immune and inflammatory infiltrated cells, and secreted protein elements of the extracellular matrix (ECM) (2). The functional and physical interactions of these tumor elements determine clinical outcomes. At the beginning of the nineteenth century, Virchow described cancer as originating from chronic unresolved inflammation (3). Many studies have demonstrated that cancer-associated inflammation plays a critical role in tumor formation, contributing to genomic instability and epigenetic modification, and regulating the creation of a protected TME to promote cancer proliferation and metastasis (4–6). Tumor-associated inflammatory cells are observed in human cancers from the earliest phases of carcinogenesis (7). The first line of defense, represented by natural killer (NK) cells and cytotoxic $CD8^+$ T cells, usually recognizes and kills malignant cells; however, a few immunogenic cancer cell variants can escape immune recognition (8).

The TME orchestrates multiple mechanisms to impair the antitumor functions of immune cells. These mechanisms include destabilizing the innate cell compartment, composed of NK cells, macrophages, neutrophils, and dendritic cells (DCs), and suppressing adaptive immune cell (T and B)-mediated antitumor activity. The presence and recruitment of atypical innate and adaptative immune cells in the tumor site are thought to occur during both the early and later stages of tumor development (6, 9). Tumor immune surveillance also fails because immunosuppression and its associated chronic inflammation further destabilize tumor-fighting immune cells, defending rather than eradicating tumors (10). The TME supports the growth of cancer-associated fibroblasts, stromal cells, and endothelial cells, contributing to tumor-associated capillary and lymphatic vessels that support tumor growth (11). These mechanisms select potentially aggressive tumor clones early during tumor development (9).

This review will discuss the crosstalk between NK cells, myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and the critical cellular and molecular mechanisms within the TME that impact tumor development, progression, and angiogenesis, as well as how current therapies target these immunosuppressive cells in the TME. We will also review different NK cell exhaustion mechanisms and novel approaches for enhancing NK cell therapeutic potential against tumors.

CYTOTOXIC T LYMPHOCYTES AND NK CELLS IN CANCER

One of the adaptive and innate immune systems' essential activities is to kill infected and tumor cells. Mounting epidemiological and experimental evidence points to a critical role for cytotoxic T lymphocyte (CTL) and NK cell-mediated effector functions in host resistance to cancer. The collaboration between innate and adaptative effector cells can lead to tumor rejection (12, 13). CTL and NK cell activity has been linked to tumor immune surveillance and protection from cancer. Both cell types can form cytotoxic immunological synapses (14), which are specialized antigen-specific cell–cell junctions with a synaptic cleft to directly communicate and transduce highly controlled secretory signals between immune cells and their target cells (14). This mechanism, also present in NK cells, improves the efficiency of cytotoxic cell-mediated killing (15). Higher numbers of tumor-infiltrating CTL and/or NK cells are a favorable prognostic indicator for many cancer types (16, 17). T cell activation occurs when a clonal T cell receptor (TCR) is triggered by a tumor-derived antigen presented on class I human leukocyte antigen (HLA-I), in combination with co-receptor ligation and co-stimulation, leading to CTL activation, proliferation, cytokine and chemokine secretion, and tumor cell killing (18). Similar to CTL cells, NK cells are also cytotoxic lymphocytes and important tumor fighters.

NK cells do not express a TCR but instead have many activating receptors and also inhibitory receptors, which bind major histocompatibility complex class I (MHC-I) (19, 20). MHC-I is often downregulated by infected and malignant cells to avoid CTL killing. NK cells sense this "lack of inhibition" and are further activated by tumor cell-expressed stress ligands that ligate NK cell-expressed activating receptors (20–22). NK cells secrete cytokines, chemokines, pore-forming proteins (perforin), and cytotoxic mediators (granzymes) that trigger target cell apoptosis upon activation. A potent NK cell-activating receptor is NK group 2 member D (NKG2D), and NKG2D ligands are commonly upregulated on tumors (23).

Substantial evidence supports the conclusion that NK cells play a crucial role in eliminating tumors and tumor metastases (24). First, low cytotoxicity in peripheral blood (PB) NK cells correlates with a higher risk of developing cancer (25). Second, in many types of cancer, NK cells exhibit an altered phenotype and hypo-functionality (26, 27). Third, in mice, resistance to NK cell killing favors polyclonal metastasis (28). Several mechanisms contribute to NK cell exhaustion, including modulating adhesion and epithelial genes, decreasing the expression of NK cell-activating ligands (28), and the suppressive effects of regulatory immune cells and soluble factors within the TME (29).

NK ORIGIN, DEVELOPMENT, AND TISSUE DISTRIBUTION

NK cells are bone marrow (BM)-derived granular lymphocytes that lyse target cells rapidly and continuously upon activation (30). In the BM, NK cells develop from $CD34^+$ hematopoietic stem cells through a common lymphoid progenitor (CLP) intermediate that can seed and develop further in lymphoid and non-lymphoid organs (31). NK cell maturation requires several cytokines, among which interleukin (IL)-15, released by BM stromal cells, is crucial for the differentiation of CLPs toward the NK cell lineage (32). NK cells are well-represented in the PB, spleen, and BM and are found in most organs, including the liver, lungs, skin, gut, lymph nodes, tonsils, uterus, thymus, kidney, pancreas, and adipose tissue. (33, 34). Their recruitment to different tissues depends on the expression of several chemokine receptors and is reviewed elsewhere (35, 36).

Human NK cells express the hematopoietic cell marker CD45, the glycoprotein CD56 and mature NK cells, and the cluster of differentiation molecule CD16 also known as Fc receptor FcγRIIIa, but they do not express any T or B cell receptors. NK cell maturation is generally assessed by the amount of NK cell-expressed CD56 and CD16 expression. Specifically, $CD56^{dim}$ and $CD56^{bright}$ subsets show profound differences in cytokine secretion, response to cytokines, and killing efficiency. $CD56^{dim}$ $CD16^{bright}$ NK cells in the PB are about 90% of the total NK cell population and include the alloreactive NK cells described as "mature" with a higher cytotoxic potential. The remaining 10% of NK cells are $CD56^{bright}$ $CD16^{dim}$ and reside in the lymphoid tissue; they are considered "immature or unlicensed." These NK cells are more sensitive to cytokine stimulation, which will activate the secretion of a variety of cytokines, including interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-5, IL-10, and IL-13 (37–39).

A sophisticated array of germline-encoded activating and inhibiting receptors regulates NK cell development and,

subsequently, their activation (40). Upon activation, NK cells employ several mechanisms of target cell killing and secrete chemokines and cytokines to interact with other immune cells (41, 42). NK cells are traditionally defined as innate immune response cells because they lack recombinase-dependent clonal antigen receptors (RAG) (43). Nonetheless, recent findings have revealed that NK cells display adaptive immunity features (44–47), including several developmental and functional characteristics of the adaptive immune system (43, 48–51). These similarities include vaccination or sensitization-dependent antigen-specific immunological memory (45, 46, 52–55). How NK memory impacts the tumor-specific NK cell response is currently unknown.

ANTITUMOR RESPONSES OF NK CELLS

Many studies have demonstrated that NK cells can kill tumor cells (56). NK cells survey their environment with a distinct receptor repertoire, including activating and inhibitory receptors, adhesion molecules, and cytokine and chemokine receptors (57). NK cells recognize the expression of HLA-I, also called MHC-I, on autologous cells. This interaction is generally inhibitory and prevents NK cells from attacking healthy host tissue. Specifically, HLA-I or MHC-I binds NK cell inhibitory receptors, including killer cell immunoglobulin-like receptors (KIRs) in humans, Ly49 in mice, and CD94/NKG2A (58, 59). In contrast to infected or malignant cells, healthy nucleated cells express robust levels of HLA-I/MHC-I molecules and escape NK cell immune attack. However, during malignant transformation or viral infection, the expression of MHC-I antigens on the cell surface can be downmodulated. This variation in the expression of MHC-I molecules on target cells (missing self) reduces the strength of inhibitory signals delivered to NK cells, thus promoting NK cell activation. NK cells survey tissues for low MHC-I molecule expression (60, 61) and for the expression of activating ligands, such as the NKG2D ligands (62, 63), and ligands for the natural cytotoxicity receptors NKp30 and NKp44 in humans and NKp46 in humans and mice (64).

NK cells also have the unique ability to exert antibody-dependent cell-mediated cytotoxicity (ADCC) upon engagement of CD16 with the Fc portion of the antibodies (65). Activation through these receptors elicits rapid target cell killing by several mechanisms. NK cells form an immunological synapse with target cells and kill them rapidly by secretion of lytic granules that contain an arsenal of effector molecules (perforin, granzymes) and cytokines (IFN-γ, TNF-α) that induce cell death in targeted cells (66–68). Perforin and granzyme are proteins that play a significant role in cell-mediated cytotoxicity. These molecules are expressed in NK cells, and several cytokines regulate their level of expression in CTLs. The role of perforin, which is involved in T cell- and NK cell-mediated target cell lysis, was demonstrated in mice lacking perforin with respect to their capability to eradicate a syngeneic lymphoid tumor mammary adenocarcinoma (69). Smyth and coworkers demonstrated that mice with lymphoma and deficient in the pore-forming protein perforin [(pfp)-deficient] showed an increased number of premalignant cells than their immunocompetent counterparts. In fact, pfp-deficient mice were 1,000-fold more susceptible to tumor (70), demonstrating that lymphocyte-mediated cytotoxicity plays an essential role in promoting host resistance to spontaneous tumor formation. NK cells also express several TNF superfamily proteins and death-inducing ligands, such as TNF-related apoptosis-inducing ligand (TRAIL) and FAS ligand (FASL), which induce target cell apoptosis via binding to their corresponding receptors (TRAIL-R and FAS). Activated NK cells also produce growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines (XCL1, CCL3, CCL4, and CCL5) l (26, 33, 71–73).

NK CELLS IN SOLID TUMORS

Genetic alterations in oncogenic pathways associated with an aberrant inflammatory milieu (74, 75), abnormal activations of transcription factors (nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB] and signal transducer and activator of transcription-3 [STAT3]) (76, 77), and hypoxia (78) may contribute to development and maintenance of the TME. The TME is responsible for tumor onset and progression by orchestrating cell growth, proliferation, malignancy, and immune escape processes. TME can impair and "polarize" the innate, adaptive, stromal, and endothelial cell compartments by several mechanisms (2, 79–82). Tumor cells, MDSCs, and tumor-associated cells, such as tumor-associated fibroblasts (TAFs) and endothelial cells (83), can contribute to tissue modifications of the ECM by matrix metalloproteases (MMPs) and fibroblast activation proteins and the release of soluble factors (basic fibroblast growth factor [FGF], platelet-derived growth factor, hepatocyte growth factor, insulin-like growth factor), chemokines (CCL2, SDF1a/CXCL12, CXCL8), and immunosuppressive cytokines, such as transforming growth factor beta (TGF-β), IL-10, and IL-6 (84, 85). Due to the adverse TME, immune cells lose their ability to reach, recognize, and target tumor cells (2, 79, 86, 87). The TME impairs immune cell homing to the lymphoid organs promoting tumor immune cell escape, invasiveness, and angiogenesis (88–90). Thus, chronic inflammation orchestrated by immunosuppressive mediators of the TME supports tumor progression by exhausting immune cells, such as NK cells (74, 78).

NK cells also express inhibitory receptors targeting non-MHC molecules on healthy cells. One of these inhibitory receptors expressed on NK cells is killer cell lectin-like receptor G1 (KLRG1), a well-conserved member of the C-type lectin receptor superfamily. KLRG1 is known for its role in NK cell maturation, development, and homeostasis (91). Recently, a new role for KLRG1 has emerged as an inhibitory receptor impacting NK cell function in tumor surveillance. Impairment in the migration and/or retention of NK cells in the BM has been observed in multiple myeloma (MM). BM localization of the more functional NK KLRG1^{-} subtypes is impaired in MM by altering the chemokine microenvironment (increasing chemokine [CXC] ligand 9 [CXCL9] and CXCL10 and reducing CXCL12 expression in BM) in a mouse tumor model of an early cancer growth

stage (88). This is due to significant dysregulation of the CXCR3 and CXCR4 chemokine receptor/ligand axes, influencing NK cell responses (88, 92). Using murine models of chronic NK cell stimulation, Alvarez and colleagues have identified a "phenotypic signature of NK cell exhaustion," characterized by upregulation of KLRG1 and downregulation of the activating receptor NKG2D (93). KLRG1 ligands, such as E- and N-cadherin, were upregulated in tumor specimens from patients with melanoma, breast, prostate, and colorectal cancer (94). The same authors showed that anti-KLRG1 antibody monotherapy in a 4T1 breast cancer mouse model enhanced tumor control compared to controls (94). Tata and colleagues also demonstrated that KLRG1-deficient mice had significantly fewer lung tissue tumors than wild-type controls (95).

Several studies have demonstrated the prognostic significance of tumor-infiltrating lymphocytes and their antitumor actions in cancer (16, 17, 96–99). It has recently become clear that CTL and NK cell cooperation are essential in many types of tumors (100–110). Using mouse models of mastocytoma (mice heterozygous for the H-2Ld/P1A35–43-specific TCR transgene: TCRP1A on the DBA/2, B10.D2; TCRP1A tg B10.D2[×DBA/2] F1; RAG-1°/°B10.D2), a study demonstrated that the frequency of cancer antigen-specific T cell precursors and the rate of antigen variants can contribute to the efficacy of adaptive T cell responses to cancer (107). Moreover, the efficiency of an effective antitumor antigen-specific T-cell response can depend on the complementary interaction between effector T cells and NK cells (107). Another study also demonstrated that the NK cell's antitumor effect requires interaction with specific activated tumor antigen-$CD8^+$ T cells (12). However, further studies are necessary to clarify the mechanism of interaction between NK cells and specific effector T cells.

In clinical trials studying solid tumors, impaired NK cell function correlated with a poor prognosis in patients with advanced disease (111, 112). The TME plays a critical role in reducing NK cell persistence and trafficking in the tumor site by inhibiting NK cell activation, leading to tumor invasion and metastasis (26). Moreover, several studies showed anergic and hypo-functional NK cells (26, 27, 113–120), including tumor-associated NK cells in the PB and tumor-infiltrating NK cells within the tumor tissue. These $CD56^{bright}$ $CD16^{low/-}$ $Perforin^{low}$ NK cells even exhibited pro-tumorigenic functions and pro-angiogenic activities (80, 121–124) and have been identified preferentially in many solid tumors (80, 87, 115–121, 123, 125–129). Some of these NK cells also downregulated their expression of NKG2D, impairing their antitumor functions further.

MDSCs AND TREGS IN CANCER

Development and Phenotypes of MDSCs

Common myeloid progenitors differentiate from hematopoietic stem cells in the BM. Later, they migrate to the peripheral lymphoid organs and differentiate into myeloid cells. This pathway involves granulocyte-macrophage progenitor and various myeloblast intermediate precursors, including common monocyte progenitors (130) (**Figure 1**). Immunological stress, as well as cancer, prolonged inflammation, trauma, and autoimmune disorders, can impair the differentiation of these immature myeloid cells (131). Tumor-associated myeloid cells are mainly represented by tumor-associated macrophages (TAMs) and MDSCs, which are one of the crucial players within the TME (**Figure 1**). The TME often subverts immunosurveillance by generating MDSCs with strong immunosuppressive activity and functional plasticity (130). MDSCs are a heterogeneous population of myeloid-derived cells represented by myeloid progenitors, immature granulocytes, DCs, and macrophages. Therefore, the characteristics that separate MDSCs from other myeloid cells are still under investigation. It is widely accepted that MDSCs are divided into two main subsets: granulocytic or polymorphonuclear (PMN)-MDSCs and monocytic (M)-MDSCs, cells showing a phenotype and morphology similar to neutrophils and macrophages, respectively (132–136) (**Figure 1**). However, MDSC subtypes can be distinguished from neutrophils and TAMs that are present in the TME (137, 138). Studies have shown additional mechanisms to describe the evolution and roles of these polarized neutrophils in the TME, and some evidence supports the idea that these cells are similar to MDSCs and could be described as PMN-MDSCs (139–141), which represent the most prevalent cells in several types of tumors (142, 143). MDSCs with granulocyte and monocyte hallmarks have genomic profiles, biochemistry, and *in vitro* properties that differ from neutrophils, monocytes, and DCs (144). Recently, whole-transcriptomic and proteomic analyses (134, 145–147) provided specific gene expression patterns for the characterization of these different cell types. Cell-surface markers have been identified to distinguish MDSC-specific phenotypes from TAMs, tumor-associated neutrophils, and neutrophils. M-MDSCs can be separated from TAMs by their differential expression of F4/80, M-CSF and $CD115^{high}$, $Ly6C^{lowtointermediate}$, $IRF8^{low}$, and $S100A9^{verylow}$ (148). In contrast, PMN-MDSCs are $CD11b^+Ly6G^+Ly6C^{loLOX-1}$ in mice and $CD11b^+CD14^-CD15^+$ or $CD11b^+$ $CD14^-CD66b$ in humans, while M-MDSCs are $CD11b^+Ly6G^-Ly6C^{hi}$ in mice and $CD11b^+CD14^+HLA\text{-}DR^{-/lo}CD15^-$ in humans (**Figure 1**). A low percentage of MDCSs (around 3%) consists of a mixture of more immature progenitors and precursors with myeloid-colony-forming activity termed "early-stage MDSCs" (e-MDSC) and are described as $Lin^-HLA\text{-}DR^-CD33^+$ (132, 144). These and other novel marker combinations are currently under further investigation (133, 134, 149).

The number of MDSCs is associated with the clinical cancer stage and metastatic disease (150). Thus, MDSCs could be a potential biomarker of disease in several types of cancer. For example, the frequencies of MDSCs change during tumor progression in glioma (151) and cervical cancer (152). The frequency of peripheral PMN-MDSCs has been correlated with cancer prognosis, while the percentage of M-MDSCs has been shown to be higher in patients with advanced cervical cancer (152). Moreover, PMN-MDSCs' rate is negatively correlated with $CD8^+$ T cells' rate (151, 152). MDSCs have been reported as prognostic markers in non-small cell lung cancer (NSCLC) (153); breast and colorectal cancer (154); gastric, esophageal, and pancreatic cancer (155); and melanoma (156). Therefore, MDSCs

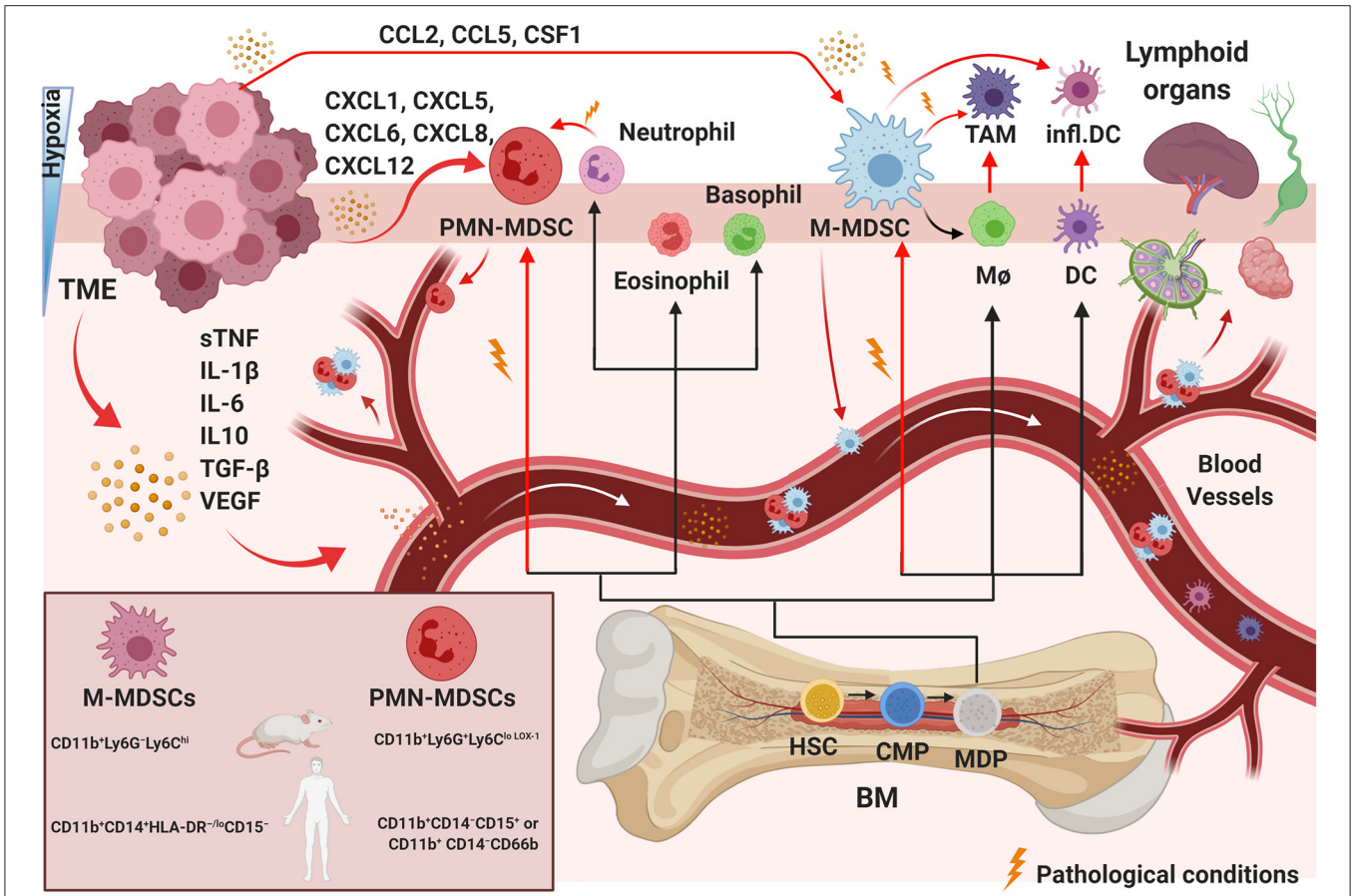

FIGURE 1 | Pathological myelopoiesis in cancer. Under physiological conditions and chronic inflammation, hematopoietic progenitor cells (HPCs) differentiate via common myeloid progenitor cells (CMPs) into monocytic/dendritic progenitor cells (MDPs). Myelopoiesis is altered under pathological conditions, such as in a tumor. Tumor-immunosuppressive factors produced in the TME alter myelopoiesis, leading to aberrant differentiation and accumulation of myeloid lineage cells. The black lines show the normal pathways of myeloid cell differentiation from immature myeloid precursor cells to dendritic cells (DCs), macrophages (MΦ), and granulocytes, as depicted. The red lines indicate the aberrant pathways of myeloid cell differentiation that arise in cancer, in which the TME promotes the development of immunosuppressive populations, including monocytic myeloid-derived suppressor cells (M-MDSCs), polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), inflammatory DCs, and tumor-associated macrophages (TAMs). The MDSC mouse and human phenotypes are shown in the left square on the bottom. BM, bone marrow; sTNF, soluble tumor necrosis factors. Created with BioRender.com.

can also be used as a predictive marker for immunotherapy. Additionally, a diminished number of MDSCs helped to eradicate metastatic disease after the removal of primary tumors in a mammary carcinoma model (157).

Recruitment and Expansion of MDSCs in Tumors

While absent in normal physiological conditions (144), MDSCs can be detected in the BM, blood, spleen, tumor, and lymph nodes in pathological conditions (**Figure 1**). MDSCs have been shown to increase significantly in early- and late-stage cancer in preclinical animal models and human tumors (6, 145, 151). Upon their recruitment, MDSCs gradually expand in the TME and support the development of an immunosuppressive tumor environment by interacting with several components of the innate and adaptive immune systems (158, 159) and by stimulation of neo-angiogenesis (160, 161). Vetsika et al. described all phases of this process (162); here, we will summarize. The network of transcriptional regulators that directs MDSC development can be combined into two partially overlapping groups: (i) factors promoting myelopoiesis and avoiding differentiation of mature myeloid cells and (ii) factors contributing to pathologic activation of MDSCs. In different types of mouse and human cancers (132, 144, 163–166), MDSCs are gradually recruited and increase in the TME. They support tumor progression through non-immune activities by stimulating pre-metastatic niche formation, invasion (167, 168), and inducing pro-tumor angiogenesis (169). Some authors have proposed a "two-signal model" for describing how MDSCs can acquire the modifications guiding their pathological activation, immunosuppressive activity, and expansion in the TME under tumor pathologic signals (170, 171). Myeloid cells exposed to pathological conditions (autoimmunity, cancer, trauma, graft vs. host disease, and infections) can be activated in response to damage-associated molecular pattern molecules, pathogen-associated molecular pattern molecules, or pro-inflammatory

cytokines (144). Because patient blood has been observed to have increased tumor-released macro- and micro-vehicles during tumor progression, tumor niches could potentially gather MDSCs from the BM by releasing exosomes. Their contents have been demonstrated to reprogram target cells in different types of cancer, increase the mobility of the progenitor myeloid population to the tumor site, and increase tumor immunosuppression (162).

MDSCs migrate in response to several chemo-attractant molecules released from cells in the TME using two main pathways: PMN-MDSC migration includes the secretion of CXCL1, CXCL5, CXCL6, CXCL8, and CXCL12, and M-MDSCs respond to CCL2, CCL5, and CSF1 (172) (**Figure 1**). The TME can guide the differentiation of incoming MDSCs in several different directions. The commitment of myeloid progenitor and precursor cells into MDSCs can be triggered by immunosuppressive cytokines released in the TME, such as soluble tumor necrosis factors (sTNF), IL-1β, IL-6, IL10, TGF-β, and vascular endothelial growth factor (VEGF) (173, 174) (**Figure 1**). MDSC phenotypes develop under conditions of acute or chronic inflammation, stress and hypoxia, high concentrations of oxidative molecules, and reduced nutrients (172, 175) (**Figure 1**). Hypoxia, specifically hypoxia-inducible factor 1 alpha (HIF-1α), appears to be one of the most critical stresses (35, 56) and was shown to be essential in M2-type TAM generation from $Ly6C^{hi}$ monocytes inside a tumor (176). During hypoxia, immunomodulatory proteins and chemokines also mediate the differentiation of TAMs or M2 macrophages (177) from M-MDSCs or/and guide later events in tumor progression (177, 178).

Because M-MDSCs have a longer lifespan (179), their differentiation has been studied more extensively than that of PMN-MDSCs. TAMs can be derived from tissue-resident macrophages proliferating *in situ* in pancreatic and mammary tumors (180, 181). Several studies have shown the ability of M-MDSCs to differentiate into TAMs after migrating from the spleen to a tumor (178, 180, 182, 183), and TAMs can be "regenerated" by the arrival of new M-MDSCs from other organs during tumor progression (184–186) (**Figure 1**). MDSCs can also differentiate into DCs and fibrocytes during cancer progression (187). Moreover, $Ly6C^{hi}$, Ly6CX3CR1, and $Ly6C^{+}CCR2^{+}$ monocytes can differentiate into TAM subsets (**Figure 1**) in mammary adenocarcinoma, lung adenocarcinoma, and lung carcinoma models (188, 189). In the chronic inflammation present in tumor tissues, IL-18 can support the function of TGF-β1 that is produced and activated by M2-polarized TAMs (190, 191). IL-18 promotes the differentiation of $CD11b^{-}$ BM progenitor cells into M-MDSCs and increases their suppressive functions, including arginase expression (ARG1) and NO secretion (192). MDSCs induced by IL-18 can inhibit $CD4^{+}$ T cell proliferation and IFN-γ production (192), contributing to the negative regulation of immune responses in tumors through immunosuppressive functions.

Treg Origin and Development in the TME

$CD4^{+}CD25^{+}$ Tregs are a subpopulation of suppressor T cells that mediate immune homeostasis, maintain peripheral tolerance, and prevent immune and auto-immune disease by suppressing autoreactive T cells (193). These cells regulate immune responses in the context of immunity and infections differently (194, 195). In cancers, Tregs are linked to the development of an immunosuppressive TME, promoting immune evasion and cancer progression and preventing antitumor immunity (196–198). Tregs represent about 1–3% of $CD4^{+}$ T cells in human tumors and about 10% in rodents. These cells express cell surface molecules associated with activated/memory T cells, CD25, FoxP3, CD45RBlow, CD62L, CD103, cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and glucocorticoid-induced TNF receptor (199). An increase in Tregs prevalence has been shown in several tumor malignances (7), and they are recruited and expand within the TME via several mechanisms (200). We will summarize these stages in the following steps. Tregs are recruited into tumors in response to chemokines secreted by tumor cells and innate immune cells. Tregs then expand and proliferate in response to tumor-derived factors (TGF-β, adenosine, VEGF, and IL-10) within the TME. TGF-β and adenosine, released from cancer cells and also MDSCs, seem to play a key role in generating suppressive $CD25^{+}$ $FoxP3^{+}$ Tregs from non-suppressive $CD25^{-}$ $FoxP3^{-}$ conventional Tregs. The recruitment of Tregs occurs at early tumor stages, as demonstrated by their presence in pre-malignant lesions, and their prevalence increases with pancreatic and breast tumor progression and worsening clinical outcomes (114, 201–203). Moreover, it has been shown that the depletion of Tregs cells in pancreatic ductal adenocarcinoma slows tumor growth and prolongs survival (204–207).

Crosstalk Between MDSCs and Tregs

MDSC expansion in PB is directly correlated with poor clinical outcomes (208–210). MDSCs can support the conversion of naive $CD4^{+}$ T cells into Tregs by secreting retinoic acid and TGF-β (**Figure 2**), promoting the trans-differentiation of Th17 cells into $Foxp3^{+}$ Tregs (211). Moreover, MDSCs can also induce Tregs immunosuppressive functions by mediating the release of IL-10 and IFN-γ (162). Tumor-infiltrating M-MDSCs express high levels of C-C chemokine receptor type 5 (CCR5) ligands and recruit high numbers of Tregs into the TME (212), establishing an additional cooperative network between MDSCs and Tregs (24, 35). Therefore, Tregs are accumulated in the TME and produce VEGF, which promotes angiogenesis (161, 213). By using light-sheet fluorescent microscopy, Siret and colleagues demonstrated direct interactions between MDSCs and Tregs in pancreatic ductal adenocarcinoma (214), and *in vivo* depletion of MDSCs significantly reduced the Tregs population in pancreatic tumors (215). Furthermore, video-microscopy and *ex vivo* functional assays have demonstrated that MDSCs can induce Treg cells by cell–cell-dependent contact at different stages of human cancer, and Tregs can also affect the survival and/or the proliferation of MDSCs (214). The molecular mechanisms guiding MDSC/Tregs interplay are not fully understood. The role of co-stimulatory molecules, protein membranes, and receptor candidates, respectively, such as PD-L1 (216), CD80 (217), and CD40 (218), is currently under investigation. Together, Tregs and MDSCs contribute to establishing an immunosuppressive TME in multiple solid neoplasms (114, 201, 202, 214).

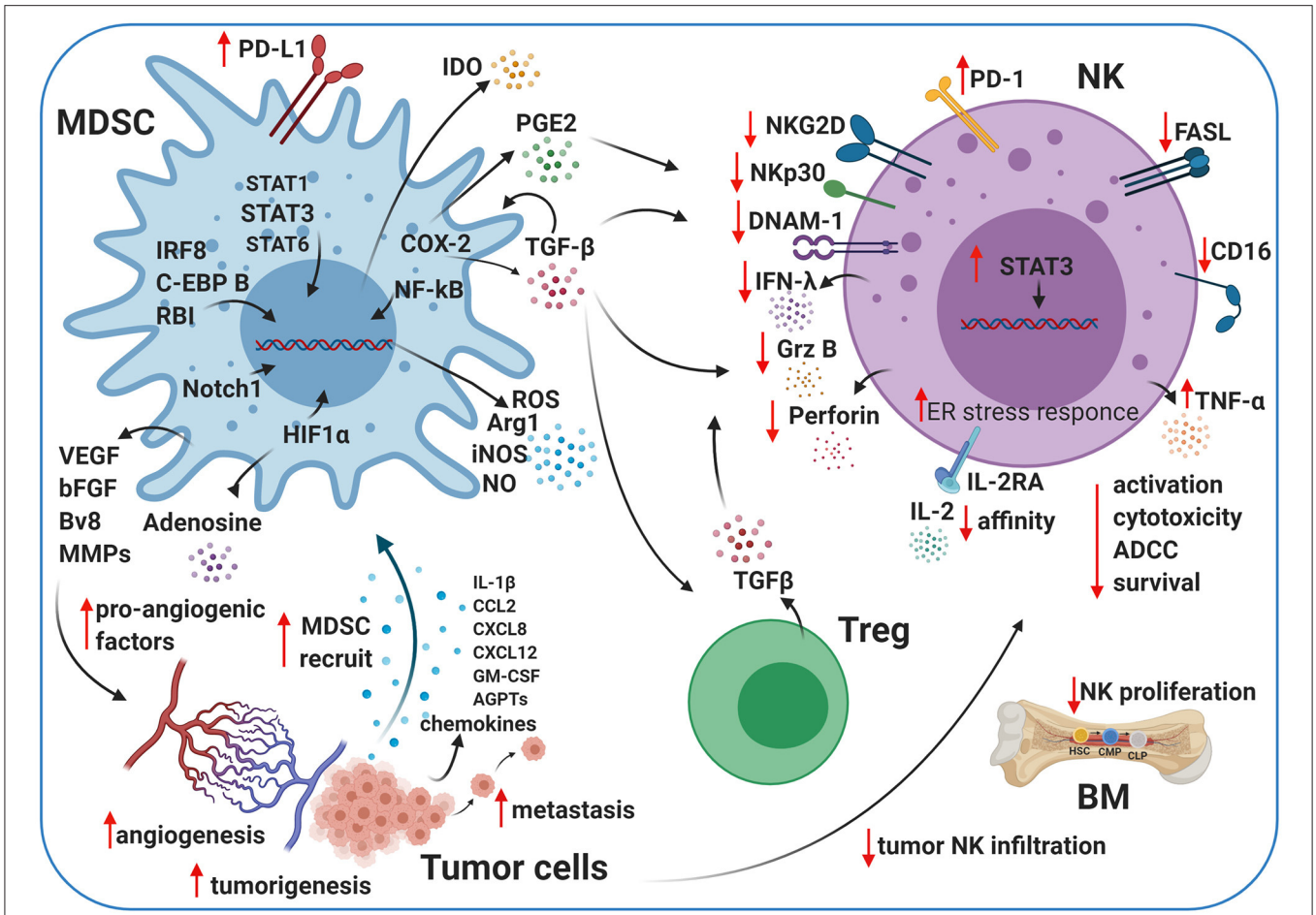

FIGURE 2 | Crosstalk between MDSCs, NK cells, and Tregs in the TME. Myeloid-derived suppressor cells (MDSCs) and natural killer (NK) cells crosstalk within the TME. The cartoon shows the immunosuppressive activity of MDSCs and regulatory T cells (Tregs) on NK cells using different pathways. MDSCs impair NK cell activation, cytotoxicity, survival, and maturation by several secreted factors, including transforming growth factor beta (TGF-β), indoleamine 2,3-dioxygenase (IDO), nitrogen oxide (NO), reactive oxygen species (ROS), and prostaglandin-E2 (PGE2). MDSCs contribute to tumor angiogenesis by releasing pro-angiogenic factors, such as adenosine and vascular endothelial growth factor (VEGF), under pathological conditions. Created with BioRender.com.

CYTOKINES AND OTHER MEDIATORS IN MDSC-MEDIATED NK CELL REGULATION

In addition to cell-intrinsic defects caused by chronic stimulation (219), an immunosuppressive TME represses CTL and NK killing (29, 220) via the recruitment of other cells types, such as MDSCs, M2 macrophages (221), and Tregs (222), which contribute to immune exhaustion via the expression of inhibitory ligands, suppressive cytokines, and tumor-promoting factors (221, 223). MDSCs display potent immunosuppressive activity and play a critical role in regulating tumors and metastasis development (144). MDSCs can impair $CD8^+$ CTLs and NK cells directly by influencing the pro-tumor TME (224). Their contribution to regulating T lymphocytes is well-described, while their interactions with other immune cells, such as NK cells, DCs, or macrophages, in the TME are less understood (158). The suppressive effects of MDSCs are mediated through cell–cell contact, as well as the secretion of soluble factors, and result in antigen-specific or antigen-non-specific suppression of T-cell responses (225). The presence of MDSCs in tumors is associated with chronic inflammation and antigen-specific tolerance by T cells (226). MDSCs can also regulate the innate immune response by inhibiting NK cell functions (227–230) and/or by modulating macrophages' cytokine production (159, 225). The immunosuppressive activity of MDSCs plays an essential role in the regulation of the NK cell response to the tumor. *In vitro* co-culture of MDSCs and NK cells showed a reduction in NK cell-mediated cytotoxicity and higher tumor cell tolerance. MDSCs inhibit antitumor responses in part through immune checkpoint inhibition (ICI), including programmed death (PD)-1/PD-L1, galectin-9/T-cell immunoglobulin domain and mucin domain 3, and CTLA-4/B7 interactions (231).

STAT3 regulates NK cell biology at several levels, including activation, cytokine/cytolytic-mediated functions, and interactions with other immune system components (232). Many growth factors and cytokine receptors signal through

STAT family transcription factors. STATs are impaired in several types of cancer and play a crucial role in innate and adaptive immunity (225, 233–235). In particular, STAT3 regulates several pathways involved in NK cell development, cytotoxic activities, and killing (232). Similarly, several cytokines, transcription factors/regulators, and signaling pathways are involved in the expansion and differentiation of MDSCs. These include interferon regulatory factor 8 (IRF8), CCAAT-enhancer-binding protein, and retinoblastoma protein; signaling pathways STAT3, STAT1, STAT6, Notch, NF-κB, and cyclooxygenase 2 (COX-2); and endoplasmic reticulum. Stress pathways are involved in MDSCs' expansion (170) (**Figure 2**) and include STAT3 activation in e-MDSC subtypes, which is critical for NF-κB activation and increasing indoleamine 2,3-dioxygenase (IDO), the release of which inhibits NK cell activation, proliferation, and effector functions (236). The molecular mechanisms regulating PMN-MDSC and M-MDSC populations differ from the afore-described pathways and are currently under investigation. Danvatirsen, a STAT3 antisense oligonucleotide, reversed the immunosuppressive TME and enhanced immune activity, as well as checkpoint blockades, in patient tumor samples from two phase I clinical trials and murine models (237).

Tumor-derived IL-1β release into the TME has been shown to enhance the recruitment of specific MDSCs during chronic inflammation (173). The augmented suppressive potential of IL-1β-induced MDSCs in mice was due to the activity of a novel subset of MDSCs lacking Ly6C expression (173). When these cells are prevalent under inflammatory conditions, they can impair NK cell development and function *in vitro* and *in vivo* by reducing the expression of the NK cell activating receptor NKG2D (173). $Ly6C^{-}$ MDSCs may be a valuable therapeutic target.

Tumor-derived prostaglandin-E2 (PGE2) may also play a crucial pro-tumor role in inducing MDSCs, mainly via COX-2 (238) (**Figure 2**). Monocytes exposed to PGE2 acquire MDSC-like functions, gaining the ability to inhibit NK cells via TGF-β (239, 240). High levels of TGF-β in the plasma were observed in patients with advanced tumors and correlated with worse outcomes (241, 242). TGF-β is released from cancer cells and can increase the expansion of M-MDSCs (243), recruit suppressive cells to the TME (MDSCs, Tregs, DCs, and stromal cells), and compromise the function of NK cells (230, 244–246), cytotoxic $CD8^{+}$ cells, DCs, Tregs, and macrophages (247). NK cells exposed to MDSCs secrete less IFN-γ and downregulate NKG2D and CD247 *in vitro* and *in vivo* (229, 230, 248, 249). TGF-β not only impairs NK cell functions by downregulating the expression of activating receptors (NKG2D and NKp30) and inhibiting their transcription but also downregulates tumor cell-expressed NKG2D ligands (250). Thereby, NK cells lose their capacity to recognize and kill tumor cells via NKG2D (250). Furthermore, TGF-β inhibits CD16-mediated IFN-γ production and ADCC in human NK cells through mothers against decapentaplegic homolog 3 (SMAD3) activation (251) and affects $CD34^{+}$ hematopoietic progenitors by inhibiting the maturation of $CD56^{bright}CD16^{+}$ NK cells fraction in the PB (246). The incubation of PB-NK cells with stromal cells, isolated from decidual tissue conditioned media, mimicked the suppressive effects of TGF-β1. NK cell interactions with CXC chemokine ligands and progesterone at the maternal–fetal interface after TGF-β1 exposure resulted in the reduction of $CD56^{bright}CD16^{+}$ NK cells and induced decidual-like NK cells that showed an exhausted phenotype (246). As such, STAT3 blockade and TGF-β inhibition improve tumor immune surveillance by NK cells (252). Specifically, tumor-infiltrating and tumor-associated NK cells from STAT3-deficient tumor-bearing mice express enhanced levels of NKG2D, CD69, FASL, granzyme B, perforin, and IFN-γ, reducing tumor growth and improving survival (252, 253). TGF-β signaling is deregulated in many diseases, including cancer. In early-stage tumor cells, this pathway has tumor-suppressor functions, including cell-cycle arrest and apoptosis (254). TGF-β signaling in late-stage cancer can promote tumorigenesis, angiogenesis, metastasis, and immunosuppression (254–256). MDSCs release TGF-β in the TME (144, 163, 175), and NK cell anergy (**Figure 2**) correlates with the marked increase of MDSCs in the liver and spleen in orthotropic liver cancer-bearing mice (230). Also, MDSCs prevent cytotoxicity, NKG2D expression, and IFN-γ production by NK cells (**Figure 2**) *in vitro* and *in vivo* through membrane-bound TGF-β (230).

IDO is an intracellular enzyme, and it regulates tryptophan catabolism into kynurenine (257, 258), which inhibits the proliferation and function of NK and T cells (259–261). IDO synthesized by MDSCs impairs NK cell activation, development, and expansion, resulting in dramatically decreased expression of NKG2D and DNAM-1 and limiting IFN-γ secretion (262, 263) (**Figure 2**).

ARG1 and reactive oxygen species (ROS), soluble factors secreted by MDSCs, also impair NK cell functions (**Figure 2**) in cancer models *in vivo* (248, 264). Moreover, several pro-inflammatory cytokines have been identified to mediate MDSC/NK crosstalk in the TME (264). These phenotypic and functional TME alterations due to MDSC/NK cell and/or T cell interactions contribute to pro-tumor, pro-angiogenic, and pre-metastatic activities in the tumor. Further studies are necessary to elucidate the mechanisms involved in MDSC/NK cell interactions to identify potential therapeutic candidates or pathways to limit NK cell MDSC-mediated suppression in the TME. MDSCs secrete high levels of soluble factors, such as ROS, inducible nitrogen-oxygen synthase (iNOS), nitrogen oxide (NO), peroxynitrate, and ARG1 (144), and show an elevated endoplasmic reticulum stress response (132, 265) (**Figure 2**).

NO is a ubiquitous, water-soluble, gaseous transmitter, which plays an essential role in various physiological conditions, inflammation, and cancer (266, 267). NO can play different roles in regulating immunity depending on the exact circumstances of its secretion. The autocrine production of NO by NK cells can improve NK cell function, but when MDSCs produce NO, it plays a vital role in mediating immunosuppression (266, 267). Co-cultures of autologous NK cells and MDSCs from patients with cancer showed that MDSCs suppress FcR-mediated function and signal transduction, leading to reduced responses to monoclonal antibody (mAb) therapies, and inhibit the secretion of IFN-γ and TNF-α by NK cells. Elimination of MDSCs or

abrogation of NO production can improve responses to mAb immunotherapy (228).

MDSCs also support tumor progression by inducing tumor angiogenesis through the release of VEGF, basic FGF (bFGF), prokineticin 2 (Bv8), and some MMPs (169, 172, 268–270) (**Figure 2**). Some studies showed that MDSCs in the TME produce high levels of MMPs, including MMP2, MMP8, MMP9, MMP13, and MMP14 (167, 268, 271, 272). MDSCs in the presence of high levels of MMP9 can promote VEGF function by raising its bioavailability (273). VEGF stimulates MDSCs via STAT3 in the TME and potentiates their immunosuppressive activity by expanding other immune cell populations (213, 274, 275) and stimulating the secretion of numerous additional angiogenic factors and chemokines, which further enhance MDSCs accumulation within tumors. IL-1β, C-C motif chemokine ligand 2 (CCL2), CXCL8, CXCL2, angiopoietin 1 and 2 (AGPTs), and GM-CSF (**Figure 2**) have been shown to contribute to MDSC-mediated angiogenesis and involve STAT3 for their expression (80, 276–278).

The major components of the TME are the endothelial cells of the blood and lymphatic vessels, fibroblasts, immune cells, and the ECM (2). During tumor development and progression, cancer and stromal cells often have restricted access to nutrients and oxygen. Most solid tumors have hypoxic regions due to abnormal vascularization and inadequate blood supply (279). The changes to cancer and stromal cells that are necessary for tumor progression in a hypoxic environment are attributed to HIF-dependent signaling. The HIF family of transcription factors includes HIF-1, HIF-2, and HIF-3. Signaling by HIF-1 and HIF-2 induces the expression of multiple pro-angiogenic factors (VEGF, angiopoietin-2 [ANG-2], phosphatidylinositol-glycan biosynthesis class F protein [PlGF], bFGF, and semaphorin 4D), and angiogenesis was promoted in MDSCs by HIF-1 through VEGF and S100 calcium-binding protein A8 (S100A8) (280).

The TME has high levels of adenosine during hypoxia and inflammation. Adenosine/adenosine receptor interactions increase immunosuppression and angiogenesis through immune cells (281, 282). MDSCs express increased levels of CD39 and CD73 under hypoxic conditions or TGF-β stimulation (283, 284). These enzymes can convert adenosine triphosphate (ATP) and adenosine monophosphate (AMP) to adenosine, resulting in increased adenosine levels in tumor lesions (**Figure 2**). Adenosine accumulation impairs IL-2 and Ly49D activation, NKp46-receptor crosslinking, and maturation in NK cells (285). It has also been shown that adenosine signaling is involved in reducing the engagement of A2A adenosine receptor (A2AR) as a checkpoint in NK cell maturation (286).

Tregs interact with different components of the TME (287) and exert their suppressive function via contact-dependent and -independent mechanisms, which have been previously reviewed (288, 289). NK cell–Treg crosstalk in the human tumor has not been studied extensively (290). However, it has been shown that peripheral Tregs isolated from healthy donors and patients with gastrointestinal stromal tumors impaired NK cells by downregulating the expression of NKG2D activated receptors and also inhibited NK cell functions via membrane-bound TGF-β (291) (**Figure 2**). A similar effect was observed *in vitro* with cervical carcinomas (292).

Through these mechanisms, MDSCs and Tregs inhibit CTL and NK cell activity, promote tumor progression, and hinder antitumor immunity (169, 172, 287, 293–296). Therefore, it is not surprising that high MDSC infiltration of tumors correlates with poor patient prognosis and resistance to immunotherapy and chemotherapy (150, 175, 297–300). MDSC and Treg numbers also positively correlate with disease stage and tumor burden (114, 132, 144, 163–166, 201–203) and are predictors of poor outcome in patients with solid tumors (301–304). Both MDSCs and Tregs increase suppressive activity via signaling pathways, and their interactions in tumors have recently been reviewed (287, 305). Consistent with these findings, pharmacological targeting of MDSCs and Tregs in animal models and cancer patients significantly improves antitumor immunity, enabling tumor control (306, 307).

TARGETING MDSCs AND THEIR LIGANDS—CROSSTALK WITH NK CELLS

The complexity of the TME impairs immune cell functions and affects their phenotype. Several strategies have been developed, investigated, and applied in clinical trials to target MDSC immunosuppression and enhance NK cells' cytotoxic activity in the TME.

Studies have indicated that the types of mediators responsible for the differentiation, inhibition, and recruitment of MDSCs into the tumor were dependent on the different MDSCs subsets and tumor models (175). Targeting MDSCs is an approach designed to limit their immunosuppression within the TME and reduce neo-angiogenesis. Many therapies are focused on blocking intratumoral recruitment and expansion of MDSCs (**Figure 3**). Other approaches are dedicated to increasing MDSC differentiation or inhibiting their immunosuppression activity (264) (**Figure 3**). MDSCs are robust immunosuppressors in the TME and are an impediment for many cancer immune therapies (161).

Low doses of chemotherapy induce MDSC exhaustion (308, 309). Chemotherapy drugs such as gemcitabine (310), 5-Fluorouracil (311), and doxorubicin (312) reduce MDSC frequency, which enhances NK-mediated antitumor cytotoxicity (313–315). Phenformin and metformin, two antidiabetic drugs, impair MDSC functions mainly by blocking 5' AMP-activated protein kinase (AMPK). These drugs also upregulate the expression of MHC class I polypeptide-related sequence A (MICA) and heat shock protein 70 (HSP70) on cancer cells through the phosphatidylinositide 3-kinase/protein kinase B pathway leading to NK cell activation (316). In addition to chemotherapy and radiotherapy, many studies have combined MDSC targeting methods with immune-based therapies to increase the antitumor effects (317). Encouraging progress has been made combining MDSC targeting with immunotherapy strategies.

Blocking TGF-β pathways is a promising strategy in some preclinical and clinical trials. Some of the approaches currently

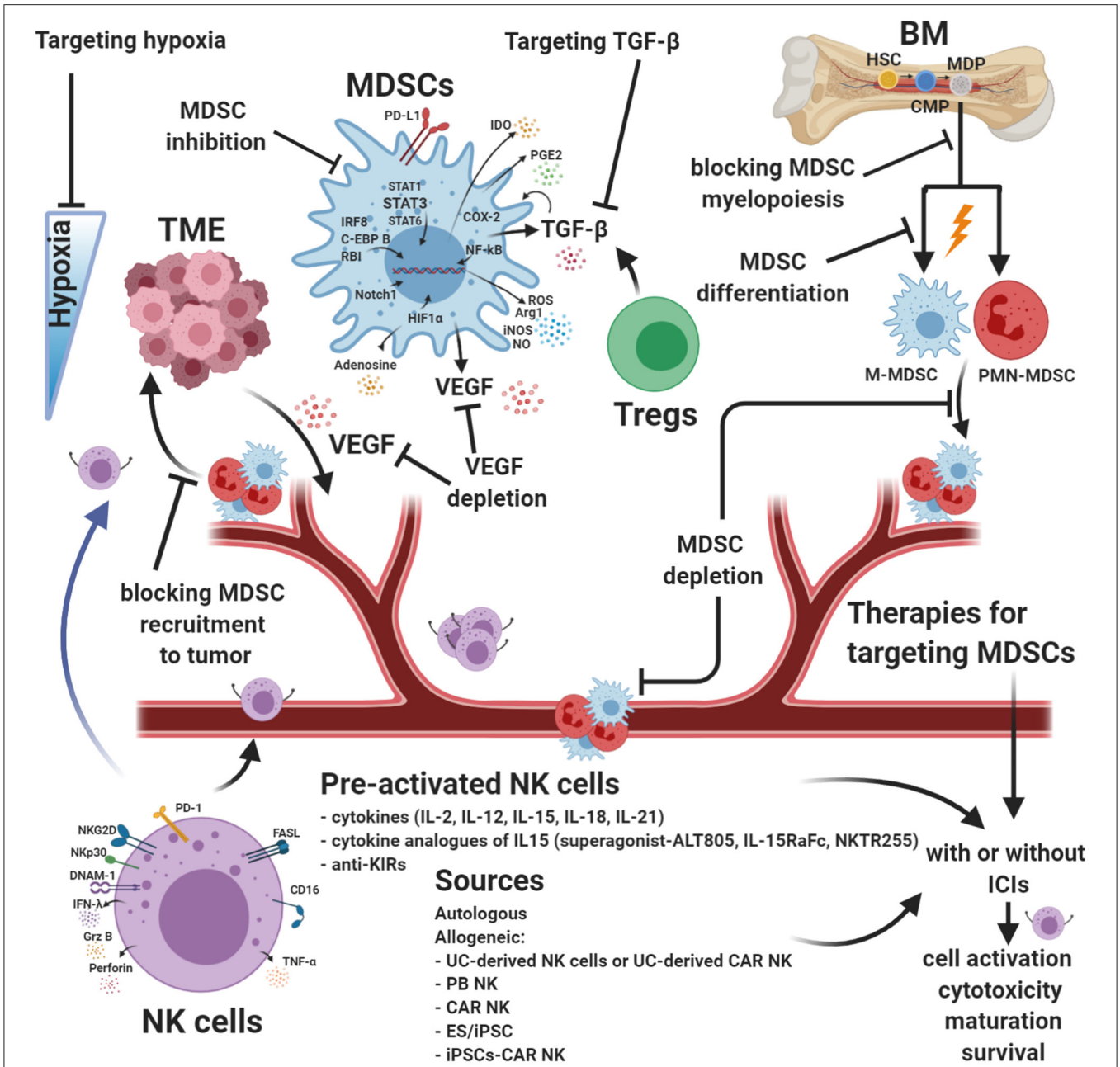

FIGURE 3 | Strategies for targeting MDSCs and augmenting NK cell functions. Myeloid-derived suppressor cells (MDSCs) are robust immunosuppressors in the tumor microenvironment (TME) and represent an obstacle for many cancer immune therapies. Targeting MDSCs is a different approach designed to limit their immunosuppression within the TME and reduce neo-angiogenesis. Many combinatorial therapies are focused on blocking intratumoral recruitment and expansion of MDSCs. Other approaches are dedicated to increasing MDSC differentiation or inhibiting their immunosuppression activity. Natural killer (NK) cell-based immunotherapy strategies have been studied in an increasing number of clinical trials. Although NK cells can be useful in some types of cancer, it is necessary to improve the efficacy of currently available NK cell products to avoid cell exhaustion. Innovative combinatorial approaches intended to improve NK cell function and block MDSCs are in development. MDSC targeting, enhanced NK cell cytotoxic activity, and drugs targeting hypoxia can be used with current cancer therapies, including immunotherapy, and may improve antitumor response efficacy. ICIs, immune checkpoint molecules. Created with BioRender.com.

under investigation in preclinical models and clinical trials consist of decreasing circulating TGF-β, blocking ligand–receptor interactions using neutralizing antibodies, and inhibiting TGF-β signaling pathways (NCT00356460 and NCT01722825) (318–323). Blocking TGF-β is a viable strategy to prevent myeloid precursors from differentiating into M-MDSCs and impair the migration and expansion of MDSCs and Tregs in the TME (**Figure 3**). Alternatively, manipulating

NK cells to block TGF-β signaling pathways is an attractive and promising strategy for solid tumors. In SMAD3-silenced NK cells (NK-92-S3KD), TGFβ1-mediated immunosuppression was blocked, inhibiting cancer progression in mouse models with human hepatoma and melanoma (324). Similarly, NK-92 cells genetically modified to express a chimeric TGF-β type II receptor (extracellular and transmembrane domains) and the intracellular domain of NK cell-activating receptor NKG2D were resistant to TGF-β-induced suppressive signaling and did not downmodulate expression of NKG2D (325). The same authors demonstrated that NK-92-TN cells inhibited the differentiation of human naïve $CD4^+$ T cells to Tregs and decreased tumor volumes *in vivo* in a hepatocellular carcinoma xenograft cancer model (325).

A recent study demonstrated that NK cell sensitivity to TGF-β can be reduced by stimulation of NK cells with IL-2 (**Figure 3**), which increases IFN-γ and TNF-α production by NK cells in the tumor, compared to NK cells that encountered acute TGF-β exposure or were not TGF-β imprinted (326). A new approach to overcoming tumor resistance mechanisms to ICIs has been tested by combining TGF-β, CXCR1/2 signaling, and PD-L1 (327). This simultaneous inhibition reduced mesenchymal tumor features and infiltration of suppressive PMN-MDSCs into the TME, improving antitumor activity by promoting immune cell infiltration and activation in tumors (327).

Cytotoxic agents and tyrosine kinase inhibitors (TKIs) deplete MDSCs and regulate myelopoiesis (264). TKIs, such as axitinib, sorafenib, and sunitinib, induce DNA damage by histone γ-H2AX phosphorylation and checkpoint kinase 1 activation, leading to senescence of human renal carcinoma cells (328). The presence of DNA damage in cancer cells also improved the identification of tumor cells by NK cells (328). TKIs can directly target VEGF and/or involve c-KIT signaling and interact with other factors, such as CSF or STAT3. These mechanisms impaired MDSC function and inhibited tumor angiogenesis (329, 330). These processes upregulate NKG2DLs and, consequently, stimulate NK cell antitumor cytotoxicity (329, 330). Several *in vivo* studies combined TKI and ICI therapies; the combination therapy improved antitumor mechanisms and simultaneously reduced immune cell exhaustion (330–333).

STATs are activated in tumor cells by multiple soluble factors. This activation causes impaired cytolytic functions mediated by perforin and granzyme B in NK cells and alters the expression of NK cell receptors NKG2D and DNAX accessory molecule-1 (DNAM-1) (232). STAT3 activation in tumor cells also represses the expression of NK cell-chemotactic factors, which reduce the migration of NK cells in the TME. TGF-β and IDO produced by tumor cells and MDSCs impair NK cell development, proliferation, and activation (232), leading to reduced NK-mediated cytotoxicity. Exhausted NK cells and T cells have a diminished secretion of IFN-γ (334). MDSCs secrete type I interferons to maintain a high level of PD-L1 expression and preserve their immunosuppressive activity in the TME as a compensatory mechanism (335); therefore, the autocrine IFNα/IFNβ-pSTAT1-PD-L1 circuit represents a pivotal pathway to targeted MDSCs (335).

STAT3 also increases the expression of PD-L1, which engages PD-1 expressed on NK cells, reducing their antitumor response (232). Janus kinase (JAK)/STAT3 inhibitors decrease MDSC trafficking in the TME, diminishing angiogenesis by inhibiting VEGFA and casein kinase 2 (336) and driving NK cell activation (336, 337). Targeting STAT3 in tumor-bearing mice leads to tumor reduction, better survival, and a significantly higher number of activated NK cells following treatment, as compared to control mice (252, 253).

Other approaches using STAT3 inhibitors combined with ICIs, such as nivolumab (NCT03647839), STAT3 small interfering RNA (siRNA), or decoy STAT3 oligonucleotide inhibitors alone or combined with ICIs (AZD9190), are in phase I/II clinical trials (NCT03421353). STAT3 siRNAs coupled to CpG oligonucleotides reduced the immunosuppression of toll-like receptor 9 (TLR9)-expressing PMN-MDSCs in preclinical data (338). Mouse and human PMN-MDSCs overexpress fatty acid transporter protein 2 (FATP2) due to stimulation by GM-CSF through activation of the STAT5 transcription factor (339). FATP2 inhibitors alone, or in combination with ICIs, delayed tumor progression in tumor-bearing mice (339). CSF-1R inhibitors, combined with anti-PD-1, improved the immune response in a mouse model of neuroblastoma (340).

MDSC immune functions are also impaired using class I deacetylase, entinostat, or ARG1 small-molecule inhibitors by decreasing iNOS and COX-2 levels (341). Some of these treatments enhanced NK killing and blocked MDSC-mediated suppression of T cells *in vitro* and *in vivo* in tumor models (342–344). One study showed that an ARG1 small peptide inhibitor combined with anti-PD-L1 slowed tumor growth (345). ARG1 inhibitors (such as CB-1158) showed encouraging preclinical results (346), increasing tumor-infiltrating NK cells and $CD8^+$ T cells (345, 347), reducing tumor burden (348, 349), and decreasing MDSCs recruitment into the TME (345).

Conventionally, phosphodiesterase-5 (PDE5) inhibitors (sildenafil and tadalafil) are used as therapies for non-malignant conditions (350). Recent evidence suggests that PDE5 inhibitors could improve antitumor cell responses by inhibiting the suppressive functions of MDSCs in the TME (306, 351, 352). Preclinical and clinical data show that the PDE5 inhibitor tadalafil enhanced the immune response in head and neck squamous cell carcinoma (HNSCC) patients through inhibition of MDSCs (NCT01697800) (352). However, another PDE5 inhibitor, Sildenafil, reduced PMN-MDSC function through downregulation of ARG1, IL4Ra, and ROS expression (248). PDE5 inhibitors also enabled NK cell antitumor cytotoxicity and reduced postoperative disease recurrence. These studies have also shown Treg reduction and enhanced CD4/CD8 T cell function in the TME (248, 306, 352). Phase I/II Clinical Trial NCT02544880 used tadalafil as a therapy for decreasing MDSCs and Tregs, improving the antitumor response. The treatment might also enhance antitumor mucin 1 (MUC1) vaccine efficacy in patients with resectable and recurrent HNSCC by promoting a permissive environment (NCT02544880).

All-trans retinoic acid (ATRA) and vitamin A metabolites (retinol) have been used to treat acute myelogenous leukemia (353–355). Several studies reported that ATRA inhibited cell

migration, metastasis, and proliferation, and promoted apoptosis of tumor cells (356–358). ATRA alone (359–361) or in combination with a DC vaccine against p53 (360) or IL-2 administration (361) showed lower MDSC frequencies and enhanced differentiation of MDSCs (359–361) into mature DCs, macrophages, and neutrophils (362). ATRA decreased the expression of immunosuppressive genes through the downregulation of TGF-β, PD-L1, IL-10, and IDO in MDSCs and upregulated MHC class I homologs MICA and MICB on tumor cells, enhancing NK cell activity (363, 364) and cytotoxicity of T cells (365) in the $CD8^-$ and $CD4^-$ immune response (362). In clinical studies, ATRA alone or combined with ipilimumab significantly reduced the level of circulating MDSCs in advanced-stage melanoma patients (366). In a preclinical breast cancer model, ATRA therapy improved the efficacy of anti-angiogenic treatments (367).

VEGF is produced by tumor cells in the TME and supports neo-angiogenesis, metastasis dissemination, and also acts as a chemoattractant for MDSCs in the tumor site (302, 368, 369). This has been shown in both NSCLC and renal cell carcinoma patients, especially under hypoxic conditions (370, 371). In a NSCLC model, VEGF attracts MDSCs from the BM to the periphery, expanding their presence throughout the individual (372). The binding of VEGF to its receptor is correlated with increased production ROS via the JAK2/STAT3 activation pathway. MDSCs can also secrete VEGF (**Figure 2**), creating, together with the tumor cells, a positive autocrine feedback loop in the TME (373, 374). Therefore, anti-VEGFR2 reduced the accumulation of intratumoral MDSCs, decreased hypoxia, and interfered with the formation of tumor microvessels through S100A8 (367). Genetic inactivation of VEGFA in MDSCs improved clearance of senescent tumor cells by NK cells, inhibited tumor regrowth after chemotherapy and, prevented cachexia in tumor-bearing mice (375). A phase II clinical trial evaluated the efficacy and pharmacokinetics of bevacizumab, an anti-VEGF recombinant human mAb, combined with capecitabine and paclitaxel chemotherapy in subjects with triple-negative, metastatic, or locally advanced breast cancer. In 77% of patients, the therapy showed an objective response rate, with complete response in 19%, and the median progression-free survival was 7.6 months (376). Several studies also demonstrated that MDSCs possess secondary pro-angiogenic mechanisms involving MMPs, as discussed in Vetsika et al.' review (162).

Because MDSCs are recruited to the TME by tumor cells, blocking their migration using a CCR5 antagonist and CCL2 inhibitors seems to be a promising therapeutic approach. Accumulation of $CCR5^+$ MDSCs with high suppressive activity, associated with increased concentrations of CCR5 ligands and tumor progression, has been shown in a tumor-bearing melanoma study model (377). The upregulation of $CCR5^+$ on $CD11b^+Gr1^+$ myeloid cells was induced *in vitro* by CCR5 ligands and other inflammatory factors. Blocking the CCR5/CCR5 ligand interaction improved survival by reducing the migration and the immunosuppressive functions of MDSCs in melanoma lesions of tumor-bearing mice (377). This strategy can also enhance the suppression of MDSCs by NK cells, as shown in a premetastatic lung animal model (378). CCL2 expression in MDSCs has been elucidated in a lung tumor model, and anti-CCL2 treatment can decrease peripheral and intratumoral PMN-MDSCs and M-MDSCs by inhibiting the ARG1 expression and iNOS (379). The same study showed enhancement in $CD4^+$ and $CD8^+$ T cell infiltration and production of IFN-γ in the TME. As a result, anti-CCL2 therapy increased the survival time of tumor-bearing mice. Anti-CCL2 therapy could be a potential approach to improve NK cell activity and the efficacy of ICI immunotherapy.

Some studies have shown that COX-2 inhibitors (celecoxib or nimesulide) reduced the expansion of MDSC subtypes and decreased cancer progression (380–382). COX-2 inhibitors also stimulate NKG2D ligand expression on tumor cells, enhancing NK cell-mediated cytotoxicity (383) and reducing angiogenic pathways via VEGF (384). A phase II clinical trial showed that perioperative treatment with a COX-2 inhibitor (etodolac) in combination with a β-adrenergic antagonist (propranolol) reduced circulating $CD14^+$ monocytes and improved NK cell activation (385). Thus, this study supports the rationale for targeting MDSCs in the perioperative period to enhance clinical outcomes. A decrease in MDSC COX-2 and PEG2 activity and NO and ROS production has been shown in animals and humans after Vitamin D3 or E treatments (386). MDSCs impair NK cell function through NO production, and clinical trials are evaluating novel therapies to block this mechanism (228). A clinical trial using celecoxib, in combination with nivolumab and ipilimumab, is currently recruiting for the treatment of colon carcinoma (NCT03026140). A colorectal cancer phase III trial combining vitamin D3 with standard chemotherapy and bevacizumab is also ongoing (NCT04094688). Vitamin E supplementation decreased PGE2 production by inhibiting COX-2 activity, resulting from decreased NO production, in mice and humans (386). It also improved the activation of T and NK cells, increased lymphocyte proliferation, and modulated DC function (386).

ROS molecules are involved in many pathways, thereby controlling a wide range of biological events, such as immunosuppression in the TME (387). Following pathogenic and inflammatory immune responses, MDSCs release ROS (387). Although ROS has toxic effects on most cells, MDSCs survive despite the elevated levels and constant production of ROS in the TME (388). The production of ROS by MDSCs is upregulated in many murine tumor models and human cancers (389, 390) and plays a crucial role in maintaining MDSCs in an undifferentiated state (387). ROS production is regulated by the nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Synthetic triterpenoid C-28 methyl ester of 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid (CDDO-Me; bardoxolone methyl) in an MC38 colon carcinoma model has been shown to reduce MDSC ROS production via Nrf2 activation (307) and IL-17D production (391). A phase I clinical trial showed encouraging results using this therapy (392). Forcing IL-17D production using Nrf2 agonists can enhance NK cell activation and recruitment, leading to tumor regression (391, 393).

The pathological intratumoral accumulation of $CXCR2^+$ PMN-MDSCs impairs the functions of NK cells in mice bearing oral carcinoma tumors by secreting TGF-β, NO, and ROS (394).

CXCR1/2 inhibitors (SX-682) significantly abrogated MDSCs trafficking within a tumor and improved tumor infiltration, activation, and therapeutic efficacy of adoptively transferred murine NK cells in the HNSCC preclinical model (394), and in combination with anti-PD-1, they improved ICI therapy (395).

HIF-1α, released in the TME from tumor cells, is another protein that has a crucial role in MDSC differentiation toward TAMs (178, 396). Some progress has been achieved targeting hypoxia using hypoxia-activated prodrugs (TH-302) (**Figure 3**), hypoxia-modulator drugs (modulating expression, DNA-binding, transcriptional activity, and degradation of HIF proteins), drugs directly modulating HIF mRNA (antisense oligonucleotides), and inhibiting pathways involved in the control of HIF-1α mRNA (397). Combination therapy of TH-302 with gemcitabine in a phase II clinical trial for advanced or metastatic pancreatic cancer (NCT01144455) (398) or with doxorubicin in a phase III trial for advanced soft tissue sarcoma (NCT01440088) (399) showed encouraging results. The combination of TH-302 therapy with anti-CTLA-4 and PD-1 cured more than 80% of tumors in a mouse prostate cancer model by reducing MDSCs and granulocytic subsets and driving T cell migration into the hypoxic tumor sites (400). Innovative combinatorial approaches, such as drugs targeting hypoxia used with current cancer therapies, including immunotherapy and MDSCs targeting, may improve antitumor response efficacy.

IL-18 and IL-33 are involved in $CD11b^-$ BM progenitor cell differentiation to M-MDSCs (192) and PMN-MDSCs (401), respectively. Targeting of $CD11b^-$ BM treated with anti-IL-18 or IL-33 has been shown to enhance T cell proliferation and IFN-γ secretion (192, 401). Other MDSC molecules targeting aminoacyl tRNA synthetase complex interacting multifunctional protein 1, TLR agonists, tumor-derived exosome-associated HSP72, and inflammasome component NLR family pyrin domain containing 3 have also been shown to contribute to MDSC differentiation and have been reviewed previously (402). MDSCs can be blocked by combining ICIs (anti-PD-1, PD-L1, and CTLA-4) with anti-IL-18 (192), inhibitors of FATP2 (339), long non-coding RNA Pvt1 (403), downregulation of the pseudogene Olfr29-ps1 (404), or deletion of the nuclear factor 1 A gene (405).

PRE-ACTIVATED NK CELL PRODUCTS FOR CANCER IMMUNOTHERAPY

Cancer immunotherapy strategies have focused on T-cell-based immunotherapy using expanded tumor-specific $CD8^+$ CTLs from tumor-infiltrating lymphocytes (TILs) (406). Adoptive transfer of TILs following lymph-depleting strategies have shown promising effectiveness in metastatic melanoma studies (407–409), but short-lived responses (410) and side effects, such as vitiligo, uveitis, and retinitis, have been reported (407, 411).

Pre-clinically and clinically, studies have identified several cytokines and other novel soluble factors that increase NK cell numbers, function, and persistence. The two most common strategies are pretreating NK cells with cytokines (before the adoptive transfer) or *in vivo* cytokine administration. IL-2 (412), IL-15 (413), IL-12 (414, 415), IL-18 (416, 417), IL-21, their combinations, and administrations with other immunotherapeutic agents have all been described in the context of regulating NK cell function, maturation, survival, and improving activation and cytotoxicity (**Figure 3**). Results are expected from clinical trials evaluating the safety and efficacy of combining IL-21 with other immunotherapeutic mediators: IL-21/anti-PD-1 against solid tumors (NCT01629758) and IL-21/ipilimumab against melanoma (NCT01489059). Cytokine analogs of IL-15, such as IL-15 superagonist ALT-803 (NCT03228667, NCT03127098, NCT03022825, NCT02384954, NCT02138734, NCT02890758, NCT02559674, and NCT03520686) and NKTR-255, are under investigation. Furthermore, multiple ongoing clinical trials are evaluating the safety and efficacy of several other immune cytokines alone or in combination with other therapeutic strategies, such as immune checkpoint inhibitors (NCT03209869, NCT03386721, NCT02627274, and NCT02350673) (**Figure 3**).

Combination therapies of PD-1 blockade and IL-15 stimulation and also IL-15 and IL-15RaFc have been reported as safe in mouse models (418) and patients with NSCLC (419). IL-15 stimulation increases the expression of the activating receptors CD16 and NKG2D on NK cells and increases the activation, proliferation, cytotoxic activity, and survival of NK cells and CTLs (420). One of our preclinical studies found that the combination of PD-1 blockade and IL-15 signaling resulted in eradication of transplanted lung adenocarcinoma (LUAD) cells in about one-half of treated LUAD-SIS-PDX mice, while the other half presented with a partial response. Notably, IL-15 alone, without PD-1 blockade, significantly reduced tumor burden in all treated LUAD-SIS-PDX animals (418). PD-1 blockade alone transiently prevented tumor growth, but tumors grew at a similar rate to untreated control tumors after 2 weeks. The addition of IL-15 to PD-1 blockade completely abrogated tumor escape from ICI, resulting in a powerful additive therapeutic effect capable of tumor eradication. These findings support a key role for adjuvant IL-15 treatment to induce an immune cell-mediated tumor attack, which can prevent tumor escape from checkpoint blockade therapy, as shown using our novel LUAD-SIS-PDX model (418).

In addition to cytokine stimulation, mAb can be used to block the activity of NK cell inhibitory receptors. The ligation of inhibitory KIRs by HLA molecules triggers NK cell inhibition (64), and anti-KIR antibodies are under investigation to improve NK cell cytotoxicity in cancer (421). Because the expression of NK cell-expressed inhibitory KIRs and PD-1 correlate in patients with solid tumors (e.g., NSCLC), combining anti-KIR antibodies with anti-PD-1 treatments to avoid immune escape of tumors in these patients may be an effective treatment strategy (422). Several clinical trials are currently evaluating anti-KIR antibodies against solid tumors combined with other immune treatments (NCT03341936, NCT03203876, and NCT03347123).

NK CELL SOURCES

NK cells do not need prior activation to target tumor cells. The fine-tuning of NK cell functions occurs during their

maturation and instills a form of tolerance. NK cells are "educated" to recognize healthy MHC-I-expressing cells with KIR receptors, resulting in inhibitory signals and preventing NK cell activation. In addition to KIRs, NK cells also express two other types of inhibitory receptors (423): leukocyte immunoglobulin-like receptors (LILRs) (424) and C-type lectin receptors (NKG2A/CD94). The balance of inhibitory and activating signals expressed on target cells mediate NK cell activation and response (425–427).

NK cells can kill tumor target cells through a variety of mechanisms, as discussed previously in this review. Since NK cells represent an important defense against tumors, NK cell infusion products have been evaluated as a possible cancer immunotherapy. Different NK cell sources have been tested in patients with tumors combined with chemotherapy (428) and, allogeneic NK cells have been selected in many studies for their increased alloreactivity, achieved by mismatching of inhibitory KIRs and tumor HLA (429) (**Figure 3**). This is referred to as a haploidentical or half-matched setting. Haploidentical NK cells are more reactive against recipient tumor cells because of reduced KIR-mediated inhibition (429). NK cells for adoptive transfers can be obtained from five different sources: autologous NK cells, allogeneic NK cells (430), umbilical cord-derived NK cells, NK cell lines, and embryonic stem cell-derived/induced pluripotent stem cell (ES/iPSC)-derived NK cells (431) (**Figure 3**), allowing for a multitude of choices to match or mismatch KIR-HLA receptor pairings.

CHIMERIC ANTIGEN RECEPTOR–NK CELLS FOR CANCER IMMUNOTHERAPY

One currently promising strategy is redirecting NK cells with chimeric antigen receptors (CARs) (**Figure 3**). Autologous CAR-T cell successes in patients with leukemia and lymphoma (432) have raised considerable interest in using immune cells as a cancer treatment. The advantage of a CAR strategy is that one CAR can be applied for many tumor types expressing the matching ligands. Modification with CAR is also proposed for reprogramming NK cells to improve their cytotoxicity. CAR-NK cells represent an exciting approach for cancer immunotherapy. NK cells can be targeted with CARs against surface molecules expressed by tumor cells and might avoid some of limitations or side effects of CAR-T cells. While conventional T cells are HLA-restricted, CAR-T cells are designed to recognize their target antigens independent of HLA expression and deliver their costimulatory signal. However, CAR-T cells are expensive and labor-intensive to generate (433), and the application of CAR-T cell therapy is often limited by intrinsic risks, such as graft-vs.-host disease (GvHD) (434). Also, off-target effects, cytokine release syndrome, and other side effects restrict their clinical applications. CAR-T cell therapy has been successful in treating blood cancers. However, a significant obstacle for the treatment of solid tumors is an extremely immunosuppressive TME that decreases the ability of immune cells to infiltrate tumors (435). CAR-T cell functions are often suppressed in solid tumors due to T cell expression of PD-1 and PD-L1 expression in the TME. These data may explain why the use of CAR-T cells has not been as useful for treating solid tumors as it has been to treat hematological malignancies (436). However, PD-1 levels expressed by NK cells are substantially lower, making NK cells good candidates for eradicating solid tumors (437).

Allogeneic NK cell transplantation rarely induces GvHD (438) and has the potential to become "off-the-shelf" products, making CAR-NK cell therapies a possible widespread product (433). Large-scale culture and genetic modification of allogenic human NK cells are feasible and could readily be used to treat a broad range of cancer patients. NK cell-expressed CARs typically include a single-chain variable fragment from a mAb, a transmembrane hinge region, and a costimulatory signaling domain, such as CD28, CD3-zeta, 4-1BB (CD137), or 2B4 (CD244) heterodimers (439, 440). These main signaling domains frequently have been derived from TCR moieties (441). Four generations of CARs are currently under development (442). The first generation of CARs usually contains only the CD3ζ activation signaling domain (443). In the second and the third generations of CARs, costimulatory molecules like CD28, 4-1BB, and CD134 are also included to increase NK cell activation (444). The fourth generation of CARs is engineered to secrete transgenic cytokine-like IL-12, which should help to remodel the tumor environment to promote therapeutic success (442, 445). Once CAR-modified NK cells recognize their specific targets, such as $CD19^-$, $CD20^-$, or $CD138^-$ cells, the CAR receptors trigger an intracellular signaling cascade that activates CAR-NK cells to kill the antigen-expressing target cell.

Methods to generate CAR-NK cells that target solid tumors include lentiviral transduction or electroporation of the NK-92 NK-like cell line (438), primary NK cells, and the differentiation of NK cells from modified pluripotent stem cells (431). NK cells express NKG2D, an activating receptor triggered by MICA/B and UL binding protein (ULBPs) expressed on the surface of stressed cells upon DNA damage, hypoxia, or viral infection (446). NKG2D ligands are often overexpressed on solid tumors and tumor-infiltrating cells like MDSCs (447). However, the NKG2D cytotoxic adapter molecule, DNAX-activation protein 10 (DAP10), is downregulated by suppressive molecules like TGF-β, which is abundantly expressed in the TME (448). NK cells and CAR-NK cells expressing the native NKG2D receptors are thus downmodulated in the TME due to the reduction in DAP10 expression. To overcome the repressive effects of the solid TME on NKG2D functions, one group (449) established a gene-modified NK cell bearing a chimeric receptor in which the activating receptor NKG2D is fused to the cytotoxic ζ-chain of the T-cell receptor (NKG2D.ζ) (450). This specific CAR has been designed to target MDSCs in the TME of solid tumors, which are refractory to other types of immunotherapy. The NKG2D.ζ-NK cells are cytotoxic against MDSCs, but unmodified NK cells are not. They also showed that NKG2D.ζ-NK cells generated from patients with neuroblastoma successfully killed autologous MDSCs in the TME, which were capable of suppressing CAR-T functions (449). CAR-NK cells have been established and engineered against several antigens for solid tumors, including epidermal growth factor receptor, human epidermal growth factor receptor-2 (HER2), egeria,

disialoganglioside, epithelial cell adhesion molecule, mesothelin, and tyrosine-protein kinase transmembrane receptor ROR1, with promising results in preclinical or clinical studies (39). In a recently published phase I/II clinical trial, HLA-mismatched anti-CD19 CAR-NK cells were administered to 11 patients with relapsed or refractory CD19-positive cancers. The majority of treated patients showed a response to treatment with anti-CD19 CAR-NK cells without developing significant toxic side effects (451). The phase I/II trial was approved for B-cell lymphoma in 2017 (NCT03056339). In another study, NK cells derived from umbilical cord blood were transfected with a CAR containing inducible caspase 9/IL-15 (iC9/CAR.19/IL-15) (452). They produce IL-15, which supports CAR-NK cell survival, and are engineered to express the inducible suicide gene caspase 9 for their pharmacologically induced elimination. Liu and coworkers showed efficient iC9/CAR.19/IL-15 cell killing of CD19-expressing tumor cell lines *in vitro* and improved clinical outcome in a xenograft Raji lymphoma murine model (452). In the clinical trial, iC9/CAR.19/IL-15 cells were used to treat patients with relapsed/refractory $CD19^+$ B lymphoid malignancies. This cell therapy was applied with high-dose chemotherapy (NCT03579927).

An alternative approach for the generation of CAR-NK cells is the use of iPSC as a platform to generate CAR-NK cells (453) (**Figure 3**). In 2018, a NK cell line was established from human iPSCs that expresses a CAR containing the transmembrane domain of NKG2D, the 2B4 costimulatory domain, and the CD3ζ signaling domain able to mediate strong antigen-specific NK cell signaling (iPSC-CAR-NK cells) (453). The combination of NKG2D-2B4ζ in this CAR construct conferred strong upregulation and activation of phospholipase C gamma, Syk-vav1-Erk, and NF-κB pathways, improving iPSC-CAR-NK cell activation, proliferation, and antitumor activity. This cell line maintains the same NK cell phenotype as wild-type cells but enhanced antitumor activity compared to CAR-T cells, iPSC-NK cells, or non-CAR PB-NK cells. In an ovarian cancer xenograft model, iPSC-CAR-NK cells significantly inhibited tumor growth and improved survival in mice and showed less toxicity than CAR-T cells (453).

A pilot study investigated the use of NKG2D ligand targeted CAR-NK cells in patients with metastatic colorectal cancer and evaluated the safety and feasibility of CAR-NK cell treatment against solid metastatic tumors (454). For these studies, autologous or allogeneic NK cells were transfected by mRNA electroporation to generate CAR-NK cells with transiently enhanced specificity and activity against NKG2D ligand-expressing cancer cells. This approach has also been used in a clinical trial involving solid metastatic tumors (NCT03415100).

About 200 clinical trials are currently using CAR-T cells, and only 20 clinical trials so far are utilizing CAR-NK cells (http://www.clinicaltrials.gov). For these trials, CAR-NK cell products are derived from either primary NK cells or NK cell lines. Only five clinical trials were conducted to evaluate the safety of CAR-NK-92 infusion products, while 15 trials evaluated CAR-NK cells from other sources. CD7 (NCT02742727), CD19 (NCT02892695), and CD33 (NCT02944162) targeted blood cancer (455); HER2 targeted glioblastoma (NCT03383978); co-stimulating conversion receptors targeted NSCLC (NCT03656705); and MUC1 targeted multiple refractory solid tumors, including hepatocellular carcinoma, NSCLC, pancreatic tumors, and triple-negative metastatic breast tumors (NCT02839954) (455).

Additional preclinical or clinical trials targeting $CD19^-$ (NCT03690310) or $CD22^-$ expressing (NCT03692767) cells are ongoing, and bivalent CD19/22 (NCT03824964) CAR-NK cell products are also being examined in patients with relapsed and refractory B cell lymphoma (456). One clinical trial targeting CD19-positive cells is testing CD19-CAR-NK cell infusions in a pediatric setting (NCT00995137), using irradiated K562-mb15-41BBL expanded PB-derived NK cells transfected with CD19-41BBz-CAR (455).

Roundabout homolog 1 (ROBO1), a member of the axon guidance receptor family (Robo1–4), is a potential target for immunotherapy (457). ROBO1 modulates the chemotaxis of T cells and tumor angiogenesis to counteract the tumor growth (458–460). Some solid tumors, such as pancreatic cancer, have increased expression of ROBO1 (461). Three clinical trials are currently studying CAR-NK cells directed against ROBO1, using CAR-NK (NCT03940820), bi-chimeric antigen receptor-NK cell BiCAR-NK (NCT03941457), and bi-chimeric antigen receptor-NK cell or T cell BiCAR-NK/T (NCT03931720) cells on patients with solid tumors (456).

Additional strategies for optimizing NK cell-mediated cytotoxicity have been employed. ADCC is a potent mechanism of cytotoxicity used by NK cells. An increasing number of mAbs are currently being examined in preclinical and clinical studies for their ability to improve antitumor ADCC (462). Limiting shedding of CD16 from NK cell membranes also enhances ADCC, and iPSC-NK cells modified to overexpress a non-cleavable version of CD16 showed improved ADCC *in vitro* and *in vivo* in a human B-cell lymphoma model (463). iPSC can also be modified to improve *in vivo* persistence of iPSC-NK cells. Furthermore, the deletion of cytokine-inducible SH2-containing protein (CISH) in iPSC differentiated NK cells improved persistence and enhanced antitumor activity in a leukemia xenograft model (464). Other relevant strategies to strengthen NK killing potential use antibody therapy to target NK cell checkpoints that inhibit NK cell activity in the TME, such as PD-1 (465), T cell immunoreceptor with Ig and ITIM domains (466), and single Ig IL-1-related receptor (467). These findings provide some flexibility in the combining of therapeutic approaches. NK cells can be modified to express CARs and/or be used in combination with ICI or additional antibody immunotherapy. While significantly more work is needed to optimize these approaches, these data nevertheless demonstrate the potential for "off-the-shelf" NK cell platforms for treating solid tumors and hematological malignancies. Further research needs to be done to identify and understand possible CAR-NK exhaustion mechanisms after transplantation in preclinical and clinical studies.

CONCLUSION

The TME plays a critical role in regulating NK cell antitumor functions and regulating NK cell trafficking, persistence, proliferation, and activation. MDSCs are one of the critical

players inducing and regulating the immunosuppressive environment of the TME. MDSCs interact with numerous innate immune cells, modulating their functions and suppressing strong tumor-specific immunity. As such, MDSCs represent an essential target in oncology. While NK cell-MDSC interactions have been investigated, few studies have evaluated how NK cell cytotoxicity can be exploited to attack both the immunosuppressive TME as well as the tumor. We have provided an updated review of several current prospective therapies for targeting the MDSC–Treg axis and improving antitumor NK cell functions (several mechanisms are summarized in **Figure 3**). These pathways have substantial implications for the tumor and are currently under investigation. The combinatorial treatments that target these pathways have been showing promising results. More work is needed to fully exploit NK cell functions to eradicate hematological malignancies and solid tumors.

AUTHOR CONTRIBUTIONS

CZ and SP conceptualized the content and wrote the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Characterization and Manipulation of the Crosstalk Between Dendritic and Natural Killer Cells Within the Tumor Microenvironment

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Cellular therapy has entered the daily clinical life with the approval of CAR T cell therapeutics and dendritic cell (DCs) vaccines in the US and the EU. In addition, numerous other adoptive cellular products, including natural killer (NK) cells, are currently evaluated in early phase I/ II clinical trials for the treatment of cancer patients. Despite these promising accomplishments, various challenges remain to be mastered in order to ensure sustained therapeutic success. These include the identification of strategies by which tumor cells escape the immune system or establish an immunosuppressive tumor microenvironment (TME). As part of the innate immune system, DCs and NK cells are both present within the TME of various tumor entities. While NK cells are well known for their intrinsic anti-tumor activity by their cytotoxicity capacities and the secretion of pro-inflammatory cytokines, the role of DCs within the TME is a double-edged sword as different DC subsets have been described with either tumor-promoting or -inhibiting characteristics. In this review, we will discuss recent findings on the interaction of DCs and NK cells under physiological conditions and within the TME. One focus is the crosstalk of various DC subsets with NK cells and their impact on the progression or inhibition of tumor growth. In addition, we will provide suggestions to overcome the immunosuppressive outcome of the interaction of DCs and NK cells within the TME.

Keywords: NK cells, dendritic cells, DC-NK cell interaction, tumor microenvironment, cellular therapies

INTRODUCTION

Tumor surveillance is achieved by a complex interplay of the components of the innate and adaptive immune system. Here, we summarize our current knowledge on the role and interaction of dendritic cells (DCs) and natural killer (NK) cells in the tumor microenvironment (TME). While circulating NK cells are efficient at identifying and eliminating tumor cells, DCs bridge the innate and adaptive

immune system via the uptake of tumor cell debris and the subsequent presentation of tumor-specific antigens to T cells (1). NK cells and DCs are currently used for immunotherapies to treat tumor patients, for NK cells exhibit the ability to directly eliminate tumor cells without prior sensitization, while DCs are able to initiate an immune response by presenting antigens and inducing tumor-antigen specific $CD8^+$ T cell (2). Moreover, when DCs and NK cells encounter each other, they are able to promote each other's activation, maturation, and functional activity (3). Within the TME, conventional type 1 DCs (cDC1s) show a bidirectional crosstalk with NK cells, increasing the selective recruitment of cDC1 together with their differentiation, and maturation as well as NK cell activation. This allows a better tumor control, patient survival and improved therapeutic responses (4, 5). However, it is known that tumor cells develop highly efficient mechanisms to impair the functionality of NK cells and DCs to escape immune surveillance in order to ensure their own survival. The tumor establishes an immune suppressive environment by the secretion of immune suppressive cytokines and chemokines, by metabolic acidification (6), by the recruitment of immune-regulatory cells, such as regulatory T cells (T regs) and myeloid-derived suppressor cells (MDSCs), by affecting the polarization of macrophages as well as by up-regulation of immunosuppressive proteins including ligands for immune checkpoint (ICP) receptors, such as PD-L1. The latter have the ability to suppress the anti-tumor activity of DCs and NK cells as well as their ability to promote each other. Moreover, DCs may even switch from a tumor-inhibiting to a tumor-promoting subtype within the TME (7). In order to overcome the suppressive effect of the TME on NK cells and DCs, the bidirectional crosstalk of NK cells and DC1 plays a very important role in the coordination of immune responses against cancer. That is why the presence of both DC1s and NK cells within the TME is primordial, and their interaction represents a potential target to improve the efficacy of cancer immunotherapy (2).

In this review, we will first give a short overview about the biology of DCs and NK cells. Next, we will describe their interaction under physiological conditions and within the TME. Finally, we will discuss possibilities to overcome the suppressive impact of the TME and to restore efficient tumor immune surveillance.

NK CELL BIOLOGY

As part of the innate immune system, NK cells have the ability to lyse virally infected and malignant cells. They play an important role at eradicating cancer cells and in shaping the response of adaptive immune cells. NK cells develop and differentiate in the bone marrow and represent approximately 10% of the lymphocytes in the peripheral blood (PB). NK cells express CD56 on their surface and lack CD3 expression ($CD56^+CD3^-$). Human NK cells are separated into two subsets, with an immune regulatory $CD56^{bright}CD16^-$ population harboring potent cytokine production capability and a highly cytotoxic $CD56^{dim}CD16^+$ population, representing the major NK cell population within the peripheral blood (90%). $CD56^{dim}CD16^+$ express the Fc gamma receptor III CD16 recognizing the Fc portion of antigen-bound antibodies allowing them to lyse the opsonized cell, a mechanism called antibody dependent cell-mediated cytotoxicity (ADCC) (8).

In contrast to T or B cells, NK cells recognize their target cells in an antigen-independent manner regulated by a variety of activating and inhibiting receptors. NK cell cytotoxicity is regulated by balanced signals of these activating and inhibitory receptors (9, 10).

Major histocompatibility (MHC) class I molecules, expressed on all healthy nucleated cells, act as ligands for the killer-immunoglobulin-like receptor-group (KIR) or natural killer group 2A (NKG2A), which are mainly responsible for providing NK cell inhibiting signals. These mechanisms ensure that NK cells become selectively activated by cells that lack MHC-I-expression, such as tumor cells, but tolerate healthy tissue in their immune response, the so called "missing-self"-theory. If MHC-I expression is absent, the inhibitory receptor signal is missing, and NK cells can be activated (11). Usually MHC-I-expression is reduced or completely down-regulated on virally infected or malignantly transformed cells as an escape mechanism from cytotoxic T cell recognition, which on the other hand renders them sensitive to NK cell killing (8, 12).

The most important signals for activation of NK cells are mediated by the family of the natural cytotoxicity receptors (NCRs), the C-type lectin-like receptor NKG2D and also certain KIR-subtypes. NKp30, NKp44 and NKp46 that belong to the group of NCRs are expressed on all activated NK cells and bind non-HLA-specific ligands like virus-derived molecules (HA/HN for NKp46, NKp44) or intracellular proteins (BAT3/BAG6 for NKp30), which are released following cell stress or transformation (13). Furthermore, MICA-, MICB- and ULPB (or RAETI)-encoded proteins bind to the NKG2D-receptor with their MHC-I-like extracellular domain. Those encoded proteins belong to a group of at least 100 different ligands which are selectively expressed on non-healthy cells for the induction of NK cell activation (14).

NK cell killing includes a variety of mechanisms. Upon engagement an immunological synapse is established between NK cells and their target cells. Subsequently, they release the content of their cytotoxic granules, including perforin and granzyme b, into the synaptic gap. While perforin penetrates the target cell's membrane, granzyme b gets inside the cell and induce programmed cell death (2, 8). Moreover, NK cells are able to induce apoptotic cell death via expression of death receptor ligands like FasL and TNF-related apoptosis-inducing ligand (TRAIL) on their surface (8, 12).

DC BIOLOGY

DCs are important regulators of immune responses (15). They are sentinels of the immune system and settle all lymphoid as well as peripheral tissues. Here, they constantly take up antigens from the surrounding and process these antigens. After migration to the local draining lymph node, they present these processed antigens as peptide MHC complexes to T cells. Depending on the environment during encounter of the

antigen, DCs either induce peripheral tolerance (steady state) or T cell immunity (inflammation) (16–18). In steady state, DCs induce peripheral tolerance by presenting antigens without costimulatory molecules leading to anergy or the deletion of antigen-specific T cells. However, in inflammatory settings and instructed by microbiota-derived cues resulting in tonic IFNAR signaling DCs can be activated by danger signals or pathogens via pattern recognition receptors such as Toll-like receptors (TLR) or C-type lectin receptors (CLR) (1, 19, 20). This leads to enhanced expression of peptide MHC complexes as well as costimulatory molecules (e.g. CD40, CD86) and the secretion of T cell polarizing cytokines such as IL-12 (17). Thereby, DCs are capable to induce and direct T cell responses. However, DCs are also able to interact with cells of the innate immune system such as NK cells (4, 21–24).

As all immune cells, except for yolk sac-derived macrophages, including Langerhans cells, DCs originate from hematopoietic stem cells (HSC) in the bone marrow (25). After differentiation of HSCs into common myeloid progenitor (MPP) cells via the steps of multipotent progenitor (MPP) and lymphoid-primed multipotent progenitor (LMPP) cells, the common DC progenitor (CDP) separates from the common monocyte progenitor (cMoP). The CDP can give rise to all DC subsets including plasmacytoid (pDC) as well as conventional DCs (cDC). Depending on the expression of specific transcription factors such as IRF8 and IRF4, cDCs further differentiate in cDC1 and cDC2, respectively (18). In contrast to cDCs, pDCs can also arise from a common lymphoid progenitor (CLP). Terminal-differentiated as well as preDCs are able to leave the bone marrow and settle all lymphoid as well as peripheral tissues.

Based on ontogeny, surface receptor expression as well as functions, DCs are subdivided into different subsets, which are mainly conserved between human and mice (17, 18, 25–27). pDCs share high transcriptional similarities between humans and mice and are identified by the expression of Siglec-H, CD45R (B220), and CD317 (PDCA-1) in mice, and by CD303a (BDCA-2), CD304 (BDCA-4) as well as CD123 (IL-3Rα chain) in humans. In both species, they share the expression of TLR7 and TLR9 and strongly react to viral infections with the secretion of vast amounts of type I IFN. However, their true role in T cell stimulation is a matter of ongoing discussions (28–31).

cDCs can be further separated into cDC1 and cDC2 in both, mice, and humans. Human and murine cDC1 share the specific expression of CLEC9A, XCR1, and CADM1 (17, 32–36). While murine cDC1 further express CD8α in lymphoid and CD103 in non-lymphoid tissues, human cDC1 are identified by a high expression of CD141 (BDCA-3) (1). In both species, cDC1 highly express TLR3 and respond to stimulation of TLR3 with production of type III IFN (37). They further produce IL-12, which is crucial for interaction with T cells as well as with NK cells. In mice, cDC1 are the only cross-presenting DC subpopulation for the activation of cytotoxic $CD8^+$ T cells. This highlights their vital role in the immune response in the context of tumors as well as viral infections (4, 38–40). Furthermore, they polarize $CD4^+$ T cells preferentially into Th1 cells. However, recent studies on human DCs suggest that all DC subpopulations are able to cross-present antigens and cDC1 excel only in the cross-presentation of necrotic cell-derived antigens, presumably due to the expression of the F-actin receptor CLEC9A (41–44).

Human and murine cDC2 transcriptionally rely on IRF4 and share the expression of CD11c and SIRPα (18). Human cDC2 can be identified by the expression of CD1c (BDCA-1), CD301 (CLEC10A) as well as the FcεR1α, while murine cDC2 express CD11b as well as CD4 and DCIR2 in lymphoid tissues (27, 45, 46). In general, cDC2 are a more heterogeneous DC subpopulation than cDC1. Recent approaches, using single cell transcriptomics, showed that both human and murine cDC2 harbor different subpopulations termed DC2/DC3 and cDC2a/b, respectively (25, 31, 46–49). In the murine system, cDC2 have a lower capacity to cross-present antigens to $CD8^+$ T cells, however they are efficient to polarize naïve T cells into Th2 and Th17 cells (50–52). In contrast, in comparison to human cDC1, human cDC2 have been suggested to harbor similar capacity to cross-present antigens to $CD8^+$ T cells. Furthermore, they have been shown to polarize $CD4^+$ T cells into Th1 cells, which can be linked to the ability of human cDC2 to secrete IL-12p70 under inflammatory conditions (27). Both, cDC1 and cDC2 have a detrimental role in the preservation of peripheral tolerance as murine cDC1 convert naïve $CD4^+$ T cells into $FoxP3^+$ T reg cells, whereas cDC2 expand already existing $FoxP3^+$ T reg cells in the periphery (53).

NK-DC INTERACTION UNDER PHYSIOLOGICAL CONDITIONS

The first key report on the interaction between DCs and NK cells to control tumor growth was published in 1999, describing the coordinated control of mesothelioma tumors in mice by both subsets (54). Since then, several groups have explored their complex relationship revealing a bidirectional crosstalk between DCs and NK cells. Human NK cells are able to induce DC maturation and IL-12 production upon co-culture with immature monocyte-derived DCs (moDCs), which was suggested to be contact-dependent and relies on the secretion of NK cell-derived cytokines like TNF-α (55, 56). In contrast, freshly isolated human NK cells are activated by lipopolysaccharide (LPS)-treated mature moDCs (55). NK cell activation by moDCs was shown to be mediated mainly by cytokines of the IL-12 family. Initial reports demonstrated that IL-12 secretion by LPS-matured human moDCs stimulates IFN-γ production in NK cells (57), and similar results have been reported for other members of this cytokine family. Further, IL-23 has been suggested to directly stimulate IFN-γ production in human $CD56^{bright}$ NK cells, and in cooperation with IL-18, in $CD56^{bright}$ and $CD56^{dim}$ NK cells (58). Additionally, LPS-matured moDC-derived IL-27 increased IFN-γ production and upregulation of NKp46- and ADCC-dependent NK cell cytotoxicity (59). The importance of DC-derived IL-12 for NK cell activation has been illustrated within an *in vivo* model of mouse cytomegalovirus (MCMV) infection. Upon MCMV

infection cDCs secrete IL-12p40 and IL-15 in a TLR9/MyD88-dependent manner leading to NK cell activation and IFN-γ production which ultimately results in MCMV clearance (60).

Moreover, other cytokines such as IL-15 or IFN-α were able to increase NK cell proliferation and survival as well as cytotoxicity, respectively (21, 61–64). Both cytokines are involved within the NK cell-mediated rejection of $CD8^+$ T cell-resistant tumors within various tumor mouse models upon treatment with the STING agonist cyclic dinucleotide (CDN). CDN activated NK cells directly via type I IFN signaling and indirectly via IL-15Rα up-regulation in DCs, which also depended on type I IFN (65). Moreover, co-incubation of human peripheral blood NK cells with autologous lung DCs from smokers suffering from chronic obstructive pulmonary disease (COPD) resulted in an IL-15-dependent increased killing of epithelial lungs cells compared to DCs from smokers without COPD. Similar results were obtained when using lung DCs from mice who were exposed to cigarette smoke compared to pure air exposed ones (66).

In addition, activated NK cells were reported to specifically kill immature moDCs (56) via NKp30 (67) and DNAM-1 (68). This process was executed mainly by $NKG2A^+KIR^-$ NK cells. This observation can be explained due to the week expression of the inhibitory NKG2A receptor ligand HLA-E on immature moDCs compared to LPS-matured moDCs, whereas HLA class I molecules, the ligands for the inhibitory KIRs, are expressed on both DCs populations (69). Similar, NK cells have been shown to interfere with the induction of Th2-biased T cell responses by killing Th2 immune response inducing moDCs in a NKp30 and DNAM-1 dependent manner (70).

Interestingly, apart from its involvement in the killing of immature moDCs, engagement of NKp30 resulted in increased secretion of TNF-α and IFN-γ by NK cells, which were able to induce moDC maturation (71). Recently a study revealed that the interaction between $CD155^+$ DCs and laquinimod-activated NK cells, which up regulated their DNAM-1 expression (recognizing CD155), led to suppression of experimental autoimmune encephalomyelitis (EAE). Interestingly, the immunoregulatory effect was not due to increased NK cell cytotoxicity against DCs, but rather due to induced reduction of HLA class II expression on DCs (72). Moreover, another group highlighted the role of the NK cell receptor NKG2D and its ligands during NK-DC interaction. They demonstrated that upon footpad injection of ectromelia virus, virus-infected murine skin-derived migratory DCs up regulated NKG2D ligands on their surface and migrated to the draining lymph nodes (dLN), where they stimulated IFNγ production by NK cells. IFNγ secretion then stimulated CXCL9 production in inflammatory monocytes resulting in an increased recruitment of circulating $CXCR3^+$ NK cells to the dLN (73).

Interaction of NK cells and DCs was also suggested to control tissue-specific autoimmunity through an innate IFN-γ–IL-27 axis resulting in the generation if IL-10-producing Tr1-like cells (74) and to promote the tumor surveillance of other immune cells, a process described as the NK cell helper function. Activated human NK cells play a role in the induction of cDC1, which are producers of high amounts of IL-12p70. In this process, $CD4^+$ T cells are primed to produce increased amounts of IFN-γ and less IL-4 favoring the induction of antigen-specific $CD8^+$ T cells (75). Furthermore, mouse NK cells, recruited via CXCR3 into local lymph nodes, provide an early IFN-γ source to induce a Th1 immune response (76). In accordance with this report, only stimuli associated with the Th1 response are able to induce IFN-γ production in mouse NK cells, but not those, which are associated with the Th2 response (77).

NK-DC INTERACTION WITHIN THE TME

The TME is a complex network comprising T reg cells, tumor-associated macrophages (TAMs), regulatory gamma-delta cells, myeloid-derived suppressor cells (MDSCs), soluble factors, the extracellular matrix and suppressive molecules expressed on tumor cells (78).

The interaction between DCs, NK cells and $CD8^+$ T cells within the TME is diverse (**Figure 1**). Here, it was shown that the maturation of DCs is depending on NK cell derived HMGB1. Further, the chemokines CCL5 and XCL1/2 mediate the recruitment of cDCs to the TME, while the NK cell derived growth factor Flt3L ensures their survival. In return, IL-15 and IL-18 released by DCs active NK cells whereas DCs derived chemokines like CXCL9 and CXCL10 recruits NK cells and $CD8^+$ T cells into the TME. Moreover, NK cells' cytotoxic abilities and cytokine production, especially IFN-γ, is enhanced by cDCs in a cell-contact dependent and independent way (79, 80).

In a murine model, Allen et al. showed that tumor-derived CCL3 contributes to attract NK cells into the TME, leading to IFN-γ, DCs accumulation and T-cell recruitment (81). Also, in mouse, Wculek et al. demonstrated the capacity of cDCs bearing dead tumor cell antigens to highly induce $CD8^+$ T cell response (82).

cDC1 express different chemokine receptors on their surface including the only receptor for XCL1, XCR1 (35) as well as receptors for CCL5 including CCR1 and CCR5 (83). Bottcher et al. demonstrated a reduced abundancy of cDC1, within tumors, when CCL5 and XCL1 was blocked in mice *in vivo*. Their data indicated that NK cell derived CCL5 and XCL1 recruits cDC1 into the TME of mouse tumors (4).

The localization of DCs within tumors is not only due to NK cell-derived chemokines but also depends on the local abundancy of growth factors like Flt3L. By depleting NK cells in tumor-bearing mice Barry et al. demonstrated that NK cell derived Flt3L regulates the amount of DCs within the tumor. These findings were further supported by the establishment of DC-NK cell conjugates within the TME (84).

It is well known that the TME induces tolerance and immunosuppression via various mechanism by dampening the functional activity of various immune cells, including NK cells and DCs (3). The TME alter the activation and cytokines production of pDCs and these pDCs are involved in tumor growth (85). Breast cancer or melanoma cells may reduce the expression of TLR or engage ILT7 (immunoglobulin like transcript 7) on pDC_s due to their BST2 expression leading to reduced IFN-α productions and release. They further induce the

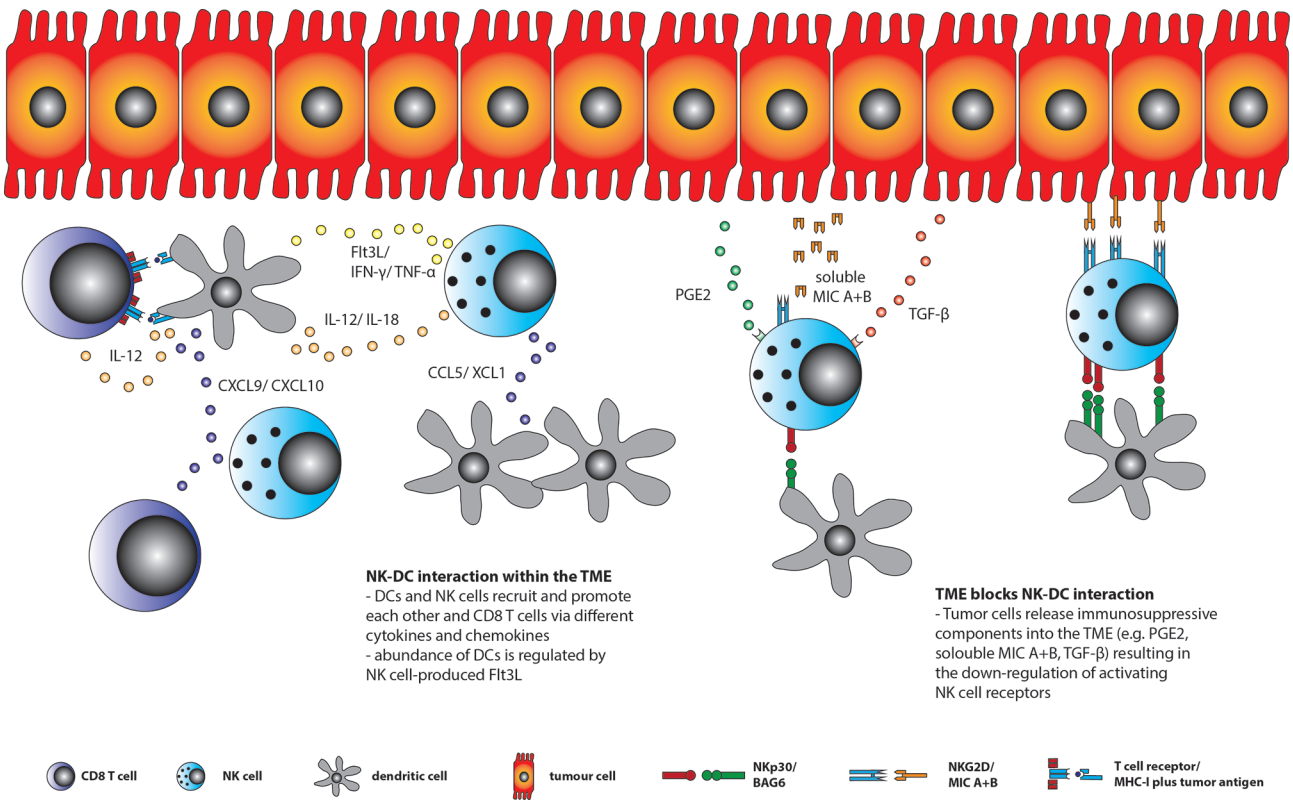

FIGURE 1 | DC, $CD8^+$ T and NK cell interaction within the TME. DCs, $CD8^+$ T cells and NK cells may recruit each other into the TME by secreting various kinds of chemokines, like CCL5/XCL1 by NK cells to recruit DCs or CXCL9/10 by DCs to recruit NK and $CD8^+$ T cells. Moreover, DCs activates $CD8^+$ T cells via tumor antigen presentation and IL-12 production as well as TNFα and IFNγ secretion by NK cells through their own production of IL-12 and IL-18. In return, NK cell derived TNFα and IFNγ induce DC maturation. In addition, NK cells are the major source of the DC growth factor Flt3L. However, tumor cells are able to secret different compounds into the TME, like TGF-ß, soluble MIC A+B or PGE_2, which are all able to down-regulate activating NK cell receptors (e.g. NKG2D and NKp30) resulting in reduced NK cell function and DC-NK cell interaction within the TME.

production of immunosuppressive TGF-β, resulting in reduced expression of activating NK cell receptors, including NKp30 and NKG2D, but not NKp46. Reduced NKp30 expression decrease NK cell cytotoxicity against immature DCs, subsequently leading to the accumulation of immature immunosuppressive DCs, probably supporting tumor growth. The release of soluble NKG2D ligands from tumor cells has been described to cause reduced expression of NKG2D on NK cells, reducing NK-DC-interaction upon IFN-stimulation. Moreover, data indicated that lower NKG2D levels on NK cells not only decreased their cytotoxic activity against NKG2D ligand-expressing tumor cells but also negatively influence IFN-α-mediated NK-DC-interactions, since NKG2D ligands are up regulated upon IFN-α stimulation on DCs. In addition, PGE_2 was demonstrated to reduce NKG2D and 2B4 expression on NK cells, which not only inhibits IFN-γ production and cytotoxicity, but potentially effect NK-DC-interactions as well. When DCs are matured with PGE_2s they were unable to recruit NK cells and were less effective in inducing IFN-γ production in NK cells. Tumor cell induced T reg cells inhibit the IL-15Rα-expression on DCs and thereby play a role in preventing the formation of a NK-DC immuno-synapse. The release of PGE_2 by tumor cells was suggested to inhibit IL-18-secretion by DCs, which was associated with a reduction of HMGB1-production by NK cells leading to reduced DC maturation (3, 86).

DISCUSSION

As pointed out the interaction between NK cells and DCs is negatively influenced by the TME in various ways. Therefore, strategies are needed to overcome these obstacles in order to restore anti-tumor surveillance within the TME (**Figure 2**).

Optimization of DC Vaccination Products

One approach to optimize DC vaccination products might be to generate large amounts of pro-inflammatory DCs *ex vivo*, pulsed with tumor antigens, to lift immunosuppression within the TME. Until today various clinical trials using different DC products to treat cancer patients have been performed. Despite encouraging clinical responses in glioblastoma, pancreatic and prostate tumor patients (87–89), only sipuleucel-T has been approved by the FDA for the treatment of metastatic hormone refractory prostate cancer so far (90). This demonstrates that further improvement

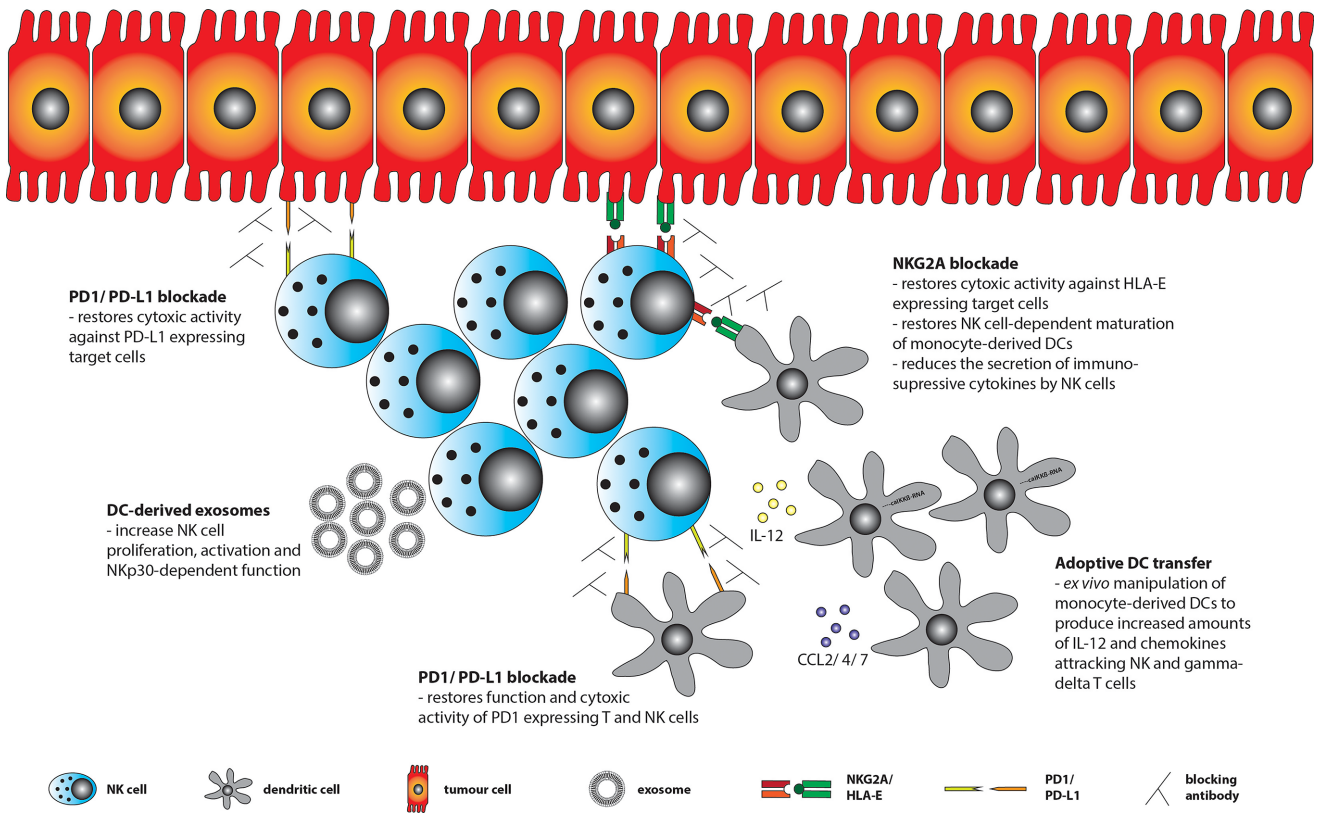

FIGURE 2 | Treatment strategies to restore anti-tumor surveillance within the TME. Tumor cells can express ligands for various immune checkpoint (ICP) receptors including HLA-E (ligand for NKG2A) and PD-L1 (ligand for PD1), of which the latter one may also be expressed by tumor-infiltrating DCs. Using blocking antibodies against ICP receptors or their ligands restores NK cell function and DC-NK cell interaction. NK cells may further be stimulated by *ex vivo* generated DC-derived exosomes (Dex). In addition, adoptive transfer of *ex vivo* generated and engineered DCs (e.g. by transfecting them with a constitutively active IKKß (caIKKß) of the NF-kB pathway to enhance IL-12 production) could improve DC-NK cell interaction and eventually lift immunosuppression within the TME.

of the DC products is needed. For example, many trials used moDCs, which are quite convenient to be generated *in vitro*, but have the disadvantage, that they do not secrete IL12p70, when matured with a standard DC maturation cocktail (91, 92). This could be overcome by using mRNA electroporation to transfect moDCs with a caIKKß-RNA (constitutively active IKKß of the NF-kB pathway) enabling the transfected cells to enhance cytotoxic activity and IFN-γ production of autologous NK cells (92, 93). Another group used mRNA electroporation to engineer moDCs to produce IL-15/IL-15Rα or IFN-α leading to increased NK cell activation and cytotoxic activity against Burkitt lymphoma cells *in vitro* (94, 95). Increased NK cell cytotoxicity *in vitro* against Burkitt lymphoma cells was also achieved by co-culturing human NK cells together with TLR-activated $CD1c^+$ myeloid and plasmacytoid DCs together instead of culturing them with only one specific DC subset alone (96). An additional approach is to optimize the DCs' capacity to attract other immune cells, such as NK cells. Here, it would be interesting to understand the influence of IL-15 addition in the generation of *ex vivo* moDCs instead of IL-4. While IL-4 generated DCs secrete Th2 and T reg cell attracting chemokines such as CCL17 und CCL22, IL-15 rather induces the secretion of chemokines such as CCL2, CCL4, CCL7, CXCL9, CXCL10 and CXCL11, with CCL4 being the most important one to recruit NK and γδ T cells (97). Of note, IL-15 derived moDCs induced a stronger NK cell activation than IL-4 generated ones did (98). Moreover, IL-15 generated moDCs, that were matured with a human papilloma virus (HPV) vaccine, demonstrated a Th1-polarized cytokine profile, and increased the cytotoxicity of NK cells against cervical cancer cell lines (99).

DC-Derived Exosomes a Vaccination Product

Nevertheless, certain obstacles remain when using DC vaccination with the aim to modulate the TME. DCs are still under the influence of the immunosuppressive milieu of the TME, need chemotactic signaling to reach their destination and a high amount of work is needed to produce and properly store them. One solution to overcome these obstacles could be the use of DC-derived exosomes (so called Dex) instead of whole DCs. Exosomes are nano-sized membrane vesicles, which can be secreted by various cell types. These exosomes are not only coated with HLA and costimulatory molecules on their surface, they also contain cytosolic proteins such as heat shock proteins and in

addition mRNAs and small RNAs (100, 101). DC-derived exosomes have already been used within clinical phase I/II trials (102–104). Within an initial phase 1 trial, 15 melanoma patients were treated with DC-derived exosomes from tumor-antigen pulsed moDCs (102). A subsequent analysis demonstrated that the used DC-derived exosomes were able to stimulate proliferation and activation of NK cells *in vitro*, due to their expression of NKG2D ligands and IL-15Rα. Moreover, DC-derived exosomes treatment increased the absolute number of NK cells *in vivo* and restored NKG2D expression levels as well as K562-specific cytotoxicity in 7 out of 14 melanoma patients (105). Similar results were observed during a phase II trial treating inoperable non-small cell lung cancer (NSCLC) patients with DC-derived exosomes from IFNγ-matured moDCs as maintenance therapy after induction chemotherapy. Here, an increase of NKp30-dependent NK cell function was observed, although NKp30 expression itself was not increased on patients' NK cells. Interestingly, NKp30-dependent function was associated with longer progression-free survival and with BAG6 expression, the ligand for NKp30, on the final DC-derived exosomes product (104). Taken together, DC-derived exosomes have the potential to induce NK cell proliferation and activation *in vivo* and *in vitro*. In addition, they have the unique property to resist the immunosuppressive milieu of the TME and to easily reach various compartments without the need of chemokine-dependent recruitment, making them an interesting option to manipulate NK-DC interaction within the TME.

Blockade of Immune Checkpoint (ICP) Receptors

One of the major breakthrough in the treatment of tumor patients during the last decade was the introduction of blocking antibodies against immune checkpoint (ICP) receptors, especially against CTLA-4 and PD1, leading to long-term tumor control in melanoma, NSCLC and renal cell carcinoma patients. An important role for ICP receptors during the interaction between NK cells and DCs has been described for PD1, NKG2A and TIGIT.

PD-L1, the ligand for the ICP receptor PD1, is expressed on murine cDC1 and cDC2 (106) as well as on tumor and peripheral cDC and pDC of lung cancer patients (107). Its expression may limit the efficacy of DC vaccination trials, since PD-L1 inhibits proliferation and cytotoxic activity of $PD1^+$ T and NK cells, respectively (108). The importance of PD1 expression on NK cells for tumor surveillance was demonstrated within the RMA-S lymphoma mouse model, which rather depends on NK cells controlling tumor growth than T cells. PD1 was strongly upregulated on NK cells within the TME and in draining lymph nodes. PD1 blockade led to a significantly reduced rate of tumor progression, which was not observed when NK cells were previously depleted. Interestingly, only a fraction of the NK cells expressed PD1. These cells were mostly activated NK cells and demonstrated higher functional activity than PD1 negative ones (109). PD1 expression on NK cells has been demonstrated as well in other tumor entities like Kaposi sarcoma (110), digestive cancers (111) and multiple myeloma (112). Using GM-CSF/IL-4 bone marrow-derived DCs (BMDCs) for vaccination in combination with pomalidomide and PD-L1 blockade, a significant tumor growth inhibition within a multiple myeloma mouse model could be achieved (113). Similar results were reported when using vaccination with GM-CSF/IL-4-derived BMDCs together with lenalidomide and PD1 blockade, which resulted in an increased functional activity of T and NK cells as well as a reduction of immunosuppressive cytokines within the TME (114). In addition, pDCs derived from the bone marrow of multiple myeloma patients expressed increased levels of PD-L1 and *in vitro* blockade of PD1 was able to enhance T cell proliferation and NK cell cytotoxicity during co-culture with myeloma-derived pDCs (115).

NKG2A is one of the major ICP receptors on NK cells recognizing the non-classical HLA molecule E (HLA-E) resulting in reduced cytotoxic activity against HLA-E positive target cells. MoDCs are able to upregulate NKG2A expression on NK cells in an IL12p70-dependent manner (116, 117). In addition, upon interaction with HLA-E expressing cells, $NKG2A^+$ NK cells secreted increased amounts of IL-10 and TGF-ß resulting in reduced activation of moDCs and DC-mediated induction of $CD4^+CD25^+$ T reg cells. However, blockade of NKG2A was able to restore the activation of moDCs (118, 119). Currently, various clinical trials are testing NKG2A blockade within tumor patients. The first published and completed phase I/II trial in patients with advanced gynecologic malignancies demonstrated that the treatment was well tolerated, but only achieved a stable disease as maximal response (120). However, in patients with refractory/recurrent squamous cell carcinoma of the head and neck, the combination of an anti-NKG2A blocking antibody (monalizumab) together with an approved anti-EGFR antibody (cetuximab) demonstrated a confirmed RECIST (Response Evaluation Criteria in Solid Tumors) partial response in 8 of 26 (31%) and a stable disease in 14 of 26 (54%) patients during the interim's analysis (121).

In addition, TIGIT is an inhibitory receptor recognizing the poliovirus receptor (PVR, CD155), such as the activating NK cell receptor DNAM1, but with a higher affinity. Importantly, binding of CD155 on moDCs by $TIGIT^+$ T cells resulted in increased IL-10 and reduced IL-12p40 production (122). Interestingly, Flt3L-derived BMDCs derived from $CD155^{-/-}$ mice were more sensitive towards NK cell killing than Flt3L-derived BMDCs from WT mice indicating a potential role for the CD155-TIGIT signaling pathway during the interaction of NK cells and DCs (123). Currently, various clinical trials are testing the potential of TIGIT blockade in tumor patients.

CONCLUSION AND OUTLOOK

We have delineated that functions of DCs and NK cells as well as their interaction with each other are severely compromised within the TME. For this reason, various strategies to counteract immunosuppression within the TME have been discussed here. Although only PD1-PD-L1 blockade and sipuleucel-T have entered daily clinical life until today, further promising treatment options are currently being tested within clinical trials. Importantly, due to the complex and multiple facets of the TME, a combination of distinct treatment approaches will be inevitable to successfully lift

immunosuppression from the TME without causing an immediate counter measurement by the tumor.

AUTHOR CONTRIBUTIONS

BJ and EU designed and coordinated the review. BJ, EU, VGe, LH, VGr, HS, and DD wrote the manuscript. All authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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