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Innate lymphocytes in tumor surveillance

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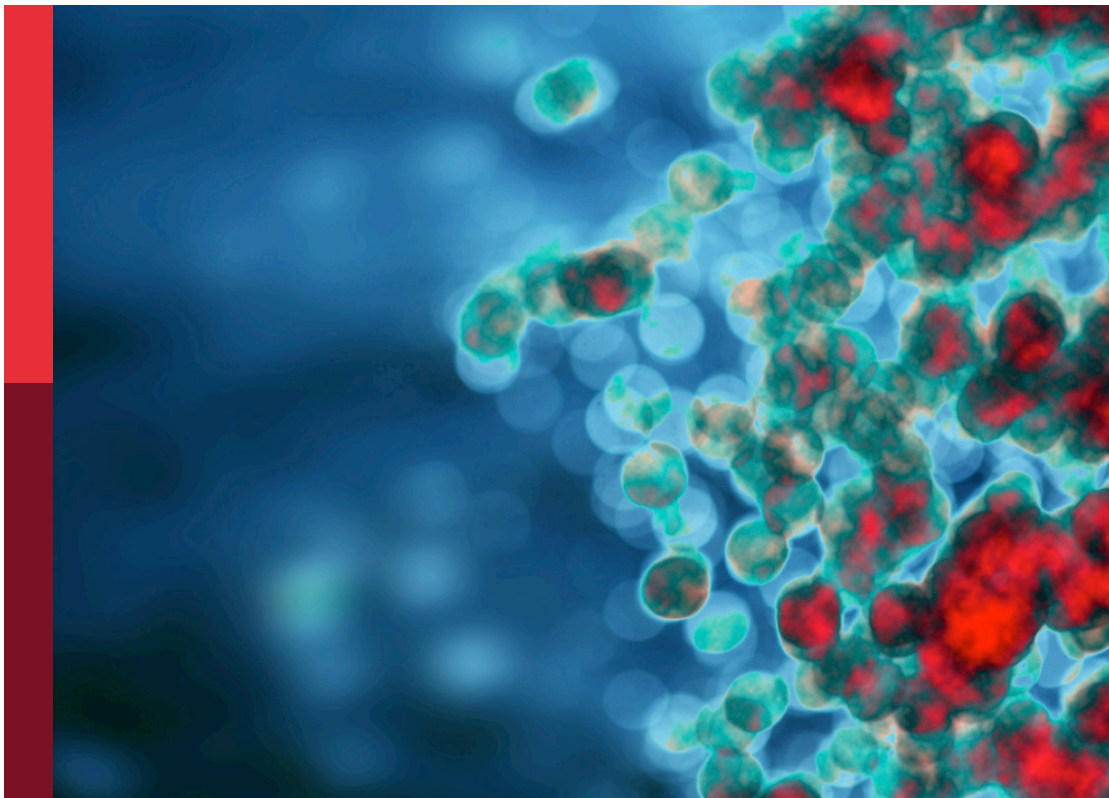

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Innate lymphocytes in tumor surveillance

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Editorial: Innate lymphocytes in tumor surveillance

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Editorial on the Research Topic
Innate lymphocytes in tumor surveillance

The multifaceted roles of Innate Lymphoid Cells (ILC) have been widely interrogated in tumor immunity. Whereas Natural Killer (NK) cells possess tumor-suppressive properties across multiple types of cancer, the other ILC family members can either promote or inhibit tumor growth depending on the environmental conditions. The differential effects of ILCs on tumor outcome have been attributed to the high degree of heterogeneity and plasticity within the ILC family members. However, it is now becoming clear that ILC responses are shaped by their dynamic crosstalk with the different components of the tumor microenvironment (TME) (1). Recent years have witnessed a significant development in the current understanding of ILCs and their roles in the innate immune system, where they regulate tissue homeostasis, inflammation, as well as tumor surveillance and tumorigenesis (2). ILCs may be classified into three subgroups depending on their phenotypic and functional characteristics: Group 1 ILCs, which include NK cells and ILC1s (3); Group 2 ILCs, which only contain ILC2s (4), and Group 3 ILCs, which comprise of LTi cells and ILC3s (5).

This Research Topic features several review and original research articles on the different facets of innate lymphocytes in the context of cancer. This includes basic underlying mechanisms of anti-tumor action as well as translational and clinical advances in this area of research. The topic spans different areas of NK cell as well as ILC research with emphasis on their role during tumor development and progression.

NK cells are the prototype innate lymphoid cells exhibiting potent cytolytic function that provide host defense against infection and tumors. They are able to kill tumor cells if these show surface markers associated with oncogenic transformation. Due to this property NK cells control tumor growth at least in the early phase of tumor development and thus they are essential in tumor surveillance. Once target cells are recognized the balance of activating and inhibitory receptor signaling regulates their effector function against tumor targets (6). These properties and their capacity to enhance antibody and T cell responses highlight the role for NK cells as anticancer agents (7, 8). Current research

is focused on investigating how NK cells may be manipulated and employed as therapeutic strategies for the treatment of cancer.

NK cells are of utmost importance in host protection during tumor development. Li and O´Sullivan review the spatiotemporal dynamics of host factors (tissue-specific and systemic) that lead to progressive dysfunction of NK cells while a tumor progresses. These include heterogeneous tumor architecture, temporal disease states, diverse cellular subsets in the microenvironment and the complex changes in NK cell states in response to all these factors. Understanding of these different signals that NK cells are confronted with may help identify new therapeutic targets to increase effectiveness of NK cell therapy for cancer.

Based on the underlying mechanisms of tumor cell evasion from NK cells a rationale for improvement of NK cell-based cancer immunotherapies is presented by Seliger and Koehl. This review highlights different factors contributing to immune escape or immunosurveillance by NK cells as well as benefits and limitations in clinical NK cell-based immunotherapies such as adoptive cell transfer-based approaches and engineered NK cell-based immunotherapies. Supported by preclinical and clinical studies additional possibilities for NK cells in cancer patient treatment are suggested *via* combination therapies, potentially leading to further clinical advances.

Tumino et al. focus on polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) and report on their strong suppressive effect on NK cell numbers and anti-tumor activity in patients with primary or metastatic lung tumors. By complementing *in vitro* cell culture experiments the authors revealed that exosomes derived from PMN-MDSCs are responsible for a reduced NK cell-mediated anti-tumor activity and suggest potential for PMN-MDSCs as prognostic marker for clinical outcome.

Concentrating on the underlying signaling pathways of tumor-NK cell interactions Witalisz-Siepracka et al. review the different roles of JAK/STAT3 signaling in the dynamic interplay between NK and tumor cells. The authors include literature on how tumor cell-intrinsic STAT3 drives evasion from NK cells but also how STAT3 may regulate NK cell cytotoxicity, cytokine production and anti-tumor responses *in vivo*.

As mentioned above multiple approaches are being investigated to relieve NK cell immunosuppression in the tumor microenvironment. Immune checkpoint blockade inhibiting the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis seems not only acting on T cells but also on NK cells. However, the role of NK cells in PD-1/PD-L1 context is still a matter of debate (9). del Rio et al. aimed to address this topic and present data from a mouse xenograft model suggesting that NK cell anti-tumor function is independent of PD-L1 expression on A20 tumor cells.

ILCs regulate immune responses by responding and integrating diverse signals within local microenvironments and as such are ideally suited to sense malignant transformation and regulate tumor immunity. However, as ILCs have been associated with anti-tumor and pro-tumor activities, they have been suggested to exert dual functions during carcinogenesis by promoting or suppressing the malignant outgrowth of premalignant lesions. Warner et al. discusses emerging evidence that shows that ILCs can impact early tumor development by regulating immune responses against transformed cells, as well as the environmental cues that influence ILC activation in premalignant lesions.

Hepatocellular carcinoma (HCC) is one of the deadliest cancers worldwide. However, the role of the ILCs in HCC is still not well defined. Bouyarou and Golub provide an overview of the known roles and actions of ILCs in HCC with an emphasis on the importance of diverse signaling pathways in the tuning of their responses.

ILCs are preferentially enriched in barrier tissues such as the skin, with emerging evidence indicating their role in the control of melanoma. Wagner and Koyasu review the current understanding of ILCs role as the first line of defence against melanoma development and progression and discuss the possibility to harness their therapeutic potential.

ILC2s are important mediators of type 2 immunity and play important roles in allergic diseases, helminth infections, and tissue fibrosis (10). Studies over the past decade have reported that ILC2s exert context-dependent roles in cancer. Wu et al. review the roles of ILC2s in solid tumors and propose that ILC2s could serve as a predictor for tumor prognosis and a new therapeutic target after immunotherapy resistance.

Despite the growing body of research on ILCs, there are still plenty of knowledge gaps of how these cells are shaped by disease- and tissue-specific cues. This Research Topic features several articles that highlight our current knowledge about ILCs in tumor surveillance, pinpoint gaps in our understanding and discuss the possibilities to harness their therapeutic potential.

Author contributions

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

Conflict of interest

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

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REVIEW
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Innate Lymphoid Cells in Skin Homeostasis and Malignancy

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Innate lymphoid cells (ILCs) are mostly tissue resident lymphocytes that are preferentially enriched in barrier tissues such as the skin. Although they lack the expression of somatically rearranged antigen receptors present on T and B cells, ILCs partake in multiple immune pathways by regulating tissue inflammation and potentiating adaptive immunity. Emerging evidence indicates that ILCs play a critical role in the control of melanoma, a type of skin malignancy thought to trigger immunity mediated mainly by adaptive immune responses. Here, we compile our current understanding of ILCs with regard to their role as the first line of defence against melanoma development and progression. We also discuss areas that merit further investigation. We envisage that the possibility to harness therapeutic potential of ILCs might benefit patients suffering from skin malignancies such as melanoma.

Keywords: innate lymphoid cells, skin, skin cancer, melanoma, immunity, immunosurveillance

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INTRODUCTION

The family of innate lymphoid cells (ILCs) comprises a heterogeneous population of immune cells harboring pleiotropic functions. Based on the expression of signature cytokines and an assembly of transcription factors, they have been divided into five subsets, namely natural killer (NK) cells, group 1 ILCs (ILC1s), ILC2s, ILC3s and lymphoid tissue inducer (LTi) cells (1). Accordingly, ILC1s secrete type 1 cytokines such as IFN-γ and TNF-α. They require the expression of T-bet, a T-box transcription factor, for development and function, but unlike NK cells are not cytotoxic and can develop in the absence of eomesodermin (Eomes), another T-box transcription factor that is homologous to T-bet and essential for NK cell differentiation (1). ILC2s secrete type 2 cytokines, including IL-4, IL-5 and IL-13 and depend on the expression of GATA3 and RORα (2, 3). Not least of all, ILC3s and LTi cells produce IL-22 and/or IL-17 and require RORγt. LTi cells, however, also produce lymphotoxin (LT), a member of the TNF family of cytokines and arise from a different developmental pathway than ILC3s (1). It should be noted, however, that in the human peripheral blood ILC3s are immature and rather represented by a population of ILC progenitors (ILCPs) (4).

The heterogeneity and diversity of ILCs might further increase owing to their plastic potential (4–6). For example, the combination of IL-1β and IL-12 has been found to induce the transdifferentiation of human ILC2s into IFN-γ-producing cells resembling ILC1s (7). It has also been demonstrated that IL-4 can reverse that phenotype converting cells reminiscent of human ILC1s back to ILC2s (8). Recent study has also reported the transdifferentiation of human cutaneous ILC2s into IL-17-producing cells resembling ILC3 (9). The plastic potential of ILCs might therefore serve as an important feature of their ability to adapt rapidly to the fluctuating levels of

environmental stimuli. A growing body of evidence indicates that environmental stimuli ingrain the phenotype of ILCs and thus their function (10, 11). With that in mind, ILCs have been equipped with receptors to sample the environment and react against threats to tissue integrity through the production of cytokines and chemokines. They are promptly activated by stress signals and various epithelial- and myeloid cell-derived cytokines, rather than by antigens as T and B cells (12, 13).

Similarly to NK cells, ILC1s require IL-15 for their development. Additionally, they are both activated by IL-12, IL-18 and IL-15 (14, 15). Whereas IL-12 and IL-18 are secreted by monocytes and activated DCs, IL-15 is produced by activated monocytes and macrophages as well as a variety of non-hematopoietic cells, including but not limited to epithelial and fibroblast cell lines (15). ILC2s, on the other hand, respond primarily to IL-33, IL-25 and thymic stromal lymphopoietin (TSLP, combined with IL-33), which are produced by numerous cell types (2, 3, 15, 16). For example, expression of IL-33 can be found in epithelial and endothelial cells, smooth muscle cells, fibroblasts, macrophages and activated DCs (15). Expression of TSLP, however, typifies epithelial cells in the barrier tissues such as the skin, whereas activated Th2 cells together with macrophages, mast cells, eosinophils, basophils and fibroblasts as well as skin epithelial cells, tuft cells, and endothelial cells produce IL-25 (15). Last in order, ILC3s and LTi cells are activated by IL-1β and IL-23 produced by activated DCs and macrophages (17, 18).

In contrast to NK cells, which circulate in the body and are particularly detected in the peripheral blood, the remaining ILC subsets are mostly tissue resident and preferentially enriched in barrier tissues such as the skin. The involvement of NK cells in antitumor immunity is unquestionable (19–22). Their abundance in the circulation correlates with decreased metastatic potential in numerous human cancers (23, 24). However, our understanding of the role and function of the remaining ILC subsets in skin malignancies is still in its infancy. The most aggressive form of skin cancer, melanoma, originates in melanocytes, which are found in the skin, eyes and hair. Although less common than squamous and basal cell carcinoma, melanoma, if left untreated at an early stage, is far more perilous because of its ability to spread more rapidly to distant organs. Melanoma has been thought to trigger immunity mediated mainly by adaptive immune responses. To what extent innate immunity, and in particular, innate lymphoid cells impact melanoma is not well understood. Here, we summarize recent insights into the unique features and functions of ILCs pertaining to their role in the protection from melanoma development and progression. We also discuss areas that require further investigation and highlight discoveries, which could have implications for the development of new therapeutic strategies.

ILCs IN THE SKIN

The skin is the largest organ of the body (25). It provides thermal insulation and physical protection from injury and infection. It also stores water, stacks the majority of the body fat and produces vitamin D. The skin is composed of three anatomically distinct layers: epidermis, dermis, and subcutis. The outermost layer of the skin, epidermis, is composed of squamous and basal cell keratinocytes, melanocytes as well as Merkel's cells, which serve as mechanoreceptors. Keratinocytes, which are the most abundant cells within the epidermis, produce the key structural material, keratin, as well as lipids responsible for the formation of the epidermal water barrier. They are also responsible for converting 7-dehydrocholesterol to vitamin D with the assistance of ultraviolet B (UVB) light from the sun. UVB light also stimulates melanocytes to secrete melanin, which is responsible for the pigment of the skin (26). The middle and by far the thickest layer of the skin, dermis (together with epidermis called cutis), is primarily composed of fibroblasts but also contains blood and lymphatic vessels, nerves as well as epidermally derived appendages including hair follicles, sudoriferous (or sweat) glands and sebaceous glands, which are deeply integrated into the fabric of connective tissue. Connected to epidermis and probably the most understudied is the deepest layer of the skin, subcutis (sometimes referred to as hypodermis), made of fat and connective tissue, which also contains extensive vasculature. However, it should be noted that mouse and human skin are quite distinct in terms of structure (27). Whereas, mouse epidermis is usually composed of only 3 cell layers (< 25μm in thickness), human epidermis is often formed of 6–10 cell layers (> 50 μm in thickness) (27). Similarly, human dermis is much thicker than mouse dermis. On the other hand, although thinner, mouse skin has more densely distributed hair follicles and contains a cutaneous muscle layer called, panniculus carnosus (27).

The skin also represents a highly specialized immunological niche with immune cells closely interacting with non-hematopoietic parenchymal cells to ensure the maintenance of the barrier function (25). For example, in an event of an insult, non-hematopoietic parenchymal cells regulate recruitment, activation and tissue residency of immune cells, whereas immune cells secrete cytokines as well as growth factors necessary for the prevention of infection and tissue reconstruction. Traversed by blood and lymphatic vasculature, dermis contains most of the immune cells in the skin, including several subsets of dermal DCs, $CD4^+$ and $CD8^+$ T cells, γδT cells, B cells, macrophages, basophils, eosinophils, mast cells and NK cells (25). Accumulated evidence has demonstrated that ILCs, which are preferentially enriched in the skin, play an important role in barrier tissue immunity (28). Beside NK cells, ILC2s were the first ILCs to be discovered in the skin, both in mice and humans (29). Although ILCs can be detected in epidermis and dermis, the majority were identified in the deeper layer of the skin with ILC2s comprising 5-10% of all $CD45^+$ cells in mice (30). Recently, transcriptome analysis of bulk and single-cell RNA-sequencing data demonstrated enrichment of ILCs expressing genes associated with ILC2s (e.g. *Gata3* and *Il5*) in subcutis, whereas ILCs in epidermis were found to predominantly express genes associated with ILC3s/LTi cells (e.g. *Rorc* and *Lta*) but also ILC2s (e.g. *Il13* and *Il2*). On the

other hand, ILCs in dermis shared resemblance with ILCs from epidermis and subcutis (31).

Tissue residency of ILCs in the skin might be governed by the tissue-derived cytokines. For example, *Il7*$^{-/-}$ mice have been found devoid of ILC2s in subcutis, whereas the number of ILCs in epidermis and dermis was moderately reduced when compared with wild-type mice. However, complete loss of ILCs has been observed in the skin of *Il7*$^{-/-}$*Tslp*$^{-/-}$ mice indicating collaborative regulation of skin residency by IL-7 and TSLP (31). The pattern of chemokine receptors expressed by ILCs might also be involved in the regulation of tissue residency. For example, ILCs that reside in the mouse epidermis express CCR6, which serves as a receptor for CCL20 that is highly expressed in the upper portion of the hair follicles (32). Hair follicles have therefore been portrayed as epicenters that recruit and position ILCs in the skin. It has also been demonstrated that hair follicles provide cytokines such as IL-7 and TSLP, which ILCs depend on for maintenance (33). Furthermore, ILCs present in the skin-draining lymph nodes that express CCR10 have been found to migrate to the skin in a CCR10-dependent manner (34).

From another angle, using mice expressing eGFP under the control of the locus of CXCR6, an analysis of the potential immunosurveillance activity in the skin revealed that ILC2s patrol their environment with an average speed similar to that of dermal DCs (i.e. 5 μm/min) (30). An analysis of the interactions with other cell types demonstrated that ILC2s strongly interact with mast cells and suppress IgE-dependent cytokine production by mast cells through the release of IL-13 (30). However, human ILC2s have also been found to induce strong proinflammatory responses following stimulation with prostaglandin D2 (PGD2) produced by mast cells. Indeed, activation of human ILC2s by PGD2 has increased their migration and upregulated the expression of IL-33 and IL-25 receptor subunits (ST2 and 17A, respectively) as well as induced production of type 2 and other cytokines such as IL-3, IL-8, IL-9, IL-21, GM-CSF and CSF-1 (18). Of note, mast cells are likely not the only source of PGD2. Although studied not specifically in the skin, ILC2s and epithelial cells have also been found to produce PGD2 (35–37).

The involvement of ILC1s and ILC3s in skin homeostasis requires further investigation. It should be noted, however, that ILC3s might play a critical role in the maintenance of tolerance towards skin microbiota (38). Nevertheless, they have also been associated with the development of psoriasis, an immune-mediated chronic disorder of the skin (39–41).

ILCs AND SKIN WOUND HEALING

The skin has evolved precise and orderly mechanisms to close breaches to its integrity in a process known as the wound healing response. Human ILC2s isolated from the skin have been typified by an increased gene expression of amphiregulin when compared with those purified from blood. Since amphiregulin aids tissue repair, it has been suggested that ILC2s in the skin are involved in wound healing response (42). This notion has recently been supported by a study, which found that ILC2s are important in the reepithelialization of cutaneous wounds. Indeed, elevated numbers of ILC2s have been found at the site of injury five days after wound induction. Importantly, impaired reepithelialization accompanied by diminished numbers of activated ILC2s has been observed in IL-33-deficient mice at the site of injury when compared with wild-type mice. However, treatment with recombinant IL-33 has significantly increased reepithelialization five days after wound induction (43).

Additionally, presence of IL-17A, which is produced among others by ILC3s, has also been found in human wounds. Interestingly, delayed wound closure has been observed in *Il17a*$^{-/-}$ mice (44). Although dendritic epidermal T cells (DETC) have been portrayed as an important source of IL-17A in the study, it is possible that ILC3s might also be engaged in the wound healing response, since impeded wound closure has been more pronounced in *Il17a*$^{-/-}$ mice when compared with *Tcrd*$^{-/-}$ mice, which lack DETC (44). Indeed, it has recently been revealed that damage to the skin activates Notch signaling, which in turn, induces recruitment of RORγ^{+} ILC3s through the production of TNF-α. Additionally, RORγ^{+} ILC3s have been found to produce IL-17F and CCL3 (also known as MIP1α) involved in the healing response through the regulation of epidermal proliferation and macrophage recruitment into dermis (45).

More than three decades ago, Dvorak suggested that cellular and biochemical processes associated with wound healing, although lost at the level of regulation, are reminiscent of the tumor stroma development. He thus coined the phrase that tumors are "wounds that do not heal" (46). Although ILCs seem to play an active role in wound healing, the nature of their responses in tumors has only recently begun to be unveiled.

ILCs IN MELANOMA

NK Cells and ILC1s

Among all ILCs, NK cells are certainly the most extensively studied mediators of immune responses against cancer (23, 47). Responses against melanoma in particular have also been detailed [Tarazona et al. for comprehensive review (19)]. Briefly, numerous studies have demonstrated that NK cells are able to distinguish and destroy melanoma cells *in vitro* (19, 48). The tumor suppressive role of NK cells has also been demonstrated using variety of *in vivo* mouse models (19). Last but not least, evaluation of NK cell alterations in melanoma patients such as down-regulation of activating receptors and exhaustion of NK cells has indicated establishment of escape mechanisms by melanoma cells to evade NK cell-mediated recognition and destruction (19, 20). Interestingly, the abundance of CD56^{bright} NK cells in the peripheral blood obtained from late stage (III/IV) melanoma patients has recently been found to negatively correlate with overall patient survival (49).

The studies of NK cells have generally stressed their cytotoxic role mediated by the release of cytotoxic granules containing perforin (PFN) and granzyme B (GrB) or by the engagement with death receptors that initiate caspase cascade. Portrayed as the first line of defence, NK cells have been viewed as innate immune cells that operate at an earlier stage than T and B cells. However, involvement of NK cells in T cell-mediated immunity has also been demonstrated (50, 51). Recently, NK cells have been found to attract $XCR1^+$ DCs that are critical for T cell–mediated immunity to melanoma tumors through the secretion of XCL1, XCL2 and CCL5 (51). NK cell frequency has also been found to correlate with cross-presenting DCs in melanoma tumors as well as with responsiveness to anti-PD-1 immunotherapy in patients and their increased overall survival (50). A broader role for NK cells, beyond their direct cytotoxic function, has therefore been proposed.

In contrast to NK cells, little is known about the role of ILC1s in melanoma. Recently, enrichment of ILC1s, although with impaired IFN-γ production capabilities, has been observed in both peripheral blood and tumor cell-infiltrated lymph nodes from melanoma patients (52). Interestingly, IFN-γ signaling in cancer and immune cells has been found to oppose each other, in order to develop a regulatory relationship that restrains both innate and adaptive immune responses. Inhibition of tumor IFN-γ signaling has been found to decrease IFN-stimulated genes (ISGs) in cancer cells and increase ISGs in immune cells by enhancing IFN-γ production by exhausted T cells. In tumors with neoantigens or MHC-I loss, including melanoma, exhausted T cells utilize IFN-γ to stimulate maturation of innate immune cells, more specifically, a population of $PD1^+TRAIL^+$ ILC1s (53). The possibility to inhibit tumor IFN-γ signaling and, at the same time, disable an inhibitory circuit impacting PD1 and TRAIL has been suggested to promote innate immune killing.

ILC2s

Although the number of studies focusing on the role of ILC2s in tumor immunity has increased, many aspects related to the mechanisms behind their antitumor function still remain to be clarified.

ILC2s have been associated with the induction of apoptosis mediated through CXCR2 signaling in melanoma tumors engineered to express IL-33 (54). Furthermore, ILC2s are known to produce IL-5, which is essential for the expansion of eosinophils, since its localized production stimulates tissue eosinophilia (20, 55). In mice, ILC2s have been found to maintain sufficient numbers of eosinophils in the lungs through the production of IL-5 in response to melanoma invasion. Additionally, genetic blockade or antibody-mediated neutralization of IL-5 has been shown to impair eosinophil recruitment into the lungs leading to an increased metastatic dissemination of melanoma cells (56). Recently, ILC2-derived granulocyte macrophage-colony stimulating factor (GM-CSF) has been shown to contribute to the recruitment and activation of eosinophils into melanoma tumors (57). Since ILC2s have been found to express PD-1, the combination of anti-PD-1 blocking antibodies together with IL-33 improved anti-tumor responses through the expansion of tumor-infiltrating ILC2s accompanied by eosinophils. Importantly, deletion of NK cells, ILC1 and/or ILC3s had no impact on either tumor growth or survival of the mice, suggesting that ILC2s play the key role in restricting the development of melanoma tumors (57).

The exact role of eosinophils in melanoma remains to be determined, however, increased tumor growth and metastatic potential have been demonstrated following an antibody-mediated depletion of eosinophils in IL-33-treated mice bearing melanoma tumors (58). The cytotoxic activity of eosinophils has been attributed to the secretory granules made of major basic protein 1 (MBP-1) and MBP-2, eosinophil cationic protein, eosin-derived neurotoxin, and eosinophil peroxidase (55). Indeed, MBP^+ eosinophils have been found to clear metastatic melanoma cells in the mouse lungs, whereas the lysates of MBP^+ eosinophils have turned out cytotoxic *in vitro* when co-cultured with cancer cells (59). Importantly, we have observed a significant correlation between an overall survival and the expression of IL-33 as well as an eosinophil marker SIGLEC8 in patients suffering from melanoma. It should be noted, however, that the expression of IL-33 and SIGLEC8 has been found to demonstrate different survival prognosis in diverse types of cancer, with better survival outcomes in melanoma patients but not in those with pancreatic adenocarcinoma and lung squamous cell carcinoma (60). Increased median overall survival has also been shown in patients with metastatic melanoma presenting a high number of eosinophils in the circulation during immune checkpoint blockade therapy (ICB). Therefore, eosinophilia has been suggested to serve as a potential prognostic marker for melanoma patients during ICB (61, 62).

A growing body of evidence indicates that immune cells can also be influenced by the metabolism of cancer cells, and the cellular and molecular mechanisms are only now becoming determined (20). We have also found that the production of lactic acid by melanoma cells greatly impairs eosinophil-mediated antitumor response regulated by ILC2s. B16F10 melanoma tumors with diminished lactic acid production have been found greatly growth delayed and highly infiltrated by ILC2s accompanied by eosinophils following treatment with IL-33. We have therefore identified lactic acid production by melanoma cells as a plausible escape mechanism to evade destruction mediated by ILC2s (60).

Additional studies are necessary to understand the mechanisms involved in the shift of ILC2s from immunosurveillance to immunosuppression associated with the promotion of tumor growth and progression (20, 63).

ILC3s and LTi Cells

ILC3s have also been accredited a role in melanoma immuno surveillance. For example, a treatment with cyclophosphamide together with an antibody targeting a native melanoma differentiation antigen, tyrosinase-related protein 1 (aTRP1), has been found to inhibit the growth of B16F10 melanoma tumors. It has also been demonstrated that the tumor-suppressing activity of this combined therapy occurs independently of adaptive immunity and NK cells, but is mediated *via* CD90 $+NK1.1^-$ ILC3s associated with intratumoral macrophage

accumulation (64). Additionally, B16F10 melanomas engineered to express IL-12 have been found to initiate local antitumor immunity by stimulating NKp46$^+$ ILC3s. Increased accumulation of NKp46$^+$ ILC3s has been associated with enhanced infiltration of CD8$^+$ and CD4$^+$ T cells as well as NK cells coupled with upregulation of adhesion molecules in the vasculature of melanoma tumors. Although T cells have been characterized as the dominant population of infiltrating leukocytes, the growth of melanomas expressing IL-12 has also been inhibited in *Rag1*$^{-/-}$ mice, which lack adaptive immunity. Additionally, antibody-mediated depletion experiments ruled out a strong contribution of NK cells in controlling the growth (but not metastatic dissemination) of melanomas expressing IL-12. It has therefore been suggested that NKp46$^+$ ILC3s might play a significant antitumor role in the presence of IL-12 (65).

In another study, it has also been revealed that the tissue microenvironment shapes the phenotype of ILC3s. Whereas ILC3s isolated from the spleen have been able to suppress the growth of B16F10 melanoma tumors expressing IL-12, intestinal ILC3s have been found ineffective. Interestingly, transcriptome analysis has revealed mutually exclusive gene expression signatures between the splenic and intestinal ILC3s regarding (but not limited to) leukocyte adhesion and activation. Increased frequencies of leukocytes have been observed in B16F10 melanomas engineered to express IL-12 and co-injected with splenic ILC3s when compared with tumors co-injected with intestinal ILC3s (66).

The involvement of LTi cells in melanoma immuno surveillance remains to be determined. However, it is tempting to hypothesize that LTi cells might contribute to the formation of tertiary lymphoid structures often observed in human melanoma (67).

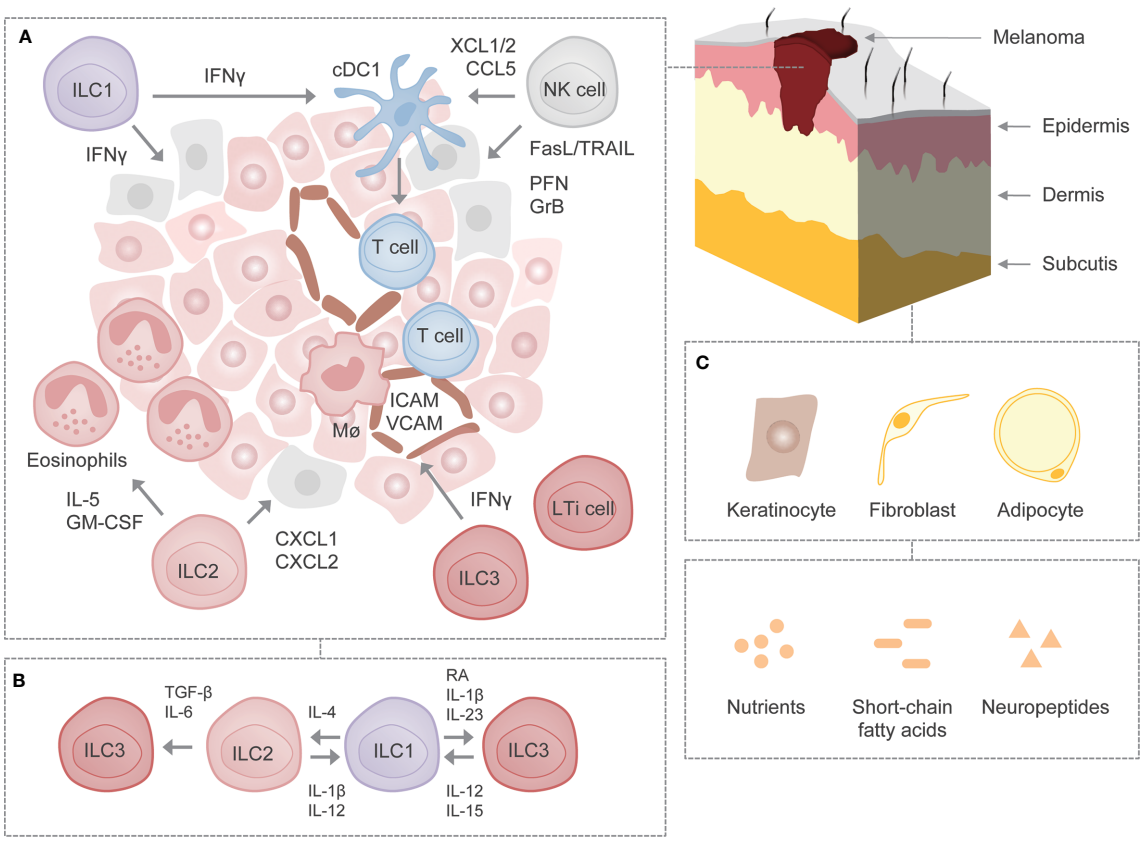

FIGURE 1 | ILCs in melanoma. **(A)** Schematic representation of cancer immunosurveillance by ILCs using mouse melanoma as a model. NK cells may induce apoptosis in melanoma cells through the release of cytotoxic granules containing perforin and granzyme B as well as through the engagement of death receptor-mediated pathways such as TRAIL and FasL. In addition, NK cells may recruit cDC1s to the tumor microenvironment by secreting XCL1/2 and CCL5 and may support their survival and maturation. ILC1s may produce IFN-γ, which exhibits direct antitumor activity or modulates activity of other immune cells. On the other hand, ILC2s may attract and activate eosinophils through the production of IL-5 and GM-CSF. ILC2s may also induce tumor cell-specific apoptosis *via* the release of CXCL1 and CXCL2. ILC3s may stimulate leukocyte recruitment to the tumor microenvironment through IFN-γ-mediated upregulation of adhesion molecules ICAM and VCAM. **(B)** Plastic potential of ILCs. Following stimulation with certain cytokines, growth factors or metabolites, ILCs exhibit potential for plasticity, although it remains to be determined whether ILCs undergo such reversible transdifferentiation in melanoma. **(C)** Interactions with parenchymal cells as well as non-cytokine factors in the skin. It remains to be deciphered whether ILCs interact with certain parenchymal cells as well as non-cytokine factors known to affect the function of ILCs in other settings. FasL, Fas ligand; GrB, granzyme B; ICAM, intercellular adhesion molecule; Mϕ, macrophage; PFN, perforin; RA, retinoic acid; TRAIL, TNF-related apoptosis-inducing ligand; VCAM, vascular cell adhesion molecule.

CONCLUSION AND FUTURE PROSPECTS

The involvement of NK cells in antitumor immunity is unquestionable, however, the role and function of other members of the family of ILCs have only recently gained attention. Given the preferential enrichment of ILCs in the skin, a deeper understanding of their contribution to the development and progression of skin malignancies is required. Indeed, a growing body of evidence indicates that ILCs play a critical role in the control of melanoma (**Figure 1**), a type of skin malignancy thought to trigger immunity mediated mainly by adaptive immune responses. However, the extent to which ILCs are engaged in antitumor immunity in general remains vague, as they have been separately associated with both tumor-suppressing and tumor-promoting activities depending on the context of tumor specificity (5, 20).

In order to improve our understanding of ILCs with regard to their role in skin malignancies, it seems imperative to determine how ILCs regulate healthy skin homeostasis. An increasing degree of heterogeneity among ILCs also necessitates their separate assessment in epidermis, dermis and subcutis. Furthermore, studying the mechanisms by which ILCs communicate with other non-hematopoietic parenchymal cells such as keratinocytes, fibroblasts and adipocytes might help to better understand the extent of their involvement in the homeostatic and pathological states (**Figure 1**) (68–70). Recently, it has been revealed that melanoma can arise from melanocyte stem cells found in hair follicles apart from melanocytes found in the bottom layer of epidermis (71). Further investigation should focus on the involvement of ILCs in the early stages of melanoma development. The same holds true for their role and function in the primary site (i.e. skin) as opposed to when confronted by metastases in another tissues.

Studying signals responsible for activation and inhibition of ILCs during malignant development and progression might help to preselect therapeutic targets. Currently, it is unknown how many non-cytokine factors, including nutrients, short-chain fatty acids and neuropeptides, which affect ILC function in other settings, shape ILC responses in melanoma (**Figure 1**). Three-dimensional (3D) spheroid-based *in vitro* models might prove useful during analysis of interactions between ILCs and malignant cells, since culture in 3D has been suggested to affect the expression of molecules involved in melanoma recognition (72–74). Not less important is further investigation of contribution of ILCs to other types of skin cancer, including squamous and basal cell carcinoma, since most of the studies (if not all) have utilized an injectable melanoma model. The methods established so far to identify and characterize ILCs in steady-state (e.g. single cell RNA-sequencing) might pave the way to properly dissect their relevance in cancer. Sophisticated imaging techniques might also allow us to better describe the spatial location of ILCs in the primary and metastatic tumor tissue. The role of specific subsets could be assessed using genetically engineered mice specifically lacking one or the other subset of ILCs. To this end, however, there is a need for identification of unique and highly specific markers. Further compounding the issue is the plastic potential of ILCs, which also merits investigation (**Figure 1**) (5, 6).

Following clarification of the role of ILCs in the skin cancer, translation of the results from mouse models to humans is necessary to fully elucidate the role of ILCs in disease pathogenesis as well as develop potential therapeutic strategies. It remains to be seen whether we can exploit ILCs to maximize its anticancer potential in the clinic.

AUTHOR CONTRIBUTIONS

All authors participated in the intellectual conception, critical review and final approval of the manuscript.

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Back to the Future: Spatiotemporal Determinants of NK Cell Antitumor Function

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NK cells play a crucial role in host protection during tumorigenesis. Throughout tumor development, however, NK cells become progressively dysfunctional through a combination of dynamic tissue-specific and systemic factors. While a number of immunosuppressive mechanisms present within the tumor microenvironment have been characterized, few studies have contextualized the spatiotemporal dynamics of these mechanisms during disease progression and across anatomical sites. Understanding how NK cell immunosuppression evolves in these contexts will be necessary to optimize NK cell therapy for solid and metastatic cancers. Here, we outline the spatiotemporal determinants of antitumor NK cell regulation, including heterogeneous tumor architecture, temporal disease states, diverse cellular communities, as well as the complex changes in NK cell states produced by the sum of these higher-order elements. Understanding of the signals encountered by NK cells across time and space may reveal new therapeutic targets to harness the full potential of NK cell therapy for cancer.

Keywords: tumor microenvironment, NK cell, immunotherapy, adoptive cell immunotherapy, solid tumor, innate lymphoid cell (ILC)

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INTRODUCTION

Cancer is a collection of dynamic diseases that arise from unique tissue types and exist in different stages over time, frequently across multiple anatomical sites in advanced stages. From the primary tumor to metastases, immune status also varies over disease progression and location. Furthermore, the cellular communities present within the tumor microenvironment (TME) likely evolve over time and space. Contemporary studies have focused on antitumor immunity at a single point in time, but evidence from comparisons of tumor-infiltrating immune populations isolated from different tumor stages suggests that differential developmental and functional immune cell phenotypes change during disease progression, the basis of which is not yet understood (1). While many of the cell-cell interactions driving immune dysfunction in the TME have been elucidated to reveal novel immunotherapeutic targets, the mechanisms are poorly contextualized in the framework of spatially and temporally dynamic cancer states.

Natural killer (NK) cells are a subset of cytotoxic group 1 innate lymphoid cells (ILCs) which have the innate ability to detect and kill virally infected or malignant cells (2, 3). While NK cells were the first lymphocytes to demonstrate natural killing of tumor cells, they have remained relatively understudied compared to the intense focus on cytotoxic T cell therapy for cancer treatment (4, 5). However, the clinical relevance of NK cells in cancer is well-established. Increased NK cell abundance has been shown to correlate with better prognosis in multiple solid tumor types (6). Additional studies have also correlated NK cell tumor infiltration, activation and developmental status, and cytotoxic capacity with enhanced antitumor activity (7–11). NK cells have proven to be critical in the initial activation of dendritic cells (DCs), enhancing tumor neoantigen presentation to effector T cells and boosting immune checkpoint blockade (ICB) efficacy by reinvigorating tumor-infiltrating T cells (12). Therapeutic approaches directly utilizing antitumor NK cells have shown promise as well. A growing number of clinical trials are bringing NK cells to the bedside using approaches ranging from chimeric antigen receptor (CAR)-NK cells to bi- and tri-specific killer engagers (BiKEs and TriKEs), all of which have demonstrated the safety and potential efficacy of NK cells as immunotherapy in both solid and hematologic tumor contexts (13). Unleashing the full potential of these therapies will require developing strategies to assist NK cells in navigating the complex immunosuppressive mechanisms of the TME. While NK cells may encounter different immunosuppressive signals than tumor-infiltrating T cells, recent studies suggest that there is overlap between immune checkpoint pathways in the two cell lineages. However, the specific microenvironmental influences, signaling pathways and downstream effects merit further study in cell- and tissue-specific contexts to fully release the therapeutic potential of NK cells in the immunosuppressive tumor milieu (14–16).

When viewed through the lens of heterogeneous tumor architecture, temporal disease states, and diverse and dynamic cellular communities, we can begin to understand the complex changes in NK cell states produced by the sum of these elements. Both preclinical and clinical evidence point to a paradigm of continuous changes in NK cell development and activation over the course of cancer progression (1, 17, 18). Comparisons of NK cell states within the TME from early to advanced disease may provide insight into specific mechanisms that can be targeted to restore antitumor NK cell function in advanced cancers. Furthermore, as our understanding of ILC heterogeneity expands, we will be able to better interpret studies describing the complex array of NK and ILC states present in the tumor that can dictate antitumor responses. Here, we describe the complex dynamics of cancer progression through different stages, tissues, and treatments as they relate to NK cell function. We also outline the network of tumor-intrinsic and NK cell-intrinsic changes during cancer that can alter effective NK cell antitumor function, as well as new approaches available to better study the sum of these interactions. A holistic understanding of the heterogeneous physical, spatial, and temporal interactions affecting NK cells in the TME may reveal new immunotherapeutic targets and engineering approaches to fully unleash the potential of NK cell therapy in cancer.

Key Variables in Tumor State-Specific NK Cell Function

While commonly conceptualized as a single homogenous mass, tumors are composed of genetically heterogenous clones, and disease progression takes place over time and space. Moreover, patients receiving NK cell-based therapies will likely have already undergone multiple cycles of cytotoxic and immune modulating therapies, which may alter the activation or developmental status of the patients' NK cells. We will refer to these broad influences of cancer progression as the "tumor state"; which includes cancer stage, prior therapies, architecture, and anatomy (**Figure 1**).

Cancer Stage

Staging is a clinical definition based on the tumor size, location, and degree of spread throughout the body. Solid malignancies are staged by the tumor-node-metastasis (TNM) system, which assigns a numerical value to the tumor size, local and regional lymph node involvement, and distant metastasis (19). TNM classifications are then grouped into stages 0-IV. While the specific tumor size and lymph node involvement criteria vary for different cancer types, in general, stage 0 describes a non-invasive tumor such as ductal carcinoma in situ; stage I and II disease are confined to the primary site or involve local lymph nodes; and stage III and IV describe disease that has spread to distant lymph nodes or metastasized to other organs, respectively. Intrinsic to this model are progressive changes to the TME as tumors gain the capacity to grow larger, evade immunosurveillance and therapy, and disseminate. Relatively few studies examine the temporal changes to the functional and developmental status of antitumor immune cells, while ICB is largely still reserved for later stage advanced, unresectable, or relapsed disease in the clinic.

NK cells isolated from invasive breast cancer patients exhibited an altered phenotype and function compared to NK cells from patients with noninvasive carcinoma *in situ* (NCIS). Comparison between healthy donor, benign mammary tumor, NCIS, and localized, locally advanced, and metastatic invasive breast cancer peripheral blood NK cells displayed progressively decreased expression of activating receptors NKp30, NKG2D, 2B4, DNAM-1, CD16, and NKp46, as well as increasing expression of inhibitory NKG2A and CD85j/LIR-1 (20). Functionally, peripheral NK cells from late-stage disease displayed decreased cytotoxicity and interferon-γ (IFN-γ) production compared to peripheral NKs from patients with benign or noninvasive tumors. Tumor tissue was enriched in immature $CD56^{bright}$ NK cells compared to peripheral blood or paired normal mammary tissue from the same patient. Compared to peripheral blood, tumor-infiltrating NK cells expressed lower levels of activating receptors and had decreased cytotoxic function *in vitro*. Notably, the premalignant NK cell phenotype was restored in patients who had undergone surgical resection of breast cancer and not

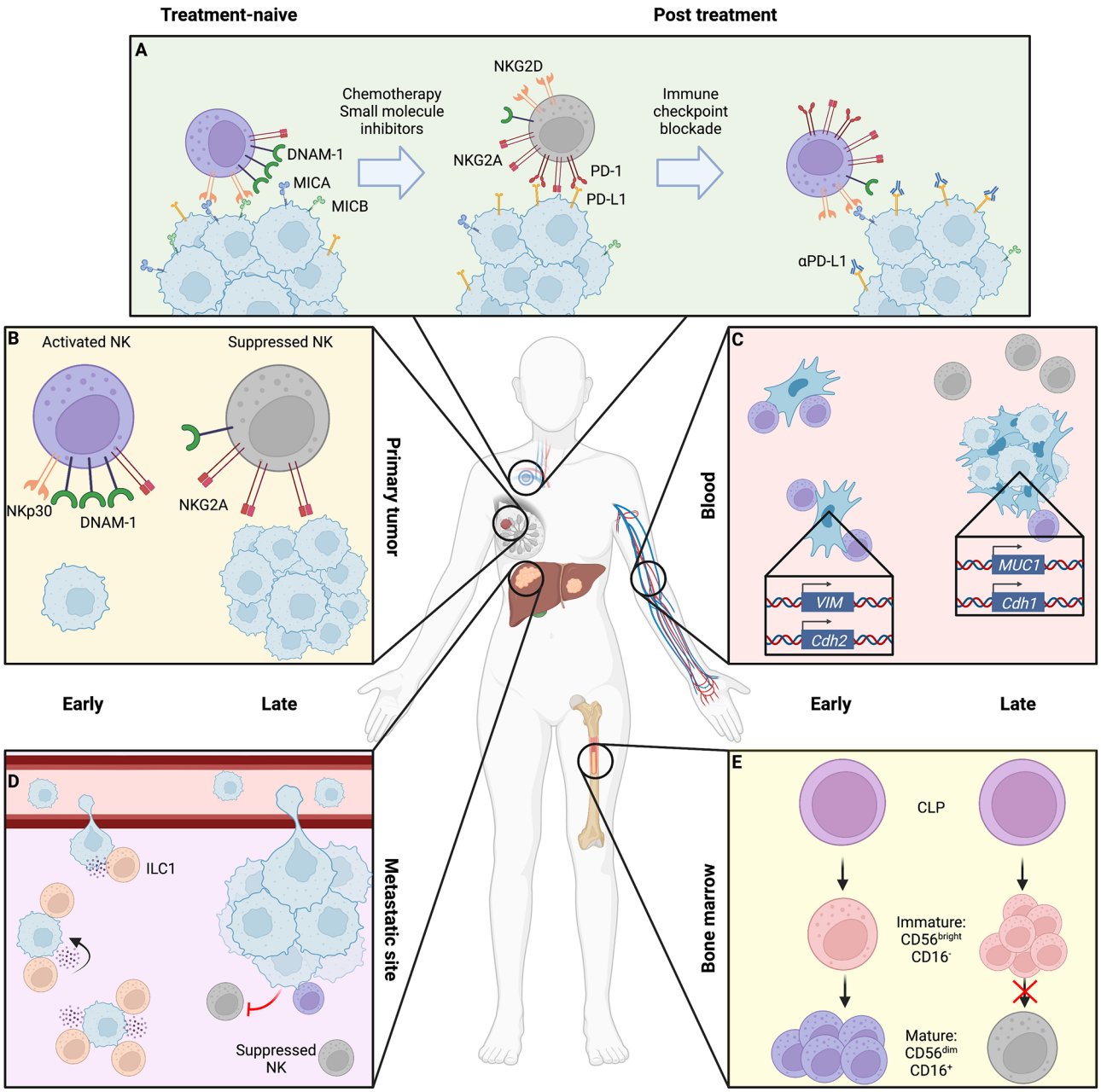

FIGURE 1 | Location- and stage-dependent changes in NK cell status and function. Magnified bubbles represent cancer therapy **(A)**, primary breast tumor **(B)**, peripheral blood with circulating tumor cells (CTCs) **(C)**, liver metastasis **(D)**, and bone marrow niche **(E)**. **(A)** During treatment, cytotoxic chemotherapy and small molecule inhibition can induce NK cell resistance through downregulation of NK activating ligands and upregulation of immune checkpoint molecules on the tumor surface, coupled with a shift in NK cell receptor repertoire toward inhibitory signaling. These may be overcome by sequential immune checkpoint blockade to release suppressed NK cells. **(B)** The late-stage primary tumor induces immunosuppressive changes in NK cells, causing reduced expression of activating receptors like DNAM-1 and NKp30 as well as cytotoxic effector molecules perforin and granzyme, while upregulating inhibitory receptors such as NKG2A. **(C)** In the blood, early-stage CTCs that have fully undergone epithelial-to-mesenchymal transition (EMT) expressing N-cadherin and vimentin are efficiently targeted by NK cells which are overcome by NK-suppressive mixed epithelial-like CTCs retaining mucin or E-cadherin expression in late disease. **(D)** At metastatic sites, ILC1s and NK cells may cooperate to respectively inhibit metastatic seeding and kill tumor cells within established lesions, though by late disease cytotoxic cells are suppressed toward an immature, dysfunctional state. **(E)** NK maturation in the bone marrow niche may be suppressed by tumor presence, arresting NK cells in an immature state. Created using Biorender.com.

relapsed for at least 5 years after surgery. These data suggest that as the tumor state progresses, there is progressive yet reversible inhibition of NK cell development and terminal maturation in both the tumor and periphery. These findings are consistent with evidence from mouse models that tumor burden leads to impairments in peripheral NK cell maturation (21). However, this study did not compare intra-tumoral NK cells between NCIS and invasive carcinoma, as the authors noted low lymphocyte yields from patient tumor samples. Future studies using single cell approaches may better resolve differences in NK cell profiles between tumor stages and detect distinct subtypes. Similar stage-specific impairment of NK cells has been seen in melanoma, as peripheral NK cells from treatment-naïve stage IV melanoma patients displayed impaired degranulation in tumor co-culture compared to NK cells isolated from earlier stage patients (22). Additionally, there was overall increased heterogeneity in NK cell expression of activating receptors NKp46, NKp30, NKG2D, and NKp44 in patients compared to healthy donors. Stage III and IV patients with locally advanced or metastatic disease had higher variance in HLA-DR expression. NKp46 expression overall decreased as disease stage progressed, while higher expression in specific patients correlated with prolonged survival. Together, these suggest that peripheral NK cell impairment in melanoma progresses in a stage specific manner. However, while peripheral NK cell activation status may have important implications for control of systemic metastasis, particularly in late-stage patients, comparison of intra-tumoral NK cells from resected tumors may provide deeper insight into TME-specific immunosuppression.

Treatment History

Although immunotherapy has come to the frontline in an increasing number of tumor types depending on tumor expression of immune checkpoint molecules, ICB is frequently used as an alternative treatment for patients with advanced disease (23, 24). Prior chemotherapy, small molecule inhibitor treatment, or immunotherapy can significantly impact the local and systemic immune landscape. Coupled with highly individualized patient treatment histories, understanding the effects of prior therapy is a crucial aspect of translating studies of tumor immune contexture to the realities of patient care.

Small molecule inhibitors are the mainstay of treatment for multiple cancers (25). Targeted inhibitors first entered clinical use for hematologic malignancies in the form of tyrosine kinase inhibitors like imatinib and dasatinib (26). NK cell lymphocytosis has been reported as a marker of favorable response to dasatinib treatment in patient with chronic myelogenous leukemia (CML) (27). However, studies investigating the functional effects of dasatinib on NK cells have revealed discordant results. A study of 8 CML patients undergoing dasatinib treatment revealed significantly elevated NK cell levels after treatment, while *in vitro*, NK cells isolated from patients who experienced lymphocytosis exhibited increased cytotoxicity in a ^{51}Cr release assay (28). These results were supported by studies indicating increased NK cell proliferation and cytotoxicity after *in vitro* dasatinib treatment of healthy human peripheral blood cells (27). Another study of peripheral blood from CML patients suggested that dasatinib treatment, but not imatinib or nilotinib, induces long-term suppression of inhibitory NKG2A on NK cells with accompanying increased cytotoxicity (29). Conversely, *in vivo* dasatinib treatment in mice resulted in decreased NK cell clearance of MHC class I-deficient RMA-S tumor cells (30). Direct comparison of these results may be confounded by differences in patient histories, dosing, and preclinical models, but further study may reveal the underlying variables that dictate the observed variability in NK cell activation or inhibition after dasatinib exposure. In melanoma, inhibitors of the common driver mutations MEK and BRAF are now frequently used in combination with ICB (31, 32). Similar to the varying effects of tyrosine kinase inhibition, there is conflicting evidence surrounding the effects of BRAF inhibition on NK cell sensitivity. One preclinical study suggested that vemurafenib-resistant melanoma cell lines were more sensitive to NK cell killing, while another study found that vemurafenib treatment led to NK cell resistance *via* decreased tumor expression of activating ligands (33, 34). While inconsistent, these data suggest that BRAF inhibition significantly modifies tumor cell interactions with NK cells and merit deeper consideration in the context of patient treatment. Other small molecules such as MEK inhibitors have been suggested to exhibit broadly proimmunogenic roles, suggesting potential for combination therapy with immunotherapy (35).

Traditional cytotoxic chemotherapy can have varied effects on the immune system due to its ability to target all rapidly-dividing cells (36). At standard doses, chemotherapy may activate antitumor immunity through induction of immunogenic cell death or modulation of tumor-intrinsic immune escape mechanisms. Low immune-modulating doses of chemotherapy can also be used to deplete specific subsets of immunosuppressive cells, for example low-dose cyclophosphamide for regulatory T cell (Treg) depletion. NK cells from stage IV melanoma patients who have undergone cytotoxic chemotherapy with dacarbazine, fotemustine, cisplatin, vincristine, or cyclophosphamide displayed heightened expression of the inhibitory receptor NKG2A as well as the activating receptor NKp46 (37, 38). Prior to treatment, NK cells were found to be NKp46^{lo}NKG2A^{lo} but converted to an NKp46^{hi}NKG2A^{hi}CD158a^{lo} phenotype after chemotherapy administration. However, NK cells derived from patients treated with chemotherapy had decreased cytotoxic functionality in coculture, potentially due to increased inhibitory NKG2A signaling. Preclinical studies indicated that cisplatin-resistant non-small cell lung cancer (NSCLC) cell lines were resistant to NK cell killing *via* upregulated programmed death ligand 1 (PD-L1) and decreased NKG2D ligand expression (39). Treatment with anti-PD-L1 ICB and MEK inhibition synergized to restore NK cell sensitivity. Consistent with these results, biopsies from NSCLC patients who had received neoadjuvant cisplatin revealed increased PD-L1 expression after cisplatin treatment, while anti-PD-L1 treatment in mouse models *in vivo* effectively inhibited tumor growth (40). Together, these data indicate that prior chemotherapy can drastically modify tumor susceptibility to NK cell cytotoxicity.

ICB, though initially developed to prevent cytotoxic T cell inhibition, has been shown to affect NK cells. Mouse NK cells activated *in vitro* upregulated the stimulatory receptor CD28 and immune checkpoint CTLA-4, which both compete for binding to ligands CD80 and CD86, and responded to receptor engagement by increasing or decreasing IFN-γ production, respectively (41). NK cells expressing CD28 and CTLA-4 were also identified in mouse RMA-S, melanoma, and lung tumors, and responded to CTLA-4 activation *ex vivo* with decreased IFN-γ production (14). Furthermore, ipilimumab (a CTLA-4 blocking antibody) efficacy in melanoma patients positively correlated with increased CD56 transcript expression in post-treatment tumor biopsies (42). In another cohort of ipilimumab-treated advanced melanoma patients, NK cells shifted toward a mature $CD56^{dim}$ phenotype expressing increased NKp46 and TIM-3 and decreased inhibitory killer Ig-like receptors (KIRs). Both $CD56^{bright}$ and $CD56^{dim}$ NK cells expressed increased CD16 and PD-1. Thus, as immunotherapy increasingly becomes a frontline treatment option and patients begin to experience sequential rounds of ICB, the effects of prior immunotherapy on NK cell status will need to be considered for future immunotherapy or cell therapy efficacy. Additionally, clinical trials combining cytotoxic chemotherapy and ICB further support the nuances of cumulative treatment effects on antitumor immunity. Combined ipilimumab and dacarbazine therapy for stage IV melanoma patients yielded increased survival compared to dacarbazine alone (43). Moreover, in both NSCLC and small-cell lung cancer (SCLC), sequential cycles of paclitaxel and carboplatin chemotherapy followed by chemotherapy plus ipilimumab were superior to the reversed sequence of ICB followed by chemotherapy, suggesting that prior chemotherapy can play a clinically significant role in immune priming (44, 45). Deeper elucidation of how NK cells respond and interface with adaptive immunity after combined chemotherapy, small molecule inhibitor, and ICB treatment will help guide logically designed combination therapy regimens.

Anatomical Location

While NK cells can be found in solid tumors, the absolute level of NK cell infiltration into the TME varies drastically by primary tumor site. In a comparison of tumor-infiltrating NK cell quantity across tumor types, tumors with the highest infiltration of NK cells included acute myeloid leukemia (AML), diffuse large B-cell lymphoma (DLBCL), and testicular germ cell tumors, while CRC, breast cancer, and melanoma exhibited low NK infiltration (46). These data suggest that there are tissue-specific determinants of NK cell infiltration in tumors, and studies of healthy tissue may guide our understanding of tumor-specific NK cell modulation. For example, mucosal epithelium exposed to the external environment, such as the lung and gut, or organs that encounter high blood flow like the liver have been found to contain specialized ILC subtypes as well as the ability to preferentially attract circulating NK cells (47, 48). This may be driven by specific inflammatory signals encountered at these different sites. The lung alveoli are constantly exposed to pathogens and external toxins and therefore demand a significant innate immune defense, provided partly in the form of lung-resident NK cells as well as increased migration of circulating NK cells into the tissue during infection (47). Recruited circulating NK cells have been found to express upregulated CD49a as well as increased IFN-γ and perforin (49). Similarly, liver-resident NK cells may be exposed to the constant flow of pathogens in blood filtering through hepatic sinusoids, whereby ligands uniquely expressed in the hepatic sinusoidal microenvironment maintain the population of liver-resident NK cells through activation of CCR5 and CXCR6 (47). Understanding similar factors that are unique to tumors based on organ of origin may reveal targets to modulate NK cell attraction and function within the tissue.

Additionally, multiple studies have indicated the existence of organ-specific ILCs and tissue-resident NK cell (trNK) populations in normal physiology (47, 50). For example, liver-specific type 1 ILCs (ILC1s) have been identified based on expression of CD200r1 and CD49a with dependence on the transcription factor Hobit for terminal maturation into cytotoxic effector cells (51, 52). Thus, it is possible that the signals responsible for tumor-type-specific ILC variation overlap with the mechanisms governing ILC specificity in healthy tissue. Indeed, unique populations of cytotoxic tumor-infiltrating ILCs have been identified in the polyoma middle T virus (PyMT) mouse model of spontaneous breast cancer (18). Tissue-specific differences in intra-tumoral NK infiltration therefore most likely differ not only in absolute quantity of NK cells but also in specific ILC subtype, and further study will be needed to identify the determinants of both.

NK cells play a critical role in metastatic control (53). In patients, metastatic load has been found to inversely correlate with quantity of both circulating peripheral and intra-tumoral NK cells in multiple cancer types (53). In hepatocellular carcinoma (HCC), the abundance of IFN-γ -responsive peripheral NK cells was predictive of recurrence risk after curative surgery or radiotherapy, suggesting that circulating NK cells actively contribute to control of residual circulating tumor cells (CTCs) with metastatic capacity after resection or resolution of the primary tumor (54). It is likely that NK cells and other ILCs exert anti-metastatic effects at different stages of the metastatic cascade, for example both controlling CTC dissemination in the blood and surveilling the site of metastatic seeding in tissues. Indeed, a recent study found that liver metastases in a mouse model were monitored by both circulating NK cells as well as tissue resident ILC1 (55). In this study, the authors found that ILC1 primarily served to limit metastatic seeding, while NK cells infiltrated metastatic nodules to exert cytotoxic effects within established lesions. The metastatic niche was reciprocally able to educate infiltrating NK cells toward unique populations of immature $CD49a^{-}$ $Eomes^{-}$ and cytotoxic $CD49a^{+}Eomes^{+}$ phenotypes in a tumor type-dependent manner. With greater understanding of metastatic control by NK cells in spatial and temporal contexts, adoptive NK cell therapy could potentially be used as an adjuvant therapy to control micrometastases or CTCs after

resection of the primary tumor. Conversely, neoadjuvant NK cell therapy for unresectable advanced disease may have the capacity to specifically reduce metastatic burden so that surgical treatment becomes a viable option.

During tumor progression, CTCs are thought to seed metastatic sites on a microscopic level. CTCs have been proposed to fall into monoclonal or polyclonal subsets, whereby monoclonal CTCs have undergone epithelial to mesenchymal transition (EMT) and exist primarily as isolated tumor cells in the blood, while polyclonal CTCs retain some epithelial characteristics, allowing them to traverse the vasculature in clusters (56). A recent study suggested that single CTCs that have fully undergone EMT were more susceptible to NK cell killing due to upregulation of NKG2D ligands and downregulation of NK-inhibiting HLA molecules, while polyclonal epithelial-like CTCs were resistant and more efficiently seeded metastases (57). Supporting this hypothesis, it has been shown that NK cells can more efficiently kill mesenchymal-like cancer cells compared to more differentiated epithelial-like cells that had not undergone EMT (58). NK cells may also suppress metastasis by maintaining disseminated tumor cells in a dormant state, as local deactivation of NK cells by activated hepatic stellate cells (aHSCs) resulted in release of dormant $p27^+$ tumor cells in the liver and subsequently increased metastatic outgrowth (59). Local NK suppression was determined to be mediated by CXCL12 released by aHSCs interacting with CXCR4 on the NK cell surface. Maintenance of tumor dormancy was found to be dependent on NK cell production of IFN-γ. Together, these suggest that NK cells are important for multiple steps of the metastatic cascade, and targeted application of NK cells to control disease spread may prove useful for treatment of cancers like PDAC that frequently present late in disease course.

Tumor Microenvironment Structure

Tumors are constructed of diverse structural parameters that affect immune function. Spatial immune infiltration has become a factor of interest for immunotherapy of solid tumors. Solid tumors have been described to exhibit both inter- and intra-tumoral spatial heterogeneity in multiple studies (60–63). One classification schema sorts areas into immune inflamed, immune excluded and immune desert, with unique molecular features to each spatial subtype (64). However, categorization of tumors based on immune infiltration to this point has focused heavily on differential T cell infiltration, with less emphasis on the spatial heterogeneity of other cell types (65). The precise spatial distribution of NK cells in patient tumors is poorly examined in the literature, though studies have confirmed that NK cells are able to localize to tumor tissue (61, 66). A study using multiplex IF staining of tissue microarrays from periampullary adenocarcinomas revealed that $CD56^+NKp46^+$ NK cells tend to be confined to the stromal compartment rather than infiltrating tumor nests (61). Similarly, studies of renal cell carcinoma (RCC) tumor microarrays also identified NK cells as excluded to the peri-tumoral region along with both helper and cytotoxic T cells (67). High grade tumors were found to contain more peri-tumoral granzyme B^+ NK cells, while tumors containing more $LAG3^+TIM3^+$ exhausted T cells tended to also contain more intra-tumoral and peri-tumoral granzyme B^+ NK cells. These data point to the complex spatial interactions that NK cells engage in the TME, and applying similar spatial approaches to other tumor types may reveal clinically relevant local immunosuppressive cell communication networks.

In many tumors, stroma makes up a major portion of tumor tissue. While stromal cells play a key role in immunosuppression, the buildup of fibrotic tissue, termed desmoplasia, is also thought to have a significant impact on tumor progression (68–70). In breast cancer, the tumor stroma to immune infiltration ratio was found to be a significant predictor of recurrence-free period (71). 87% of patients with high levels of infiltrating cytotoxic T cells and NK cells combined with low density of tumor stroma were recurrence free 10 years post tumor resection, whereas only 17% of patients with low immune infiltrate and high stroma remained free of disease recurrence. Likewise, a TME risk score calculated for gastric cancer based on high/low NK cell infiltration and high/low stroma was predictive of chemotherapy response and survival in gastric cancer patients (7). Patients with tumors that had high NK cells and low stroma had the best prognosis while NK low/stroma high patients had the worst outcomes of the studied group. While this study identified NK cell and tumor stromal abundance as independently prognostic factors, they could also be linked through direct effect of NK cells on fibrosis. In the liver, NK cells have been shown to have a direct antifibrotic role, though their activity is antagonized by aHSCs. NK cells have been suggested to attenuated diet- or carbon tetrachloride-induced liver fibrosis in mice in an IFN-γ-dependent manner, and antibody-mediated blockade of NKG2D or TRAIL abrogated NK cell antifibrotic activity (72). In hepatitis B and C patients with liver cirrhosis, aHSCs were able to undergo emperipolesis with antifibrotic NK cells in a TGF-β manner, creating cell-in-cell structures in which NK cells underwent apoptosis (73). It has yet to be determined if NK cells display antifibrotic activity within desmoplastic tumors. Furthermore, considering the previously described dynamics of aHSC-metastatic niche formation and antimetastatic NK cell function, it will be interesting to examine the interplay between fibrosis, metastatic seeding, and NK cell surveillance.

As tumors grow, the accompanying growth of new vasculature is essential for nutrient delivery to rapidly-proliferating tumor cells (74). Tumor vasculature and lymphatics play multiple clinically relevant roles: vessels supplying the tumor serve as entry points for intravenously administered chemotherapies and biologics, avenues for trafficking immune cells and conduits for CTCs leaving the primary tumor to seed metastases. Tumor vasculature is known to develop in a disordered manner due to its arising from an overload of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) rather than being guided through the normal process of vasculogenesis. As such, tumor vasculature is leaky, tortuous, and fragile (75). Over disease development, poorly perfused solid tumor cores often resultantly become acidic, hypoxic, and necrotic, with

significant effects on immune function within the TME. Local acidity and hypoxia-induced molecules such as hypoxia-inducible factor 1-α (HIF-1α) have significant suppressive effects on NK cells, as previously reviewed (2). Antiangiogenic therapy targeting pro-angiogenic molecules have become a useful therapy for certain diseases, such as bevacizumab for glioblastoma multiforme (GBM) (76). Clinical trials combining bevacizumab with anti-PD-L1 therapy have shown promise in multiple studies (77). Considering the evidence that ICB both modulates and relies on NK cells for full effect, it is likely that further study will implicate NK cells in the efficacy of these anti-angiogenic approaches.

Tertiary lymphoid structures (TLSs) are histologically identifiable structures within peripheral tissues that are thought to serve as sites of lymphoid development and activation functionally similar to secondary lymphoid organs (SLOs) like the spleen. Tumor TLSs have drawn interest as a potential biomarker of response to immunotherapy or even as a concentrated source of potentially inducible antitumor immunity (78). Increased density of TLSs in biopsies was prognostic of better outcomes in multiple solid tumor types independent of stage (78). Interestingly, TLSs appear to be primarily populated by B and T cells and are devoid of NK cells, yet TLS presence correlates with higher non-TLS $CD57^+$ NK cell infiltration in oral squamous cell carcinoma (79). The mechanism of association between NK cell-devoid TLSs and NK cell infiltration has yet to be characterized but may provide further insight into how NK cells proceed to activate adaptive antitumor immunity. Notably, maintenance of TLSs in influenza infection required DC-derived lymphotoxin β (LTβ), CXCL12 and 13, and CCL19 and 21, while the presence of mature DCs in tumor TLSs was associated with increased effector-memory T cells and patient survival. These data suggest that DCs may be important for the maintenance and function of tumor TLSs, potentially *via* initial recruitment by the recently described NK-DC axis (12, 80, 81).

Unique molecular features in different tumor types may also mold the development of specific immune environments. Solid tumors have been extensively molecularly subtyped, and multiple cancers have been identified as harboring a unique common oncogenic driver (82–84). For example, >90% of pancreatic ductal adenocarcinomas (PDAC) are driven by mutant KRAS, while ~50% of melanomas house BRAF driver mutations (24, 85). Specific oncogenes can induce differential inflammatory states that may alter NK cell and other immune dynamics within the tumor (86). Oncogenic KRAS may be pro-inflammatory *via* induction of IL-6, CCL5, NF-kB, and other immune modulating molecules (87). Similarly, mutant BRAF in melanoma stimulates MAPK signaling and can result in expression of IL-6 and IL-10, promoting immune-tolerant DC maturation and inhibiting cytotoxic T cells (88). Thus, tumor-intrinsic oncogenes and mutations could represent critical upstream mediators regulating NK cell suppressive ligands expressed on multiple cell types in the TME through tumor-derived factors (87). Immunosurveillance can additionally be influenced by varying degrees of mutational load, microsatellite instability, and copy number alterations between tumors (89). To add to the complexity, the prevalence of certain molecular features within a patient's disease evolves during disease progression, likely as a product of clonal selection through treatment and immunoediting (90). Specific enrichment for intrinsically less immunogenic and more immunosuppressive tumor clones in late-stage disease may partly explain the progressive NK cell dysfunction described in previous sections. If this is true, immunotherapy at earlier timepoints as well as rational combination treatment with immunotherapy and small molecule inhibitors of driver oncogenes may prove to be useful therapeutic strategies.

Cell-Cell Interactions in the Tumor Microenvironment

The importance of the interactions between tumor and non-tumor cells that make up the TME has become increasingly clear in both research and clinical settings. Rather than focusing solely on tumor cell-intrinsic signaling, recent efforts have focused on diverse cellular components in the TME and how they interact to promote or arrest immune tumor surveillance and tumor growth (91–95). As NK cell activation is determined by the combination of activating and inhibiting signals from their environment, the cell-cell interactions that NK cells encounter in the TME have a direct impact on their functionality (**Figure 2**). While the abundance of specific ligand-receptor interactions likely varies based on key variables influencing NK cell activation within the tumor as discussed previously, we will discuss notable cell type-specific interactions which influence NK cell function in the TME.

Tumor Cells

Transformed malignant cells are natural targets for NK cells due to tumor expression of stress ligands such as MICA/MICB that interact with NKG2D on the NK cell surface (96). MHC class I normally inhibits NK cells by binding to inhibitory killer Ig-like receptors (KIRs) (96). NK cells can be activated by cells which have lost MHC class I, such as tumor cells utilizing MHC downregulation as an escape mechanism (96). Tumor cells have also evolved other methods to escape NK cell killing by expression of other inhibitory ligands. For example, tumor cells can express CD155 which interacts with both the activating receptor DNAM-1 and inhibitory receptor TIGIT on NK cells. Within the tumor, CD155 is largely overexpressed by tumor cells, though it can be also expressed on myeloid-derived suppressor cells (MDSCs) (97). Paradoxically, $CD155^+$ primary ovarian carcinoma cells have been found to decrease DNAM-1 expression on NK cells through direct contact, rendering NK cells less responsive to DNAM-1 activation (98). Another study demonstrated that membrane CD155 triggers DNAM-1 internalization and degradation, decreasing DNAM-1 on the NK cell surface and pushing the receptor balance toward inhibitory interactions with TIGIT (99). Tumor cells may also produce soluble CD155 to inhibit $DNAM\text{-}1^+$ NK cells without direct contact (100). In metastatic melanoma, high tumor expression of CD155 was predictive of poor response to anti-

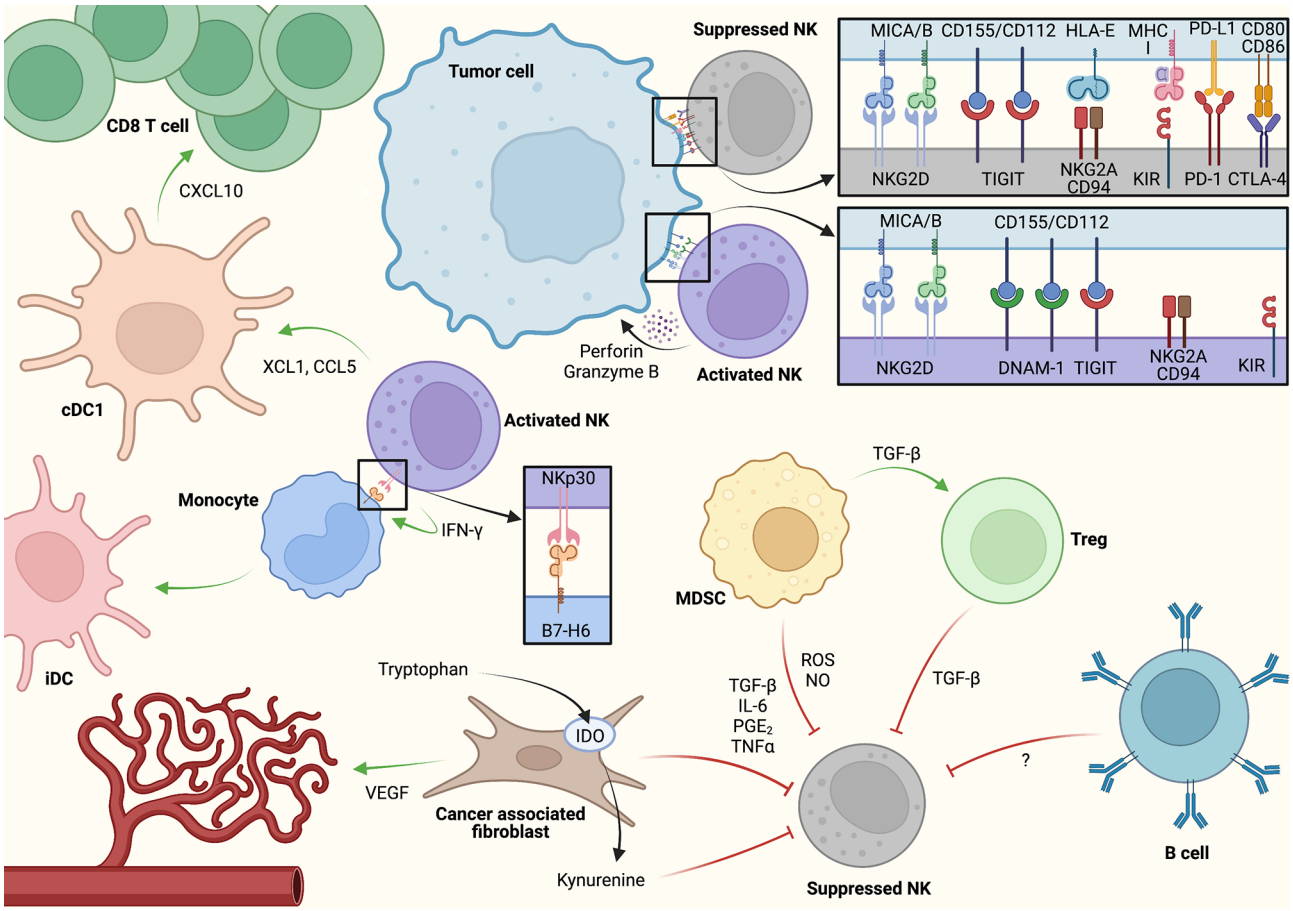

FIGURE 2 | A model for cell-cell interactions and key signaling molecules associated with NK cell activation and inhibition within the tumor microenvironment (TME). NK cells may induce adaptive antitumor immunity *via* direct conventional dendritic cell (cDC1) activation as well as through monocyte polarization toward inflammatory dendritic cell (iDC). At the same time, immunosuppressive crosstalk between cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and potentially B cells/B regulatory cells through soluble factors restrain antitumor NK cell activity. Created using Biorender.com.

PD-1 treatment (101). Furthermore, CD112 on the tumor surface has been found to function similarly to CD155, downregulating DNAM-1 and inhibiting NK cell activation, highlighting CD112 as another potential clinically relevant target (102). HLA-E, a noncanonical human leukocyte antigen (HLA) molecule, has also been shown to inhibit NK cell killing *in vitro via* interaction with the NKG2A/CD94 heterodimer found on the NK cell surface (103). HLA-E was identified as a significant negative regulator of tumor NK cell sensitivity in combined analysis of two high-throughput screening methods (104). Inhibition of NKG2A expression in another study allowed NK cells to regain cytotoxic function against HLA-E^{+} tumor cells (105). In patient tissues, HLA-E was found to be highly expressed primarily on tumor cells in neoplastic compared to healthy tissue (106). Furthermore, in gynecologic cancers, high HLA-E expression in patients abrogated the survival benefit of high levels of cytotoxic tumor-infiltrating lymphocytes, suggesting that tumor HLA-E expression is a relevant factor when considering ICB or adoptive cell therapies for patients (107). In addition to regulation of NK cell function *via* cell surface interactions, tumor modulation of NK cell function may also be partly due to chromatin alterations. In T cells, epigenetic profiling has been suggested to be a more robust method of subset stratification than transcriptional signatures (108). Accordingly, it is possible that the same is true for NK cells, and that tumor-induced epigenetic states may denote bona fide NK cell subsets. During NK cell development, expression of NKG2A is regulated by DNA methylation status (109). Furthermore, expression of the activating NKG2D receptor as well as subsequent inflammatory function is regulated by histone methylation status *via* Ezh2 histone methyltransferase and Jumonji demethylases such as UTX and JMJD3 (110–112). NK cells differentiated from hematopoietic stem cells treated with an Ezh2 inhibitor exhibited increased antitumor activity, an effect also seen in cytotoxic T cells (113). Furthermore, direct NK cell exposure to breast tumor organoids was found to induce a

"resting" NK cell state characterized by increased LAG3, KLRG1, and TIGIT expression as well as downregulation of genes involved in immune cell proliferation, adhesion, and activation (114). Functionally, resting NK cells exhibited decreased cytotoxicity and metastatic control *in vitro* and *in vivo* which was reversed with anti-TIGIT, anti-KLRG1, and DNA methyltransferase inhibitor treatment. Together these studies suggest that tumor cell contact can induce an epigenetically determined and potentially reversible dysfunctional NK cell state which could be leveraged to increase anti-tumor NK cell activity.

Stromal Cells

Cancer-associated fibroblasts (CAFs) are an abundant and active population of mesenchymal stromal cells in solid tumors, distinguished from normal tissue fibroblasts primarily by expression of specific markers including α-smooth muscle actin (SMA), fibroblast activating protein (FAP), or fibroblast-specific protein-1/S1001A4 (94). CAFs are prolific producers of soluble factors implicated in tumor immunosuppression such as transforming growth factor beta (TGF-β), indoleamine-2,3-dioxygenase (IDO), prostaglandin E2 (PGE_2), interleukin-6 (IL-6), tumor necrosis factor a (TNF-α), and VEGF (2). The effects of these molecules on tumor-infiltrating NK cells have been previously reviewed (2). In coculture experiments combining primary healthy donor NK cells with CAFs isolated from metastatic melanoma or healthy skin samples, the presence of CAFs attenuated IL-2-induced NK cell activation and upregulation of activating receptors NKp44, NKp30, and DNAM-1, while also mildly decreasing expression of perforin and granzymes (93). Direct CAF-NK cell contact was required for DNAM-1 inhibition, while NKp44 and NKp30 downregulation was found to be dependent on CAF-derived PGE_2. While CAFs cultured alone spontaneously released PGE_2, CAF-NK cell coculture significantly increased CAF PGE_2 release, suggesting that this may be an immunosuppressive mechanism prevalent within the TME. In the liver, CAFs may develop from aHSCs, a significant component of both normal liver parenchyma and the HCC TME. While the distinction between activated stellate cells and CAFs is not well defined, it has been shown that aHSCs accompanying the formation of liver metastases can secrete the chemokine CXCL12 and induce NK cell quiescence through CXCR4 binding, as previously noted (59). CAFs can also cooperate with other immunosuppressive cells of the TME to alter NK functionality. In colorectal cancer (CRC), CAFs were found to promote tumor-monocyte interactions, polarizing macrophages into an immunosuppressive M2-like phenotype (115). CAFs in CRC were also found to directly inhibit NK cell expression of activating receptors and effector molecules such as CD69, NKG2D, DNAM-1, NKp30, NKp44, perforin, and granzyme B in coculture, suggesting that CAFs in the TME can act both directly and indirectly to mediate NK cell suppression (116).

Dendritic Cells

Recently, an NK-DC axis that plays a critical role in tumor surveillance and ICB response has been described (12). In multiple COX-deficient mouse tumor models, conventional type 1 dendritic cell (cDC1) accumulation and subsequent $CD8^+$ T cell infiltration was determined to be dependent on the presence of $NK1.1^+CD3^-$ NK cells (12). Mechanistically, XCL1 and CCL5 derived from intra-tumoral NKs recruited cDC1s into the tumor, which then produced CXCL10 to attract T cells. PGE_2, the production of which depends on COX enzymatic activity, impaired both CCL5 and XCL1 production from activated NK cells and expression of the corresponding chemokine receptors, CCR5 and XCR1, in cDC1s, suggesting an immunosuppressive mechanism that may be therapeutically targeted to boost activation of adaptive immunity. These data additionally augment previous evidence that human NK-DC interactions enhance DC cross-presentation of tumor antigens and provide further mechanistic insight within the TME (117). Furthermore, specific ILC subtypes may preferentially interact with DCs, as a recent study identified a group of ILC1-like NK cells characterized as $CD27^+CD62L^-CD160^+$ virus-responsive $Ly49H^+$ NK cells during mouse CMV (MCMV) infection (118). These cells exhibited transcriptional and functional signatures of both ILC1s and NK cells but interestingly were also marked by high expression of Batf3 and XCL1. ILC1-like NK cells were found to be resident in the spleen and were necessary for preferential clustering of cDC1s to prime antigen-specific $CD8^+$ T cells, though it has yet to be determined if these cells were truly tissue-resident or circulating NK cells that had transiently extravasated into the tissue during infection. It will be interesting to study the presence and potential role of these ILC1-like NK cells in the tumor, as these new subtypes suggest that subset-specific NK-DC priming in the TME may provide ways to improve DC-based immunotherapies.

Studies of NK-DC interactions in other inflammatory states further suggest that the function of these cell types are closely linked (119). Human NK cell interaction with $CD14^+CD16^-$ monocytes was found to influence monocyte differentiation into DCs *via* cell-cell interactions stabilized by NKp30 (120). Early on in this process, some monocytes were killed by NK cell cytotoxicity, but remaining monocytes were polarized toward DC differentiation *via* IFN-γ. Early monocyte culling by NK cells seen in this study is also reminiscent of evidence suggesting that cytotoxic NK cells edit the pool of immature DCs to remove less immunogenic precursors and make room for expansion of more immunogenic DCs (121, 122). In these studies, human DCs matured from an NK cell-edited pool exhibited a higher capacity to activate cytotoxic T cells. While these data further support the importance of an NK-DC axis in bridging innate and adaptive immunity, it is likely that unique cytokine milieus within the TME may alter the potency of NK-derived cytokines driving DC activation, and further study could guide the design of adoptive cell-delivered cytokine payloads for local modulation of cytokine balance and DC function. With the understanding of NK-DC-T cell cross-activation in tumor surveillance, one outstanding question is how the timing of these interactions affects optimal antitumor activity. In inflammatory states, NK cells are thought to be rapid responders existing in a poised epigenetic state to facilitate rapid expression of effector chemokines such as IFN-γ (123). If NK cell activation is required to induce the maximal

antitumor response from the adaptive immune system downstream, it is possible that NK cells play a primary role in surveillance during early tumorigenesis, after which the responsibility of tumor control transitions to activated cytotoxic T cells in later disease stages. Understanding these dynamics may provide the basis for rational design of immunotherapy regimens that selectively modulate innate or adaptive immunity based on cancer stage.

Myeloid Cells

Myeloid cells in the TME exist largely as MDSCs and tumor-associated macrophages (TAMs) (124). MDSCs are subdivided into phenotypically and functionally different polymorphonuclear and monocytic MDSCs (PMN-MDSCs and M-MDSCs, respectively). PMN-MDSCs more commonly populate peripheral lymphoid organs, while M-MDSCs are more commonly found in the tumor itself and promote monocyte differentiation into TAMs. These cells suppress antitumor immunity through a variety of mechanisms, generally involving the production of reactive oxygen species (ROS) or soluble factors similarly to CAFs. The effects of these soluble inhibitory factors on NK cells have been previously reviewed (2). MDSCs can also inhibit NK cells and other immune effector cells through direct contact *via* immune checkpoint molecules or CD155-TIGIT interactions (125, 126). Clinically, MDSC accumulation is associated with disease progression in multiple tumor types, as well as being a prognostic marker in NSCLC, breast cancer, gastrointestinal malignancies, and melanoma (127).

In PyMT mice, TAMs were found to inhibit NK cell cytotoxicity in a TGF-β-dependent manner (128). Coculture with TAMs induced a $CD27^{-}CD11b^{+}$ terminally differentiated phenotype. Adoptive transfer of splenic MDSCs from tumor-bearing mice were similarly able to suppress NK cell antitumor function in a proposed contact- and STAT5-dependent manner, suggesting that both intra-tumoral and peripheral MDSCs possess spatially and mechanistically distinct modes of NK cell suppression (129). In another study, TAMs isolated from human gastric cancer had a similar capacity to impair NK cell IFN-γ and TNF-α responses as well as proliferation (130). "Adaptive" human NK cells that exhibit features of memory response to cytomegalovirus (CMV) infection were found to be uniquely resistant to patient-derived MDSC suppression due to decreased TIGIT expression (126). MDSCs increased CD155 and CD112 expression in an ROS-dependent manner, and CD155-TIGIT engagement inhibited ERK and ZAP70 signaling in NK cells. These data appear to support studies in advanced melanoma suggesting that cytokine-induced memory-like NK cells can exhibit superior antitumor function (131). Additionally, TIGIT-blocking ICB may be a promising new avenue toward releasing MDSC inhibition of NK cells in the TME. Indeed, primary analysis of the CITYSCAPE trial examining combined anti-TIGIT antibody tiragolumab plus anti-PD-L1 atezolizumab for locally advanced or metastatic NSCLC suggested a significant improvement in overall response rate and progression-free survival (132). As a significant component of MDSC immunosuppression is derived from crosstalk with other suppressive members of the TME, for example induction of Tregs *via* TGF-β, targeting MDSCs as a communication hub of immunosuppression may be an efficient method to abrogate the induction of multiple NK-suppressive cell types (127).

Regulatory T Cells

Tregs in the TME restrain antitumor NK cells. Regulatory T (Treg) cell and NK cell function are inversely correlated in multiple solid tumors including gastrointestinal stromal tumor (GIST), CRC, and prostate carcinoma (133). In cervical carcinoma, tumors were enriched for TGF-β expressing Tregs compared to peripheral blood, and Tregs were able to suppress NK cell function *ex vivo (*134). In patients with HCC, decreased $CD56^{dim}CD16^{+}$ NK cells were found in tumor regions associated with increased $CD4^{+}CD25^{+}$ Treg infiltration (135). Tregs can inhibit NK cell function *via* multiple mechanisms. In mice, diphtheria toxin-mediated ablation of Tregs resulted in increased NK cell cytotoxicity against missing-self targets *via* increased availability of IL-2 (136). Adoptively transferred Tregs actively suppressed the ability of NK cells to control metastasis *via* TGF-β in T cell-deficient *Rag1*$^{-/-}$ mice, while Treg depletion combined with IL-12 therapy similarly boosted NK cell control of primary and metastatic mammary carcinoma (137). Multiple approaches to Treg depletion *via* low-dose cyclophosphamide, depleting antibodies, ICB, and small molecules have been trialed in preclinical and clinical studies, with varied results (138). Further progress in Treg depleting strategies may depend on improved specificity, while combination therapy with other immune-activating treatment modalities will warrant caution due to the potential for exacerbated immune adverse events.

B Cells

B cells are potent activators of the immune system but the role of tumor-associated B cells is poorly understood. Combined antigen receptor and scRNA-seq analysis of B cells isolated from triple-negative breast cancer compared to peripheral blood revealed that intra-tumoral B cells exhibit greater clonal diversity and more differentiated memory and plasma B cells with higher rates of somatic hypermutation (SHM) (139). The presence of germinal center, marginal zone, and class-switched memory B cells suggested the presence of active tertiary lymphoid structures (TLSs) within tumors. Additionally, higher transcript levels of CD20 and naïve and memory B cell signatures from tumor samples correlated with both improved overall survival and disease-free survival. Other studies support the role of B cells in maintaining TLSs *via* cytokine and chemokine production in inflammatory settings such as chronic obstructive pulmonary disease or in gut lymphoid structures (140–142). NK cells and B cells may engage in functionally relevant cross-talk. NK cells can enhance production of IgG and IgM by preactivated B cells and initiate early steps of B cell class switching *via* IFN-γ secretion and direct CD48-CD2 interactions (143). While NK-B cell interactions within tumors have not been well-characterized, a recent study showed that r PD-L1 and CTLA-4 expressing regulatory B cells

can regulate NK cell activity (140). Further study may reveal novel pathways of NK cell reactivation, particularly in the context of TLSs.

NK Heterogeneity and Developmental Arrest in the TME

Normal NK cell development

The process of NK cell development in both mice and humans is well defined (112, 144). In brief, common lymphoid progenitors (CLPs) in both species originate from hematopoietic stem cells in the bone marrow. CLPs destined to become NK cells then undergo a commitment and maturation process defined by the gain and loss of species-specific cell surface markers over time. In mice, $CD122^+$ precursors gain NKG2D expression, followed by NKG2A, DNAM-1, NK1.1, NKp36/NCR1, L-selectin, and leukosialin to become immature NK cells (144). Mature NK cells express CD51, CD49b, and KLRG1, while terminal mouse NK cells go on to acquire CD43, CD11b, and expression of Ly49 subsets such as Ly49H. Mouse NK cell maturation is commonly assessed by expression of markers CD27 and CD11b, where maturing cells progress through double negative $CD27^-CD11b^-$, $CD27^+CD11b^-$, double positive $CD27^+CD11b^+$, and finally "mature" $CD27^-CD11b^+$ stages. Similarly, human NK cells undergo a maturation pathway in which CD122 expression denotes CLP commitment to the NK lineage (144). Maturing human NK cells express CD56 and gradually gain expression of NKG2D, NKp36/NCR1, NKp30/NCR3, and finally NKP80/KLRF. $CD56^{bright}NKp80^-$ cells express maximal levels of NKG2D, NKp36, NKp30, NKG2A, and CD161, after which they mature into a $CD56^{bright}NKp80^+$ inflammatory cytokine-producing state. The final stage of human NK cell maturation is traditionally thought to be the more cytolytic, less inflammatory $CD56^{dim}CD16^+$ state, though there is controversy regarding whether the progression from $CD56^{bright}$ to $CD56^{dim}$ represents a linear progression of maturation, two differing terminal stages, or plastic interchangeable NK cell states (145).

NK Cell Development in Cancer

Multiple studies have observed NK cells in an immature and less activated state in tumors (1, 20, 21). Tumor presence has been shown to alter peripheral NK cell maturation in primary and secondary lymphoid organs (SLOs) (21). In mice, the presence of thymoma, breast, colon, and melanoma cell line-derived subcutaneous tumors led to inhibited NK cell development in the bone marrow, as evidenced by reduced mature $NK1.1^+CD11b^+$ NK cell fractions in tumor bearing mice compared to controls (21). Spleens from tumor-bearing mice also showed a decreased fraction of mature NK cells, though to a lesser degree than in the bone marrow. This peripheral maturation arrest was independent of T cell presence or bone marrow metastasis. Functionally, tumor-induced immature NK cells had decreased ability to control tumor growth *in vivo* and exhibited decreased IFN-γ production in response to *in vitro* stimulation with IL-15. Within the tumor itself, studies using PyMT mice showed that breast tumor-infiltrating NK cells in late-stage tumors exhibited an increased proportion of immature double negative $CD27^-CD11b^-$ cells compared to the spleen, suggesting that factors in the TME may inhibit NK cell maturation or cause de-maturation of mature NK cells in the local environment as well (1). These data raise the question of how spatial and temporal factors regulate tumor-induced NK cell maturation dysfunction, whether through de-maturation of mature NK cells in the TME or preferential attraction of immature NKs. Further studies will be important to understand where and when immature NK cells are generated during tumorigenesis and what combination of mechanisms is responsible, providing insight toward developing strategies of restoring intra-tumoral NK cell maturation.

In human disease, examination of metastases and lymph nodes from patients with advanced melanoma revealed that infiltrating NK cells were predominantly immature and less cytotoxic $CD56^{bright}CD16^-$ subsets, while mature cytolytic $CD56^{dim}CD16^+$ tumor-infiltrating NK cells that were present expressed lower levels of granzyme B and perforin compared to NK cells in peripheral blood (131). This suggests that the TME can affect both maturation and effector function, though the discussion surrounding the origin of $CD56^{bright}$ versus $CD56^{dim}$ NK cells raises the question of whether these cells are developmentally arrested or represent differential immunosuppression of two distinct terminal NK cell types. Other studies have found enrichment of $CD56^{bright}Perforin^{low}$ NK cells in breast and lung tumors, while colon cancer tissues contained an increased proportion of $CD56^{dim}CD16^+$ tumor-infiltrating NKs similar to melanoma (146). NK cells isolated from NSCLC tissues exhibited an inhibited phenotype, with decreased activating receptors NKp30, NKp80, CD16, NKG2D, ILT2, and DNAM-1 and increased NKp44, NKG2A, CD69; these cells were also functionally impaired compared to circulating peripheral blood NK cells based on decreased tumor killing and degranulation in tumor cell co-culture (147). Likewise, intra-tumoral NK cells from invasive breast cancer expressed less NKp30, NKG2D, DNAM-1, CD16, CD25, CD57, perforin, granzyme, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), as well as higher NKG2A and NKp44 compared to both normal breast tissue and carcinoma in situ (20). Together, these data suggest that NK cells in a wide range of human tumor types are locally rendered immature and dysfunctional, even compared to peripheral blood NK cells from the same patient. Interestingly, increased NKp44 observed on NK cells in NSCLC and breast cancer may alternatively suggest that these cells have been activated by the TME, attempted to respond to tumor, and have now entered a state resembling immune exhaustion. Indeed, NK cells from mice exposed to continuous IL-15 stimulation for 15 days expressed decreased NKG2D, DNAM-1, TRAIL, and FasL alongside increased NKG2A, similar to the phenotype seen in patient tumors (148). However, It may also be possible that these cells represent activated immature NK cells in the TME, or even an activated phenotype of other ILC lineages. Clinically, accumulation of immature NK cells was associated with worse outcomes in HCC, suggesting that NK cell maturation status is a

clinically relevant feature of cancer that merits further study (149). While the precise mechanisms that influence the observed dysfunctional cell states of intratumoral NK cells are not well understood, understanding the origins of these cells may reveal new targets for NK-specific ICB, thus prolonging the NK cell antitumor response with the potential to simultaneously enhance activation of adaptive antitumor immunity.

ILC Heterogeneity in the TME

NK cells represent only one type of group 1 ILC, a heterogeneous group of cell types that still remain controversial (50). Group 1 ILCs have been broadly defined by their ability to produce Th1 cytokines such as IFN-γ and encompass NK cells, tissue-specific trNKs, and non-NK ILC1s. In mice, NK cells and trNKs are defined by their expression and dependence on the transcription factor Eomes for development, while ILC1s can be broadly identified as Eomes$^-$CD200R$^+$. Tissue resident cells have previously been identified by markers CD69 and CD49a, though these markers can be differentially expressed during development, infection, and cancer (2). Additionally, mouse studies have shown that tissue-specific ILC1 populations may require unique developmental programs, such as Hobit-dependent liver ILC1s, further complicating study of specific ILC1 subtypes in mice (52, 150). In humans, precise identification of specific ILC subsets and their developmental origins has proven even more elusive due to limitations of studying human samples *ex vivo* and the inability to generate *in vivo* developmental knockout models (151, 152). Human group 1 ILCs have been thought to include NK cells as well as CD127(IL-7Rα)^{hi} and CD127^{lo} ILC1s. CD127^{hi} ILC1s largely inhabit the lamina propria, lack expression of EOMES or human NK cell markers, and predominantly produce cytokines, while CD127^{lo} ILC1s resemble intraepithelial CD8$^+$ T cells and exhibit cytotoxic function as well as retained expression of EOMES, which could represent a subset of trNK cells (152). Similarly, unique populations of human trNKs have been identified in the liver, lung, thymus, and uterus, with some overlap with mouse ILC1 markers (47).

Recent studies utilizing high-dimensional cytometry and unbiased single-cell genomics have been invaluable in parsing out the complex diversity of tissue- and activation- specific ILCs (153). For example, an early effort to identify tissue-specific human ILC subtypes at a higher resolution using mass cytometry suggested that CD127^{hi} ILC1s could not be identified in any of the tissues examined (154). In contrast, scRNA-seq analysis of human ILCs in blood, tonsil, lung, and colon indicated the presence of *EOMES*$^-$ and *EOMES*$^+$ ILC1s in blood, tonsil, and lung. However, this data set was limited by a relatively low number of cells analyzed, and the identified ILC1 populations lacked *TBX21* expression (155). Subsequently, scRNA-seq analysis of lean and obese human adipose tissue at a higher cell number was able to distinguish ILC1s, immature, mature, and tissue-resident NK cells, with markers verified by flow cytometry (156). In this study, CD200R1 was identified as a reliable marker distinguishing ILCs from mNK in the adipose tissue. Within CD200R1$^-$ NK cells, mNK, iNK, and trNK could be identified by gating on EOMES versus PERFORIN, revealing EOMES^{int}PERFORIN^{hi} mNK, EOMES^{lo}PERFORIN^{int} iNK, and EOMES^{hi}PERFORIN^{int} trNK. Among CD200R1$^+$ ILCs, ILC1s in the adipose were distinguished by expression of *ZNF683*, *TBX21*, and *CXCR6*, as well as TBET expression by flow cytometry. RNA velocity analysis revealed putative ILC developmental pathways within the adipose, identifying a unique shared ILC3 and ILC1 progenitor population. Applying unbiased genomic approaches to studying ILCs within the TME may reveal similar stage- and tissue-specific alterations to ILC development and function as well as new markers to better identify specific subpopulations and/or lineages.

Because ILC subtypes are not well characterized, studies of ILCs in the TME have been confounded by difficulties identifying true distinct cell types rather than immature or activated NK cells. One study previously suggested that the TME may directly convert conventional NK cells into a functionally impaired double-positive CD49a$^+$CD49b$^+$ "intermediate ILC1" phenotype (157). However, unbiased characterization of ILC development in mouse models of solid tumors using scRNA-seq revealed that these double-positive NK cells were likely not bona fide tissue-resident CD49a$^+$ cells but rather TGF-β-suppressed circulating NK cells expressing CD49a (153). Another recent study using scRNA-seq was able to identify a unique Hobit-dependent developmental trajectory of ILC1s in the mouse liver from an immature stage to a cytotoxic effector stage, demonstrating that these unbiased methods may allow us to accurately distinguish between developmental stages versus distinct group 1 ILC cell lineages in the TME (51). Similar studies such as these may allow us to more accurately identify the relative contributions of different ILC subtypes to tumor control both in mouse models and human disease.

DISCUSSION

NK cells entering the TME encounter a rich and complex community of immunosuppressive factors. As we begin to understand the distinct mechanisms by which NK cells are regulated by their neighbors and their environment, it will be possible to identify pathways that can be directly modulated by therapeutic strategies to improve clinical outcomes. Already, multiple approaches are being tested to relieve NK cell immunosuppression in the TME. As previously mentioned, ICB blocking the PD-1/PD-L1 or CTLA-4 axis is beginning to be understood as not only acting on T cells but also antitumor NK cells. Administration of immune agonists such as CD40 ligand presents another route of promoting continued NK cell function in the TME and may synergize with other anticancer therapies (35, 158, 159). Similar to the "armored CAR" approach being applied to CAR-T cells, CAR-NK cells engineered to produce IL-15 as they persist within the TME have been tested in preclinical and clinical scenarios with some promise (160–164). Another CAR-based approach to bypassing immunosuppression utilized a chimeric

extracellular TGF-β receptor domain with the intracellular activating domain of NKG2D, thereby converting a suppressive signal to an activating signal (165). Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 editing of either autologous cord-blood NK cells or the readily-available NK-92 human NK cell line may be an alternate method of producing NK cell therapy that is intrinsically resistant to evolving immunosuppressive mechanisms over time, though this will depend on identification of key NK cell repressor molecules to be edited.

While NK cells are a comparatively rare cell type in the TME compared to other immune populations, emerging methods will enable higher resolution analysis of NK cell dynamics and ILC heterogeneity within the tumor. scRNA-seq atlases of healthy and tumor tissue have provided the resolution to identify rare and new cell subtypes (166) while advances in single cell ATAC-seq have provided insight into unique epigenetic ILC states. Evidence in T cells suggests that differential chromatin landscapes may uniquely identify immune subtypes compared to transcriptional signatures, while distinct chromatin accessibility in NK cells can distinguish between differentiated and "memory" subsets (108, 167). Combining scRNA-seq and scATAC-seq will be a powerful tool to fully survey changes in the spectrum of intratumoral NK cell and other ILC subset roles based on combined transcriptional and epigenetic profiles in an unbiased manner. Analysis of these data can also reveal putative cellular interactions within the TME based on predicted ligand-receptor interactions (168, 169). Once identified, combining these computationally-identified signaling partners with high-dimensional spatial validation will offer deeper insight into novel NK cell-TME interactions and the relevant signaling pathways that dictate NK development and function within tumors. Multiple groups have developed multiplex IHC and IF approaches to surveying tumor tissue samples, revealing striking clinical correlates (60). Multiplex imaging using upwards of 10 colors can be used to identify the spatial colocalization of heterogeneous cell types in the tumor and provide confirmation of cell-cell interactions computationally inferred from scRNA-seq. In addition, spatial sequencing techniques combining spatial cellular localization data with transcriptional data have the potential to inform where in the TME transcriptionally-identified cell types reside and who they interact with. While commercially-available approaches such as

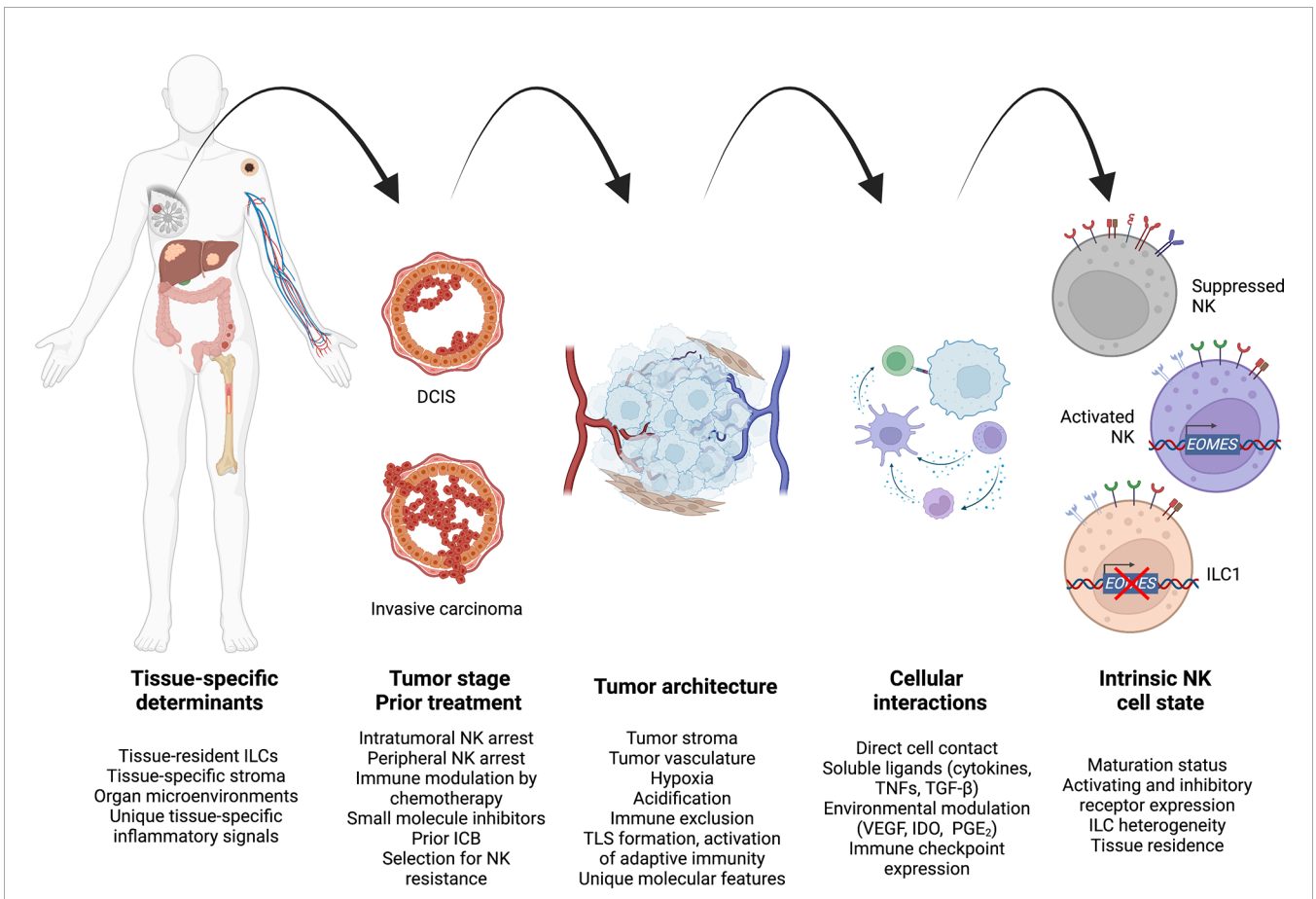

FIGURE 3 | A unified model to integrate high-level spatiotemporal and intracellular factors governing antitumor NK cell regulation during cancer progression. Created using Biorender.com.

10x Visium or Nanostring GeoMx do not yet provide spatial resolution down to a single cell level, recent single-cell spatial sequencing methods will prove to be a powerful tool to assess the TME once further refined (170, 171).

NK cell dysfunction within the TME is a progressive process that occurs heterogeneously across tissues and cancer stages (**Figure 3**). Applying new understandings of the interplay between innate and adaptive immunity may reveal novel applications of cell therapy, for example sequential NK and CAR-T cell therapy to maximize T cell activation and neoantigen presentation to the endogenous pool of antitumor T cells. At the same time, further study may allow us to identify the ideal treatment methodology to harness specific NK cell functions such as metastatic control, though long-term suppression of disease spread may require genetic modification to enhance NK cell persistence. As therapeutics specifically targeting NK cells undergo further preclinical and clinical study, studies defining how immunosuppression evolves will inform rationally optimized NK cell treatment design and treatment combinations for cancer therapy.

AUTHOR CONTRIBUTIONS

Both JL and TO'S planned, wrote, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Polymorphonuclear Myeloid-Derived Suppressor Cells Are Abundant in Peripheral Blood of Cancer Patients and Suppress Natural Killer Cell Anti-Tumor Activity

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Tumor microenvironment (TME) includes a wide variety of cell types and soluble factors capable of suppressing immune-responses. While the role of NK cells in TME has been analyzed, limited information is available on the presence and the effect of polymorphonuclear (PMN) myeloid-derived suppressor cells, (MDSC). Among the immunomodulatory cells present in TME, MDSC are potentially efficient in counteracting the anti-tumor activity of several effector cells. We show that PMN-MDSC are present in high numbers in the PB of patients with primary or metastatic lung tumor. Their frequency correlated with the overall survival of patients. In addition, it inversely correlated with low frequencies of NK cells both in the PB and in tumor lesions. Moreover, such NK cells displayed an impaired anti-tumor activity, even those isolated from PB. The compromised function of NK cells was consequent to their interaction with PMN-MDSC. Indeed, we show that the expression of major activating NK receptors, the NK cytolytic activity and the cytokine production were inhibited upon co-culture with PMN-MDSC through both cell-to-cell contact and soluble factors. In this context, we show that exosomes derived from PMN-MDSC are responsible of a significant immunosuppressive effect on NK cell-mediated anti-tumor activity. Our data may provide a novel useful tool to implement the tumor immunoscore. Indeed, the detection of PMN-MDSC in the PB may be of prognostic value, providing clues on the presence and extension of both adult and pediatric tumors and information on the efficacy not only of immune response but also of immunotherapy and, possibly, on the clinical outcome.

Keywords: natural killer, myeloid-derived suppressor cell, immunoscore, biomarker, lung tumor

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INTRODUCTION

Lung tumor is the second most common cancer in both men and women (1). Almost 60% of all lung cancers are metastatic at diagnosis and metastases occur in various tissues and organs. Traditional therapeutic options for lung cancer treatment are surgery, chemotherapy and radiotherapy. However, given the overall poor prognosis, strategies to improve the efficacy of these treatments are strictly needed, especially for tumors in advanced stages (2). Significant therapeutic progresses have been achieved over the years leading to an improved prognosis. Thus, the 5-year survival probability of metastatic disease is now significantly higher but far from being satisfactory (3). In recent years, immunotherapy with checkpoint inhibitors revealed as a particularly promising approach, however anti-tumor immunity is frequently hampered by tumor-mediated immunosuppression and immune evasion which strongly compromise the clinical efficacy (2, 4–6). The impact of the tumor microenvironment (TME) has recently been emphasized also in the context of resistance to treatment (7, 8). Various types of immunosuppressive cells are involved, including tumor associated macrophages (TAMs) (9), regulatory T cells (Treg) (10), myeloid derived suppressor cells (MDSC) (11), mesenchymal stromal cells (MSC) (12). Among these cells, increasing attention has been paid on the effect of MDSC on the treatment and on the prognosis of lung cancer (13, 14).

MDSC represent a heterogeneous population composed of both immature and mature activated myeloid cells capable of inhibiting both innate and adaptive immune responses. Thus, it has been shown that both human and murine MDSC are capable of interfering with T and NK cell proliferation and/or function (15). On the basis of surface markers expression, human MDSC can be divided in two major subsets, namely, monocytic MDSC (Mo-MDSC) and polymorphonuclear MDSC (PMN-MDSC). Thus, Mo-MDSC are $CD45^+Lin^-(CD3^-CD19^-CD56^-)$ $HLA\text{-}DR^{-/low}CD33^+CD11b^+CD14^+CD15^-CD66b^-$ while PMN-MDSC are $CD45^+Lin^-HLA\text{-}DR^{-/low}CD33^+CD11b^+CD14^-CD15^+CD66b^+$ (16).

Expansion/accumulation of these immunosuppressive cells may be due to a partial block in their differentiation from immature myeloid cells. An expansion of MDSC during acute/chronic viral or bacterial infection has recently been reported (17). In addition, previous studies revealed the presence, in the TME of different tumors, of suppressive cell types that compromise anti-tumor immune responses and favor the expansion of MDSC (18–26). Importantly, both Mo- and PMN-MDSC have been detected at the tumor site and even in peripheral blood (PB) of tumor patients (27–29). Notably, their presence has been associated with a poor prognosis (20). In addition, they have been detected in the PB of patients with sepsis or GvHD and also in healthy donors who received G-CSF for HSC mobilization (27).

MDSC have been shown to suppress immune cells by different mechanisms. For example, nitric oxide synthase 2 (NOS2) is produced by Mo-MDSC while reactive oxygen species (ROS) by PMN-MDSC. Together with arginase 1 (ARG1), they induce suppression of T cell proliferation consequent to inhibition of CD3ζ chain expression and to induction of T cell apoptosis. Additional mechanisms of MDSC-mediated immunosuppression are due to Indoleamine 2,3-dioxygenase (IDO)-derived L-kynurenine (a tryptophan catabolite), and to prostaglandin E2 (PGE2), which cause both T and NK cell dysfunction (19, 30). Also cytokines produced by MDSC, such as TGF-β and IL-10, have been shown to inhibit NK cell cytotoxicity and to induce Treg (31). As recently reported, another mechanism by which MDSC may exert suppression is by secreting exosomes, known as important players in intercellular communications (32, 33).

NK cells play a relevant role in the control of tumor growth and metastatic spread (34). They are able to efficiently kill tumor and virally-infected cells thanks to their ability to release cytolytic granules and pro-inflammatory cytokines (35). However, these NK-mediated effector functions can be compromised by cells or soluble factors present in TME. In this context, in a previous study we showed that PMN-MDSC derived from G-CSF-mobilized donors, undergoing apheresis for hematopoietic stem cell transplantation (HSCT), are able to strongly suppress the anti-tumor cytotoxicity and cytokine production of NK cells, thus compromising their important role in graft versus leukemia activity (GvL) (27, 36, 37).

In the present study, we show that the subset of PMN-MDSC is present not only at the tumor site, but also in the PB of patients with primary or metastatic lung tumors. Since these cells were enriched in cancer patients, they could represent a useful marker revealing tumor presence. Importantly, they impact on the frequency of mature NK cells present in patient's PB and compromise their function. This inhibitory effect is primarily mediated, by cell-to-cell contact and PMN-MDSC-derived exosomes. It is conceivable that PMN-MDSC may play a primary role in the inhibition of the NK-mediated anti-tumor activity. Moreover, this effect may impair immunotherapy and the overall survival of patients with cancer as also suggested by our recent study in pediatric patients with neuroblastoma. This study offers an important clue for therapeutic interventions focused on targeting PMN-MDSC in order to block their immunosuppressive activity in tumor tissues and also in the periphery.

RESULTS

Presence of PMN-MDSC in TME and PB of Patients With Primary or Metastatic Lung Tumor

TME may contain different cell types capable of inhibiting the anti-tumor activity of effector cells, thus favoring immune evasion and tumor growth (38). In particular, we assessed whether MDSC were present in the TME of lung tumors using a tissue microarray (TMA). Twenty different tumor tissue samples from patients with lung adenocarcinoma, were analyzed by immunohistochemistry (IHC) for the expression of S100A9, a suitable marker for PMN-MDSC identification (39). As shown in **Figure 1A**, in the majority of these samples (14 out

of 20) S100A9$^+$ cells were highly represented (cell mean > 230 per mm^2 of S100A9$^+$ cells, from 70 to 909 per mm^2 of S100A9$^+$ cells) while in the other 6 cases were present in lower but sizable percentages (cell mean range from 21 to 55 per mm^2 of S100A9$^+$ cells). These results indicate that PMN-MDSC accumulate in TME where they may exert immunosuppressive activity on tumor-infiltrating immune effector cells.

The Overall Survival analysis performed by using the Kaplan Meier method on lung adenocarcinoma patients retrieved from TCGA revealed that a higher expression of S100A9 significantly correlates with a poor clinical outcome. Notably similar results were observed analyzing CD15 marker expression indicating a possible association between CD15$^+$ or S100A9$^+$ cells and poor prognosis (**Figure 1B**). These data suggest that CD15 and S100A9, markers both strictly related to PMN-MDSC cells, were involved in the overall survival of lung tumor patients. We also performed the expression analysis of markers (i.e. NCR1, FCGR3A, FCGR3B, NCR3, KIR3DL2, KIR3DP1), specific for NK cells using the same dataset. As shown in **Supplementary Figure 1A**, it is possible to speculate that a higher accumulation of NK cells occurred in normal as compared to lung tumor tissues. We also analyzed the expression of S100A8, S100A9 and CD15, myeloid markers that individually cannot specifically identify PMN-MDSC. S100A9 and CD15 are both partially down-regulated in normal samples with respect to lung tumor samples while S100A8 is higher in normal tissues (**Supplementary Figure 1A**). Moreover, the hierarchical clustering and heatmap for genes specific for NK cells and PMN-MDSC in 57 lung adenocarcinoma samples and 57-matched normal samples retrieved from TCGA indicated that

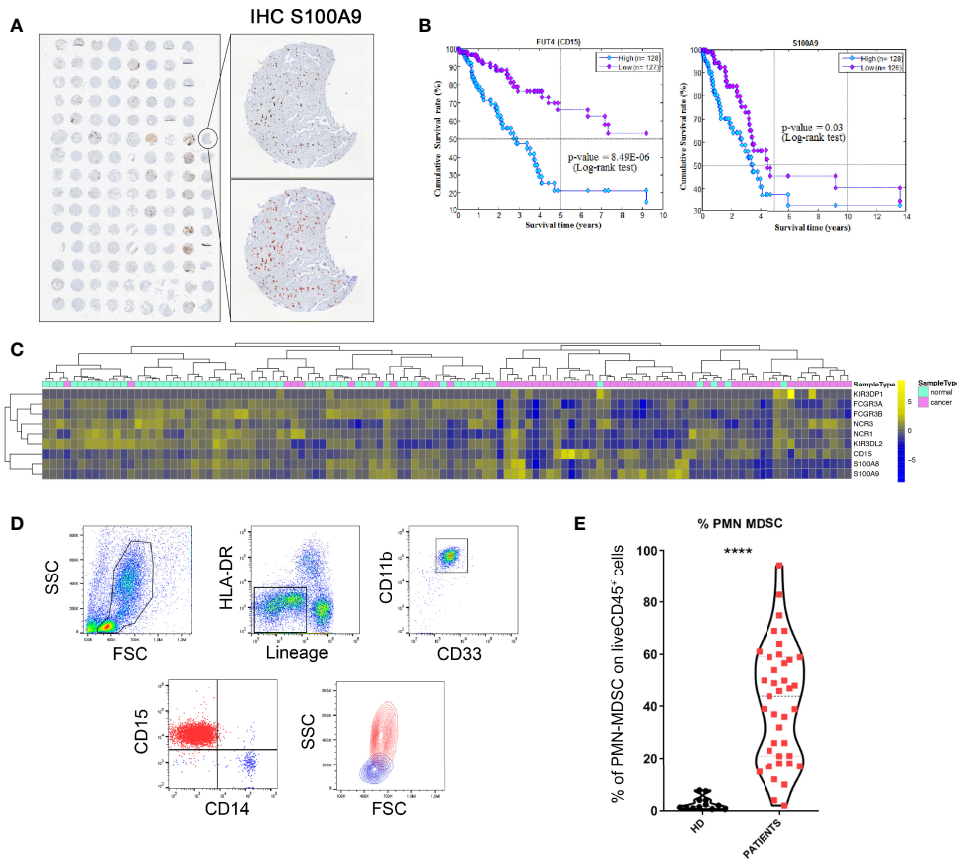

FIGURE 1 | PMN-MDSC are present in high frequency in PB of patients with primary and metastatic lung tumor and correlate with their overall survival. **(A)** TMA of lung adenocarcinoma cases stained with S100A9 showing positive cells digitally quantified within tissue cores. The magnification shows S100A9 (upper panel) and S100A9$^+$ cells automatically indicated with red circles (lower panel) to perform a cell count in each core using QuPath (absolute numbers of S100A9$^+$ per mm^2). **(B)** Kaplan-Meier survival analysis. Kaplan-Meier analyses to evaluate the correlations between the expression level of CD15 and S100A9 and the Overall Survival in lung adenocarcinoma patients retrieved from TCGA. Low- and high-expression groups refer to patients with expression levels lower than the 25th percentile (violet curve) and greater than the 75th percentile (cyan curve), respectively. **(C)** Hierarchical clustering and heatmap for KIR3DP1, FCGR3A, FCGR3B, NCR3, NCR1, KIR3DL2, CD15, S100A8, and S100A9 in 57 lung adenocarcinoma samples (violet bars) and 57-matched normal samples (water blue bars) retrieved from TCGA. The expression profiles of genes are clustered according to rows (genes) and columns (samples) by using the Pearson correlation as the distance metric and the complete-linkage as clustering method. The colors represent different expression levels that increase from blue to yellow. **(D)** PBMC were analyzed *ex-vivo* by flow cytometry for the expression of specific markers that allow the identification of PMN-MDSC. One representative experiment out of 34 performed. **(E)** Percentages of PMN-MDSC (CD15$^+$ cells) in the PB of healthy donors (HD) and lung tumor patients (n=34). ****p ≤ 0.0.00005.

genes specific for NK cells were enriched in normal tissue while genes specific (S100A9 and CD15) for PMN-MDSC were more expressed in the tumor tissues (**Figure 1C**).

In a second set of experiments, we investigated whether PMN-MDSC were present in PB of patients with primary or metastatic lung or pleural tumors. To this end, we applied a gating strategy allowing to identify and characterize, by flow-cytometry, the different MDSC subsets (16). In particular, we analyzed the PB of 34 patients with tumors (**Table 1**) for the presence of PMN-MDSC identified as $CD45^+$ Lin^- $HLA\text{-}DR^{low/-}$ $CD11b^+$ $CD33^+$ $CD14^-$ $CD15^+$ (**Figure 1D** and **Supplementary Figure 1B**). As shown in **Figure 1E**, we could detect relevant proportions of PMN-MDSC in the PB of tumor patients while these cells were virtually absent in PB of healthy donors (HD) indicating a possible correlation between the presence of PMN-MDSC in PB and the occurrence of a cancer.

PB-Derived PMN-MDSC Inhibit the Anti-Tumor Activity of NK Cells

Since NK cells are known to display an important role in the anti-tumor activity, we first investigated a possible association between PB-PMN-MDSC and NK cells in our cohort of patients by correlating the proportion of PMN-MDSC and that of NK cells. As shown in **Figure 2A**, a statistically significant inverse correlation exists between the percentages of PMN-MDSC and that of NK cells. This result suggested that, in our cohort of tumor patients, PMN-MDSC could exert their immunosuppressive activity also by influencing the proportion of circulating NK cells.

We further analyzed whether PMN-MDSC could impair the anti-tumor activity of effector cells not only at the tumor site, but also in peripheral tissues. In order to analyze in more detail the immunosuppressive activity of PMN-MDSC, these cells were isolated from PB of tumor patients and co-cultured with fresh, short-term and long-term IL2-activated allogenic NK cells at 1:1 ratio, referred in the text as "conditioned" NK cells (cond.). After 48 hours, NK cells were isolated from co-cultures (by magnetic depletion of $CD15^+$ cells) and assessed for their cytolytic activity against tumor cells. Notably, in order to better mimic the possible inhibitory effect of PMN-MDSC in the lung TME, primitive tumor cells, isolated from mesothelioma and adenocarcinoma patients, were also used. NK cells cultured in the absence of PMN-MDSC were comparatively analyzed. As shown in **Figures 2B, C** and **Supplementary Figure 1C**, target cell killing was strongly inhibited in conditioned NK cells analyzed at different effector:target (E:T) ratios. In addition, as shown in **Figure 2D**, PMN-MDSC could also inhibit the cytolytic activity of short-term-IL-2 activated allogenic PB-NK cells. Next, we investigated the effect of patient's PMN-MDSC on autologous NK cells. To this end, both PMN-MDSC and NK cells were isolated from PB. These PMN-MDSC displayed a strong inhibitory effect on the cytotoxicity of autologous NK cells (**Supplementary Figure 1D**). We then assessed whether PMN-MDSC could inhibit also the production of pro-inflammatory cytokines and the degranulation (CD107a expression) of NK cells. Results indicate that a sharp reduction of IFN-γ, TNF-α and CD107a expression occurred also in conditioned NK cells (**Figure 2E** and **Supplementary Figure 1E**). Taken together, these results suggest that, in patients with lung tumor, PB-PMN-MDSC can compromise the anti-tumor effector function of NK cells.

TABLE 1 | Features of patients included in the study.

Patients with primary lung or pleural tumor
Epithelioid mesothelioma
Female: n. 3 - median age 76 (range 68-86)
Male: n. 6 - median age 72,5 (range 60-82)
Non-small cell lung cancer
Female: n. 7 - median age 69.4 (range 60-81)
Male: n. 6- median age 70.3 (range 58-77)
Small cell lung cancer
Male: n. 2 - median age 70.5 (range 69-72)
n- 24 - female: 10m male: 14 –median age 71.7 (range 58-86)
Patients with secondary metastatic lung plural tumor
Intestinal Carcinoma
Male: n. 1 - age 74
Uterine Carcinoma
Female: n. 1 - age 63
Chorio-Carcinoma
Female: n. 1 - age 41
Vescical Carcinoma
Female: n. 1 - age 57
Brest Carcinoma
Female: n. 2 - median age 57.5 (range 53-62)
Kidney Carcinoma
Male: n. 1 - age 73
Primary tumor unknow
Female: n. 2 - median age 83 (range 80-86)
Male: n. 1 - age 64
n. 10 -female: 7, male: 1- median age 65.3 (range 41-86)

PMN-MDSC Influence the Gene Expression Profile of PB-NK Cells in Lung Tumor Patients

Since PMN-MDSC, present in high numbers in PB of tumor patients, exert a potent inhibitory activity on NK cells, we investigated whether NK cells present in patient's PB were modified in their gene expression profile. Thus, by PCR array, we compared the expression of a wide panel of genes involved in NK cell biology between PB-NK cells from patients and HD. In particular, we focused on genes involved in NK cell immuno-effector function or in their development. As shown in **Figure 3A**, patient-derived NK cells showed a markedly decreased expression of CD16 and a trend of decreased expression of several other genes known to play a role in NK-mediated anti-tumor activity, including genes encoding for NKp46, CD69, CD62L, DAP10 and GZMB. Of note, in patient-derived PB-NK cells, a higher expression of the inhibitory receptor TIGIT was observed.

To verify the contribution of PMN-MDSC on the peculiar transcriptional profile of patient's NK cells, we further analyzed the gene expression of IL-2-activated allogenic HD-NK cells conditioned or not with patient-derived PMN-MDSC. Notably, conditioned NK cells displayed a decrease expression of several genes associated to NK cell activity as compared to unconditioned ones (**Figure 3B**). On the other hand, upon

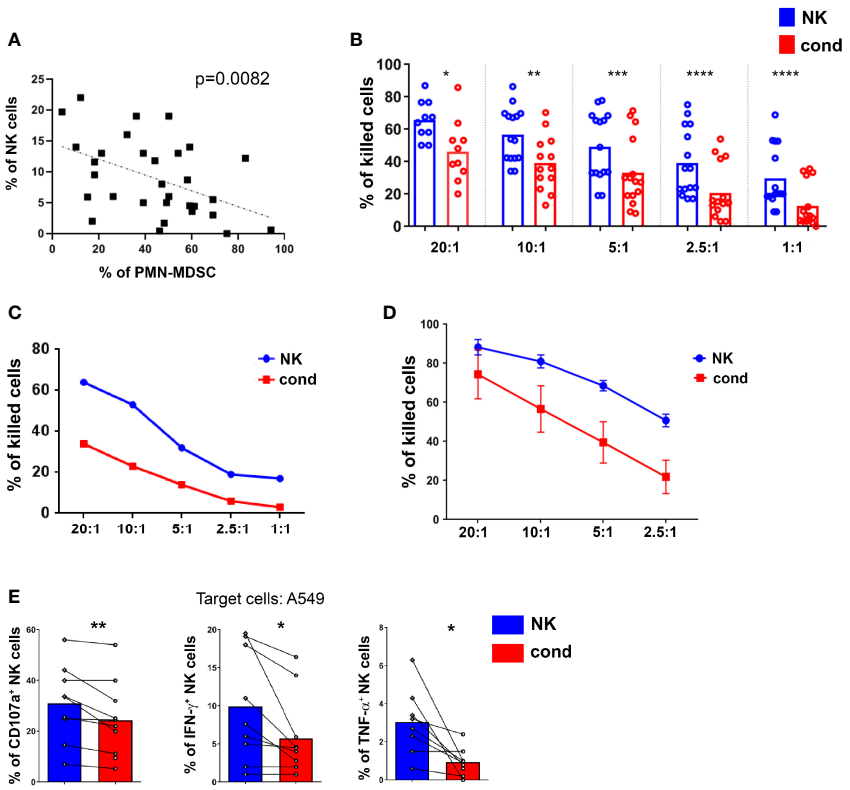

FIGURE 2 | PMN-MDSC impair the anti-tumor activity of NK cells. **(A)** Correlation between the frequencies of PMN-MDSC and NK cells present in PB of lung tumor patients. P = 0.0082 (n = 31) **(B–E)** NK cells activated *in vitro*-expanded or *ex vivo* were cultured alone (NK) or in the presence (ratio 1:1) of PMN-MDSC (cond.) derived from PB of lung tumor patients. After 48h of co-culture, PMN-MDSC were depleted from 1:1 co-cultures and the resulting NK cells used as effector cells in the different functional assays. **(B, C)** Percentages of killed NALM-18 target cells. **(B)** Statistical analysis of 15 independent experiments. **(C)** One representative experiment out of 15 performed. **(D)** Percentages of killed NALM-18 target cells of short-term NK cells conditioned or not with PMN-MDSC derived from PB of lung tumor patients (n = 3). The different Effector/Target (E/T) ratios are indicated. **(E)** Cytokine production and degranulation capabilities of NK and cond. cells were analyzed after 4h of co-culture with A549 target cells. Bars indicated percentage of median of cytokines production (IFN-γ and TNF-α) and degranulation (CD107a) of NK and cond. cells (n = 9). *p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005; ****p ≤ 0.0.00005.

conditioning, the checkpoint inhibitor SIGIRR (40) resulted slightly increased. Similar data trend were observed by analyzing in flow cytometry the expression of proteins encoded by some of these genes (**Supplementary Figures 2A, B**). Despite some differences, a similar trend was observed in gene expression by NK cells isolated from patients confirming that PMN-MDSC present in PB of lung tumor patients may represent a cellular player responsible for TME-induced immunosuppression.

Exosomes and Cell-to-Cell Contact Are Involved in the Inhibition of the NK-Mediated Anti-Tumor Cytolytic Activity of NK Cells

Previous studies showed that PMN-MDSC can suppress immune cell function by exploiting different mechanisms, including cell-to-cell contact and release of soluble factors (41). In order to identify the nature of the inhibitory mechanism(s) that impair NK-cell function, a first set of experiments was performed using trans-well chambers as illustrated in **Figure 4A**. The cytolytic activity was partially inhibited under trans-well conditions, suggesting that a soluble mechanism is involved in NK cell-mediated suppression (**Figure 4B**). Previous studies provided evidence that NK cell function may be compromised by IDO-derived catabolites (in particular L-kynurenine) and/or PGE2 (42) and TGF-β. Thus, co-culture experiments were performed using competitive inhibitors or blocking antibodies specific for IDO, PGE2, and TGF-β as well as for other inhibitory factors known to be released by PMN-MDSC (e.g. Arginase, catalase, nitric-oxide synthase). As shown in **Figure 4C**, none of these inhibitory pathways was involved in PMN-MDSC-mediated immunosuppression of NK cells. As shown in **Supplementary Figure 2C**, PMN-MDSC derived from lung tumor patients expressed different molecules that could be involved in modulation of immunoresponse (43–45). In addition, we further evaluated the involvement of other soluble elements and, in particular, we investigated the possible effect of exosomes released by PMN-MDSC (33). To this end, PMN-MDSC isolated from patient's PB were cultured for 48 hours in exosome-free-media. Exosomes were then isolated from supernatants by ultracentrifugation. Flow cytometric analysis

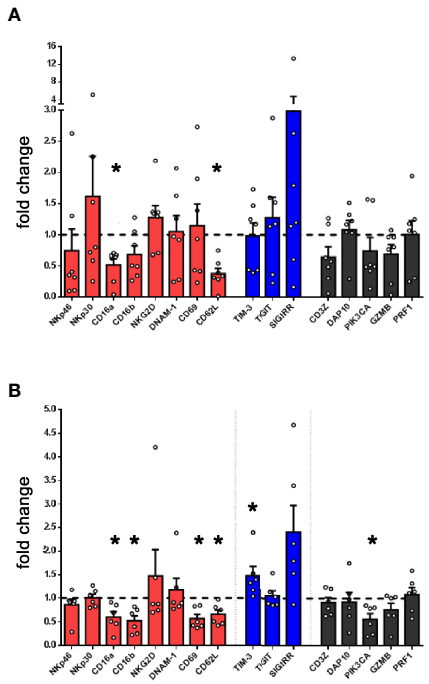

FIGURE 3 | Lung tumor NK cells display an impairment of their activation status and inversely correlate with PMN-MDSC. **(A)** The expression of a panel of selected genes associated to NK cell function was evaluated by PCR array in PB-NK cells derived from lung cancer patients (n = 7). Values are expressed as fold change with respect to NK cells from HD (n = 5). Bars represent SEM. **(B)** The expression of genes associated to NK cell function was evaluated in short-term IL-2 activated NK cells upon co-culture with lung cancer PMN-MDSC. Values are expressed as fold change with respect to NK cells cultured with IL-2 only, used as control (n=6). *p ≤ 0.05.

confirmed their expression of the exosome-specific markers CD81 and CD63 (data not shown). To assess their immunomodulatory potential, IL-2-activated HD-NK cells were cultured either in the presence or in the absence of PMN-MDSC derived-exosomes. The cytolytic activity of NK cells cultured in the presence of exosomes was significantly lower than that of NK cells cultured alone (**Figure 4D**). Notably, the degree of inhibition mediated by either exosomes (**Figure 4E**) or cell-to-cell contact (**Figure 4F**) was comparable at different E:T ratios. All these results suggested that PMN-MDSC-derived-exosomes represent important inhibitory mediators on anti-tumor NK cell function. In addition, we observed that PMN-MDSC-derived exosomes contain a set of miRNA (**Figure 4G**) with immunomodulatory properties (46).

DISCUSSION

NK cells have been shown to play an important role in the control of viral infections and tumor growth and metastases. Despite their strong anti-tumor activity, in tumor patients their effector function is frequently impaired by soluble inhibitory factors and/or immunosuppressive cells present in TME. Of note, evaluation of the immune infiltrate (immunoscore) in tumor lesions represents a valuable tool to stratify patients in different prognostic categories (47–49). However so far, immunomodulatory cells detectable in TME, have not been included in the immunoscore. MDSC represent a cell population derived from a common myeloid precursor present in the bone marrow. Under pathological conditions characterized by the presence of inflammation, a partial block of myeloid differentiation may favor the accumulation of MDSC, both in PB and tissues. Although different subsets of MDSC display morphological heterogeneity, they share the ability to suppress both innate and adaptive immune responses. MDSC exert a potent immunosuppressive activity and their presence has been documented in tumor patients (18–20, 50). Primary inflammatory cytokines such as IL6 and IL1β that may be present at high levels in TME and may drive the accumulation of MDSC favoring their immunosuppressive activity.

In this study, we show that PMN-MDSC are present not only in TME but also in PB of patients with primary or metastatic lung or pleural tumors. Remarkably, the detection and the numbers of PMN-MDSC in PB of these patients (since they are virtually absent in HD) may provide a clue for the presence and, possibly, the progression of a tumor. Of note PMN-MDSC could exert a strong inhibitory activity on NK cells in the periphery, further compromising the anti-tumor activity of these potent effector cells. In this context, we show that PMN-MDSC isolated from patients PB can exert a potent inhibitory effect *in vitro* on NK cell cytotoxicity, degranulation and cytokine production. Remarkably, the existence of an inverse correlation between the frequencies of PMN-MDSC and that of NK cells in the PB of lung tumor patients was compatible with the concept that the inhibitory effect may occur also in peripheral tissues. In addition, a significant correlation exists between the high frequency of PMN-MDSC and a poor clinical outcome in lung tumor patients.

Our data clearly show that even NK cells isolated from PB of tumor patients are impaired in their functional activities. Of note, they exhibit a peculiar gene expression profile. These data are in agreement with the downregulation of major activating NK receptors and consequent impaired anti-tumor effector function. Importantly, we provide evidence that these changes in gene expression profile can be induced by the interaction with PMN-MDSC.

Previous studies revealed different mechanisms by which PMN-MDSC suppress immune cells including release of soluble factors and cell-to-cell contact. Of note, we could not detect the occurrence of classical immunosuppressive mechanisms including Arginases, NO, IDO, TGF-β, PGE2 and ROS (51). Since exosomes were recently reported as an additional immunomodulatory mechanism, we assessed the possible contribution of PMN-MDSC-derived exosomes (51). Indeed, exosomes produced by PB-PMN-MDSC, isolated from lung tumor patients, contain a set of miRNA with immunomodulatory properties (46) that could inhibit the cytolytic activity of NK cells. These data indicate that the immune-modulatory activity of PMN-MDSC in lung tumor

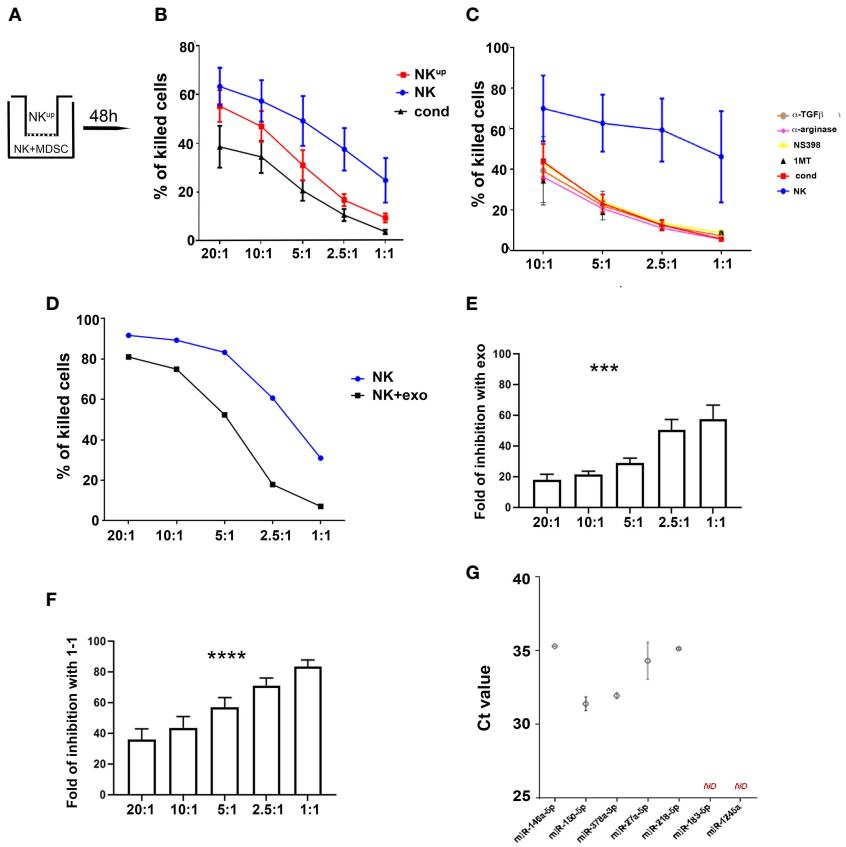

FIGURE 4 | PMN-MDSC-mediated inhibitory mechanisms of NK cell function. **(A–C)** Activated NK cells were cultured alone (NK) or with PMN-MDSC (ratio 1/1) under cell-to-cell contact or transwell (NK^{UP}) condition. After 48h of co-culture, PMN-MDSC were depleted in the 1/1 condition and the resulting NK cells were used as effector cells (cond.) in the functional assays. **(A)** Schematic culture conditions. **(B)** Percentages (mean ± SEM) of killed NALM-18 target cells at different E/T ratios in NK cells cultured alone (blue), in cell-to-cell contact with PMN-MDSC (black) and in the transwell chamber (red) (n = 6). **(C)** Percentages of mean ± SEM of killed NALM-18 target cells by NK cells cultured alone or in the presence of PMN-MDSC either in the absence or in the presence of indicated inhibitors and blocking mAbs (n = 3). **(D)** Activated NK cells were incubated with 20 ug of exo-derived PMN-MDSC. After 48h their cytolytic potential was assessed against NALM-18 cell line. Percentages of mean ± SEM of killed NALM-18 target cells at different E/T ratios. One representative experiment. **(E)** Fold of inhibition of NK cell killing capability upon incubation with 20ug of PMN-MDSC derived exosomes at different E/T ratios (n = 4). **(F)** Fold of inhibition of NK cell killing capability upon co-culturing with PMN-MDSC at different E/T ratios (n = 9). **(G)** Expression of immuno-modulatory miRNAs in lung cancer PMN-MDSC-derived exosomes. Real time PCR analysis for the indicated miRNAs in lung cancer PMN-MDSC exosomes. Threshold cycle (Ct) values for each miRNA are reported. Bars indicate SD (n = 2). ND, Non Detected. ***$p \leq 0.0005$; ****$p \leq 0.0.00005$.

patients may be exerted both by their direct contact with effector cells and by the release of exosomes.

Of note, PMN-MDSC are present in the PB of pediatric patients affected by tumors with severe prognosis, such as neuroblastoma, and may compromise the effectiveness of immuno-therapies (i.e. chimeric antigen receptor-T cells, CAR-T cells, directed to GD2 antigen) (52). These findings further underscore the need to antagonize or targeting these cells to achieve a successful therapy.

In conclusion, our data clarified an important mechanism of immunosuppression occurring in primary and metastatic lung or pleural tumors, offering a clue to implement the immunoscore with the evaluation of PMN-MDSC numbers at the tumor lesion. The inhibitory effect of PMN-MDSC present in PB in normal versus pathological conditions reflects major differences in frequency rather than qualitative differences in the suppressive capacity of PMN-MDSC (**Supplementary Figure 2D**) and of their exosomes. Thus, identification of these cells in PB may provide a novel marker revealing the presence/extension of a tumor. Moreover, the assessment of their size in PB could indeed contribute to provide useful information on the clinical status and on prognostic aspects in different tumors (52). Importantly, it is also conceivable that targeting PMN-MDSC may offer a new strategy in the treatment of these type of tumors, complementary to other immune-therapies, including the use of checkpoint inhibitors or CAR-T, contributing to restore effective anti-tumor responses.

MATERIALS AND METHODS

Samples, and Ethical Statements

34 patients with primary or metastatic lung tumor were enrolled at ASL3, Ospedale Villa Scassi, Genoa, Italy and analyzed at the

time of diagnosis. Details on patient characteristics are summarized in **Table 1**. Peripheral blood mononuclear cells (PBMC) were obtained from patients and healthy donors (HD). PBMC were obtained after density gradient centrifugation (Ficoll-Lympholyte, Cederlane) as described before (53). This study was approved by Azienda Sanitaria Locale 3 (ASL, Genova, Italy) ethics board (N9-13, 2013) and after by Regione Liguria Ethics Board (Ethics Board id 4975, 2020). All patients gave written informed consent in according to the Declaration of Helsinki. PBMC of healthy donors were obtained from buffy coat (UO Centro Trasfusionale, IRCCS Ospedale Policlinico San Martino, Genova and IRCCS Bambino Gesù Children's Hospital, Rome).

Tissue Samples and Immunohistochemistry (IHC)

For each case of adenocarcinoma, all hematoxylin and eosin–stained slides were reviewed for confirmation of diagnosis; one block was then selected for adenocarcinoma tissue microarrays (TMAs) construction. For each block, five cores with a diameter of 1 mm were obtained from diverse areas of the tumor and randomly numbered from 1 to 5. From each TMA 5-μm sections were cut and stained with S100A9 (clone D5060, Cell Signaling Technology, Danvers, MA) on an automated staining platform (Benchmark Ultra, Ventana Medical Systems). An OptiView DAB IHC Detection Kit (Ventana Medical Systems). Stained sections were scanned with a Ventana iScan HT slide scanner (Ventana Medical Systems). The absolute numbers of S100A9-positive cells per mm^2 were automatically counted in each core using QuPath version 0.2.0 (54).

Antibodies and Flow Cytometry

For the evaluation of surface antigen expression the following monoclonal antibodies (mAbs) were used: CD3-APC-A700, CD19-APC-A700, CD56-ECD, PC7 and APC-A700, CD11b-FITC, CD33-PC7, HLA-DR-PE, CD14-ECD, CD45-KrO, CD66b-APC, CD15-APC (all Beckman Coulter), CD107a-eFLUOR660 (Invitrogen), CD275-, CD155-, CD85j-, Ceacam1-, CD39-APC (Miltenyi biotec). For intracellular evaluation the following mAbs were used: anti-IFN-γ-PE (BD, biosciences), anti-TNF-α-eFluor450 (Invitrogen). For MDSC immunostaining a custom Duraclone platform (Beckman Coulter) was used in order to standardize the protocol. After staining procedures cells were acquired at Cytoflex S and LX (Beckman Coulter) and analyzed with Cytexpert software (v2.2, Beckman Coulter), and FlowJo 10 (Starlab).

Cell Isolation and Co-Culture Experiments and Cell Lines

PMN-MDSC cells were isolated by CD15 microbeads kit (Miltenyi Biotec) following manufacture instruction. NK cells were isolated as previously described (55), using NK isolation kit II (Miltenyi Biotec) or RosetteSep (StemCell technologies) (purity >95%). Freshly isolated NK cells either from patients or HD were immediately used or were cultured in 10% serum-supplemented RPMI 1640 medium (Lonza) supplemented with only IL-2 (100U/ml, Proleukin) for 48h referred as "short-term". To obtain "activated" NK cells we performed long-term cultures (15-20 days) as previously described (56, 57).

Co-culture experiments were performed using effector cells (NK cells) cultured alone or in combination (1:1) with autologous or allogenic PMN-MDSC or in the presence of exosomes-derived PMN-MDSC (20ug). Co-culture experiments were performed in the absence or in the presence of α-TGF-β blocking mAb (R&D), α-arginase (N-hydroxy-nor-L-arginine, NOHA, 500mg/ml, Calbiochem, Germany), 1-Methyl-D-Tryptophane (1-MT indolamine-2,3-Dioxygenase inhibitor, 0,25mM Sigma Aldrich) and NS398 (N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide, PGE2 inhibitor, 5μM Sigma Aldrich). After 48 hours PMN-MDSC were depleted from co-culture (as described before by CD15 microbeads kit) and the resulting NK cells were used to perform phenotypical and functional assays.

Functional Assay

To assess the degranulation activity and cytokine production, NK cells were incubated with NALM-18 a Childhood B acute lymphoblastic leukemia cell line or A549 lung adenocarcinoma cells, as target cells at 1:1 effector:target (E:T) ratio for 4 hours in the presence of Monensin (2mM BD, GolgiStop) and Brefeldin A (1μg/ml BD, GolgiPlug) and CD107a mAb. To detect intra-cytoplasmic cytokines, after incubation, with target cells, NK cells were stained for surface markers, fixed and permeabilized with Fixation and Permeabilization Kit (BD Biosciences, New Jersey USA) and incubated with specific intracellular mAbs. To detect spontaneus degranulation a control sample without target cells was included.

Cell cytotoxicity assays was performed using as target cells NALM-18 cell line and as effector cells (short-term- or activated-NK cells) at different E:T cell ratios. In order to distinguish effector cells from target cells, NALM-18 cell line was stained with FITC cell tracker following manufacture instructions (Invitrogen). Iodure Propidio (PI) was added at the end of the co-culture (4 hours) in order to identify the percentage of target cell lysis. The calculation of specific lysis of NK cells was performed as described in (58).

RNA Extraction and Gene Expression Analysis in NK Cells

Total RNA extraction from purified NK cells was performed with miRNeasy micro kit combined with on-column DNase I digestion following the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). For mRNA quantification, 300 ng of total RNA was reverse transcribed with random primers by using Super Script IV first-strand synthesis system following manufacturer's instructions (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). To explore a wide panel of genes, 150 ng of cDNA template per fill reservoir was loaded in 384-well TaqMan array microfluidic cards (Applied Biosystems, Foster City, CA, U.S.A) with a custom configuration focused on 92 human genes implicated in NK cell biology. Real time PCR analysis was performed with TaqMan Fast Advanced Master Mix

(Applied Biosystems, Foster City, CA, U.S.A) on a QuantStudio 12k Flex instrument (Applied Biosystems, Foster City, CA, U.S.A). Expression values was calculated applying the relative threshold algorithm (Ct) with ΔΔCt method. Gene expression data were normalized by global normalization method using Relative Quantification app in Thermo Fisher Cloud (Thermo Fisher Scientific, Wilmington, DE, U.S.A.): the median Cq of all the assays in the PCR array card was calculated by software as the normalization factor, on a per sample basis.

Exosome Isolation and Analysis

MDSC cells were plated at $4x10^6$ cells/ml in RPMI 1640 supplemented with 10% exosome-depleted Fetal Bovine Serum (FBS). After 48h, conditioned medium was collected and centrifuged at 300 x g for 5 min. Following centrifugation at 2000 x g for 15 min, supernatants were passed through a 0.22 μm filter and then exosome were pelleted by high-speed centrifugation (100'000 x g for 2 hours) (Optima X Optima XPN, Beckman, California, USA). Collected exosome were then washed with a large amount of Phosphate Buffer Saline (PBS) for an additional 1 hour and resuspended in PBS. Exosome samples were stored at -80°C until use. Exosome protein concentration was determined by Bradford Assay. For miRNA analysis exosomes were purified from culture supernatants by ultracentrifugation and RNA was extracted with miRNeasy micro kit (Qiagen). For each sample, the same amount of exosomal RNA (20 ng) was reverse transcribed with miRCURY LNA RT kit (Qiagen) and 3 μL of 1:30 diluted cDNA was amplified with miRCURY LNA miRNA Sybr Green system (Qiagen). For each sample, the synthetic spike-in UniSP6 was added as internal control to monitor cDNA synthesis and amplification efficiency. Outlier samples for UniSP6 expression were discarded from the analysis. All the real time PCR reactions were carried out in triplicate on a QuantStudio7 Flex instrument and data was analyzed with baseline threshold algorithm using QuantStudio Real time PCR software (Thermo Fisher Scientific).

Expression Analysis on TCGA Dataset

RNA-sequencing expression data from 511 tumor samples of lung adenocarcinoma (luad) and 57 normal samples were retrieved from The Cancer Genome Atlas (TCGA) (59). The analysis was restricted to 57 individuals for which the complete sets of tumor and matched normal (normal tissue taken from the same patient) profiles were available, for a total of 114 samples.

We compared the NCR1 expression level on 57 luad samples and 57 matched-normal samples by applying a Student's t-test for paired samples. Hierarchical clustering for KIR3DP1, FCGR3A, FCGR3B, NCR3, NCR1, KIR3DL2, CD15, S100A8, and S100A9 in luad samples and matched normal were obtained by clustering the expression profiles of genes according to rows and columns by using the Pearson correlation as distance metric and the complete-linkage as clustering method.

Kaplan Meier Analysis on TCGA Dataset

To analyze the correlation between the expression level of genes and patient overall survival (OS), we exploited the RNA-sequencing data obtained from TCGA to split the entire cohort of lung adenocarcinoma patients (511 samples) into two groups (called low-expression and high-expression group) according to the upper and lower expression quartile. In particular, low- and high-expression groups refer to patients with expression levels of the given gene lower than 25th and greater than the 75th percentile of the expression levels distribution, respectively. For each patient cohort, the cumulative survival rates were computed for each gene according to the Kaplan-Meier (KM) method (60) on the clinical metadata provided by TCGA. For each gene, the survival outcomes of the two patients groups were compared by the log-rank test, showing a statistically significant p-value (< 0.05) if there exists a significant difference between the population survival curves.

Statistical Analysis

Statistical analysis were performed with GraphPad Prism software (La Jolla, CA). In **Figures 1E** and **3A** we used nonparametric Mann–Whitney test; **Figure 2A** simple linear regression; **Supplementary Figure 1A** Student's t-test for paired samples. **Figures 2B, E** and **3B**, **Supplementary Figures 1C, E** show the nonparametric Wilcoxon tests; **Figures 4E, F** one-way ANOVA plus post test for linear trend. A p value ≤ 0.05 was considered statistically significant. *$p \leq 0.05$; **$p \leq 0.005$; *** $p \leq 0.0005$; **** $p \leq 0.0.00005$. Where not indicated the data were not statistically significant.

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 Azienda Sanitaria Locale 3 (ASL, Genova, Italy) ethics board (N9-13, 2013) and after by Regione Liguria Ethics Board (Ethics Board id 4975, 2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Designed experiments NT and PV. Performed the experiments NT, FB, SM ADP, AP, PFF, EM, and GB. Analyzed the data NT, PV, SM, ADP, and AP. Interpreted the results NT and PV. Wrote the manuscript NT, PV, SM, and LM. Provided samples from the patients FS and MGC. Followed patients enrolled in the study FS. Performed the molecular data set analysis PP and GF. Critically revised the manuscript LQ, PP and MCM. Provided intellectual input and revised the manuscript LM. 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.2021.803014/full#supplementary-material

Supplementary Figure 1 | (A) Boxplots of NCR1, FCGR3A, FCGR3B, NCR3, KIR3DL2, S100A8, S100A9, and CD15 expression level (logarithmic scale) in 57 lung adenocarcinoma samples (violet box) and 57 matched-normal samples (water blue box) retrieved from TCGA. P-value was obtained by applying a Student's t-test for paired samples. **(B)** PBMC were analyzed *ex-vivo* by flow cytometry for the expression of specific markers that allow the identification of PMN-MDSC. Two representative experiments corresponding to 2 different patients with high and low percentage of PMN-MDSC. **(C, D)** NK cells were cultured alone (NK) or in the presence (ratio 1:1) of autologous or allogenic PMN-MDSC (cond.) derived from PB of lung tumor patients. After 48h of co-culture, PMN-MDSC were depleted from 1:1 co-cultures and the resulting NK cells used as effector cells in the different functional assays. **(C)** Percentages of killed mesothelioma or adenocarcinoma target cells isolated from lung tumor patients. Statistical analysis of 6 independent experiments. **(D)** Percentages of killed NALM-18 target cells by NK cells in autologous setting. One representative experiment out of 3 performed. **(E)** Cytokine production and degranulation capabilities of NK cells conditioned or not. Cells were analyzed after 4h of co-culture with NALM-18 target cells. Bars indicated percentage of median of cytokines production (IFN-γ and TNF-α) and degranulation (CD107a) of NK and cond. cells (n = 11). *p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005; ns, not significant.

Supplementary Figure 2 | (A, B) Mean ± SEM of the indicated markers evaluated by flow cytometry on PBMC from **(A)** ex-vivo HD vs Lung tumor patients (lung) (n = 3) and **(B)** HD-NK cells cultured alone (NK) or after co-culture (48h) with PMN-MDSC (NK 1/1) of lung tumor patients (n = 4). **(C)** Expression of the indicated markers on PMN-MDSC of lung tumor patients by flow cytometry. **(D)** Fold of Inhibition of killing mediated by NK cells co-cultured with PMN-MDSC isolated from G-CSF mobilized HD (white bars, n = 4) and from PB of lung tumor patients (black bars, n = 10).

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Signaling Pathways Tuning Innate Lymphoid Cell Response to Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the deadliest cancers worldwide and its incidence continues to rise globally. Various causes can lead to its development such as chronic viral infections causing hepatitis, cirrhosis or nonalcoholic steatohepatitis (NASH). The contribution of immune cells to HCC development and progression has been extensively studied when it comes to adaptive lymphocytes or myeloid populations. However, the role of the innate lymphoid cells (ILCs) is still not well defined. ILCs are a family of lymphocytes comprising five subsets including circulating Natural Killer (NK) cells, ILC1s, ILC2s, ILC3s and lymphocytes tissue-inducer cells (LTi). Mostly located at epithelial surfaces, tissue-resident ILCs and NK cells can rapidly react to environmental changes to mount appropriate immune responses. Here, we provide an overview of their roles and actions in HCC with an emphasis on the importance of diverse signaling pathways (Notch, TGF-β, Wnt/β-catenin…) in the tuning of their response to HCC.

Keywords: HCC, NASH, NK cells, ILC1, liver, inflammation, Notch and TGF-β pathways

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for up to 90% of all cases of primary liver cancers while the intrahepatic cholangiocarcinoma represents roughly 10% (1, 2). HCC is one of the deadliest cancers worldwide and its incidence continues to rise globally (3). Various causes can lead to the development of an HCC. Chronic viral infections by hepatitis B virus (HBV) or hepatitis C virus (HCV) still account for more than half its cases (4). However, antiviral therapies against HCV have considerably been improved over the last decades and HCV clearance is effective is most patients (5). HBV infection remains for life but can be prevented by vaccination (6). On the other hand, cirrhosis is the highest risk for progression towards HCC with 40% of the patients developing liver cancer. Cirrhosis can be alcohol-related or can result from metabolic syndrome-associated non-alcoholic steatohepatitis (NASH). The latter is thought to become the most leading cause of HCC and is already the fastest growing aetiology in western countries where the unbalanced food diets are responsible for a rise in obesity (7).

HCC is frequently diagnosed at later stages and there is no curative therapy for an advanced HCC. Thus, the early diagnosis is one of the biggest challenge (8). Over the past few years, the early diagnosis of HCC has relied on surveillance with ultrasonography (US) and serological assessments of alpha-fetoprotein (AFP). However, the specificity and sensitivity of US/AFP is not satisfactory enough to detect early onset HCC. HCC is mostly treated with surgical resection and liver

transplantation (9, 10). Systemic therapies targeting the immune system have emerged and offer an alternative to the conventional therapies (11). Thus, it remains important to continue improving our knowledge of the immune mechanisms in the context of HCC.

The liver is an organ that contains a large number of immune cells. The contribution of T and B lymphocytes to HCC (12–14) as well as that of myeloid populations (15–18) have been extensively studied but less is known about the involvement of innate lymphoid cells (ILCs) in HCC. ILCs are the innate counterpart of T lymphocytes but lack antigen receptors. They are divided into five subsets based on their developmental pathway, phenotype and function: NK cells, helper ILC1s, ILC2s and ILC3s that mirror Th1, Th2 and Th17 lymphocytes, and the lymphoid tissue-inducer (LTi) cells. Canonical signaling pathways such as Notch, Wnt/β-catenin and TGF-β pathways, were shown to be involved in ILC differentiation and functional activation. Thus, in addition to discussing the roles of ILCs in the HCC context, this review aims at highlighting how these signaling pathways might impact the functions of ILCs in liver cancer and what therapeutic strategies could be considered.

PRESENTATION OF ILC SUBSETS

The current ILC nomenclature is based on their phenotype and functions (19, 20). ILC1s and NK cells share a lot of similarities with Th1 lymphocytes. They both produce type 1 cytokines such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) upon activation by interleukin (IL)-12, IL-15 and/or IL-18 (**Figure 1A**). They are involved in immunity to intracellular bacteria and viruses and play a protective role in cancer. They both require the expression of the T-box transcription factor T-bet however, NK cells also rely on eomesodermin (Eomes) expression for development and function (21) (**Figure 1A**). Contrary to most ILC1 subsets, liver ILC1s can exhibit

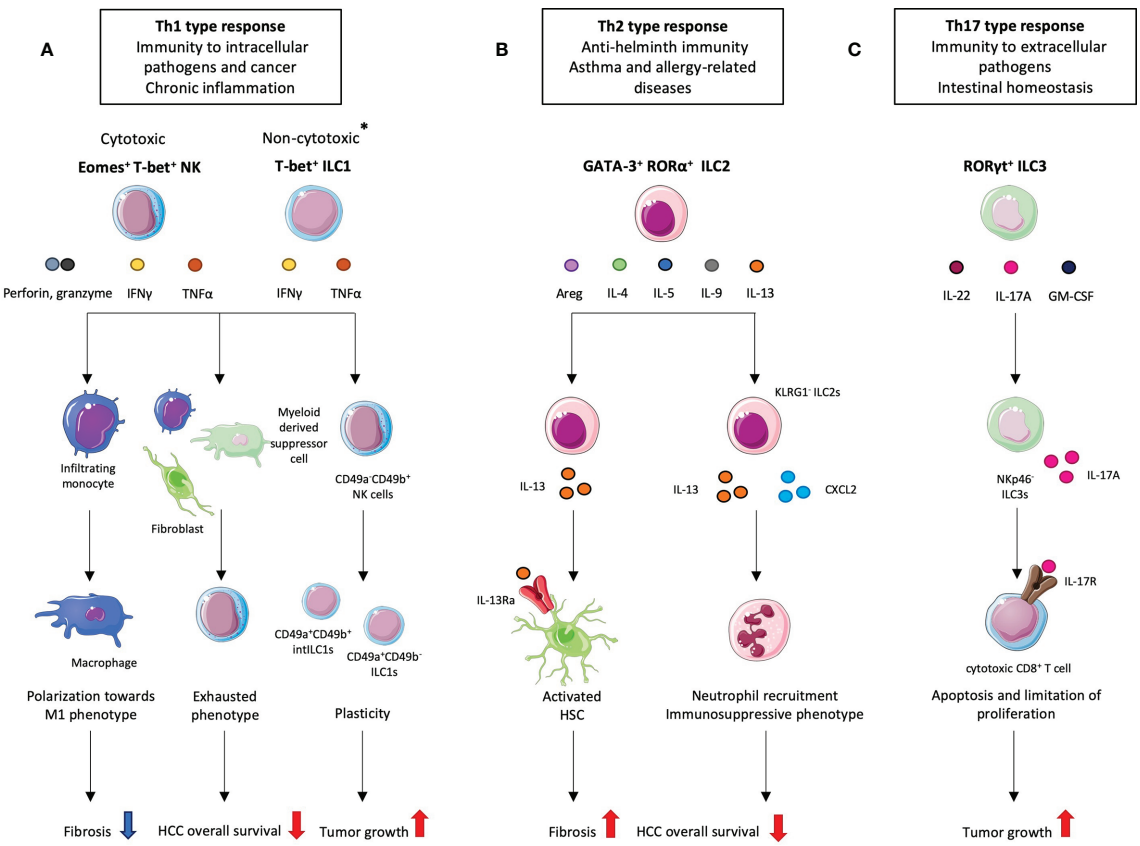

FIGURE 1 | Different ILC subsets and their pro- and anti-tumoral roles in hepatocellular carcinoma (HCC). *Most ILC1 subsets are non-cytotoxic however hepatic ILC1s can secrete perforin and granzyme B. **(A)** Given their cytotoxic activity, liver NK cells and liver ILC1s appear as good candidates in the anti-tumor response. Indeed, at the fibrosis stage, which precedes HCC development, NK cells exhibit a protective role by promoting macrophages towards an M1 phenotype. However, in HCC, NK cells display an exhausted phenotype resulting from interaction with cancer-associated fibroblasts and/or myeloid-derived suppressor cells which correlates with decreased patient overall survival. NK cells also convert to intermediate (int) ILC1s. **(B)** ILC2s are implicated at the fibrotic stage where they play a detrimental role. IL-33-activated ILC2s produce IL-13 which activates hepatic stellate cells (HSCs) that start secreting collagen. At the HCC stage, ILC2s tend to promote its progression by recruiting neutrophils. This effect is mediated by the loss of KLRG1 and increased secretion of CXCL2 and IL-13. **(C)** NKp46$^-$ ILC3s have been shown to promote HCC through the IL-23/IL-17A axis. ILC3-produced IL-17A directly inhibits CD8$^+$ T cell proliferation and cytotoxic activity which subsequently lead to tumor growth.

cytotoxic activities like NK cells with the expression of TNF-related apoptosis-inducing ligand (TRAIL) as well as granzyme b and perforin (22). ILC2s mirror Th2 lymphocytes. They depend on the expression of the transcription factors GATA-3 and RORα (23, 24) and secrete type 2 cytokines such as IL-4, IL-5, IL-9, IL-13 and amphiregulin (Areg) (25, 26) (**Figure 1B**). Their activation is dependent on the cytokines IL-33, IL-25 and on the thymic stromal lymphopoeitin (TSLP). They are essential in the anti-helminth immune response but were shown to be detrimental in asthma and allergy contexts. The role of ILC2s in cancer is an emergent field of study with pro-tumor and anti-tumor actions depending on the context (27, 28). Adult ILC3s are enriched in the intestine, rely on the expression of retinoic acid related orphan receptor (ROR)γt encoded by the *RORc* gene and are activated by IL-23 and IL-1β. They produce Th17-related cytokines namely IL-17A and IL-22 as well as GM-CSF (**Figure 1C**). ILC3s are further divided into three subsets: one that expresses the natural cytotoxicity receptor (NCR), first discovered on NK cells and ILC1s, and the other one that does not. NCR^+ ILC3s arise from the NCR^- ILC3s and mainly produce IL-22 while the NCR^- subset produces high amounts of IL-17A (29, 30). ILC3s are involved in the defense against extracellular pathogens and in intestinal homeostasis but were also shown to play a role in chronic intestinal inflammation (31, 32). Patients suffering from Inflammatory bowel disease (IBD) are at high risk of developing cancer (33) and ILC3s are known to be central players in the aggravation of IBD (34) which suggests that these cells can also be involved in cancer progression.

Under specific circumstances, plasticity between the different subsets was demonstrated in many studies. Cellular plasticity was first described by Helen Blau in 1985 (35). In her studies, muscle cell identity was regulated by extracellular factors. Cell identity was no longer fixed but subjected to regulation by environmental cues. In mice, $CCR6^-$ $ROR\gamma t^+$ ILC3s can downregulate their expression of *Rorc* to the benefit of *Tbx21* coding for T-bet during *Salmonella enterica* infection. These "ex-ILC3s" exhibit ILC1-like properties with a T-bet-driven production of IFNγ that contributes to the protection of the epithelial barrier during the infection (36). This conversion can also be dependent on extrinsic signals such as the production of IL-12 by dendritic cells and monocytes (37–39). Interestingly, the opposite plasticity was shown as possible with an IL-23- and IL-1β-mediated *in vitro* transdifferentiation of human ILC1s into ILC3-like cells (39). ILC2s also displayed plastic properties in several studies. In response to IL-12, human peripheral blood ILC2s can upregulate T-bet expression and start producing IFNγ both *in vitro* and *in vivo* (40–42). In some studies, the expression of GATA-3 and Type 2 cytokine production were maintained giving a mixed ILC1/ILC2 phenotype to the cells (40, 42). In another one, the Th2-related pathway was downregulated in human ILC2s following *in vitro* culture with IL-1β and IL-12 (41). Interestingly, *in vitro* culture of human ILC2s with IL-4 was able to prevent such conversion indicating its crucial role in maintaining the ILC2 identity (41). The importance of these two cytokines in ILC2 plasticity was corroborated by the fact that ILC2 frequency is decreased in tissues from patients with severe chronic obstructive pulmonary disease (COPD) displaying an IL-12 signature while patients with chronic rhinosinusitis with nasal polyps (CRSwNP) displaying elevated eosinophil-derived IL-4 showed accumulation of ILC2s (41).

In contrast to NK cells that can circulate throughout the body, the other ILC subsets are mostly tissue-resident and are enriched at the barrier surfaces where they maintain tissue integrity in the steady-state. Thus, ILCs are considered as important players in the immune response that can rapidly react to tissue disruption or infection by the secretion of cytokines.

PRO- AND ANTI-TUMORAL ROLES OF ILCs IN HCC

NK and ILC1s

In the liver, NK and ILC1s represent 30-50% of total lymphocytes in humans and around 10% in mice. Murine liver NK cells display a $CD49a^-$ $CD49b^+$ T-bet^+ $Eomes^+$ phenotype. They are located in the sinusoids and can recirculate. Mouse liver ILC1s are characterized as $CD49a^+$ $CD49b^-$ T-bet^+ $Eomes^-$ and are considered as liver-resident. In humans, liver NK cell population comprises $CD56^{dim}$ and $CD56^{bright}$ subsets. The $CD56^{bright}$ population express high levels of Eomes and could thus be considered as conventional NK cells. However, they also express residency markers such as CXCR6 and CD69 (43–45). Human hepatic ILC1s were not successfully identified probably due to a lack of specific marker. Nonetheless, Marquardt et al. proved the existence of a unique subset of human liver $CD56^{bright}$ NK cells expressing CD49a but not CD49b and whose lineage depends on T-bet but not on Eomes. After *in vitro* stimulation, these cells showed enhanced cytokine production but decreased degranulation capacity compared to $CD49a^-$ NK cells (46). These results suggest that $CD56^{bright}$ $CD49a^+$ $CD49b^-$ NK cells could be the human counterparts of mouse hepatic ILC1s.

In obesity-associated NASH mouse models, one of the most rising cause for HCC, NK cells have contrasting roles depending on the diet and the various parameters such as diet kinetics. In methionine and choline deficient diet (MCD)-induced NASH, $NKp46^+$ $NK1.1^+$ $CD49b^+$ cell numbers are increased and were shown to prevent fibrosis by polarizing infiltrating monocytes towards M1 type macrophages in the liver (47, 48) (**Figure 1A**). This suggest a protective role for NK cells keeping the disease from progressing towards fibrosis and subsequently to HCC. Nonetheless, other studies showed a detrimental role for NK cells in the development of NASH. IL-15 knockout NK-deficient mice displayed an attenuated NASH in response to high fat diet (HFD) (49). Wang et al. showed that murine NK cells secrete higher levels of pro-inflammatory cytokines in NASH which subsequently activate hepatocytes through JAK/STAT and NF-kB signaling. This induces hepatocyte damage and apoptosis while NK cell depletion resulted in alleviation of the disease (50).

In HCC, the role of NK cells has been extensively studied given their cytotoxic capacities. The number of infiltrating $CD56^+$ NK cells in the liver of patients suffering from HCC was positively correlated with the overall survival and cancer cell

elimination suggesting a protective role (51, 52). However, decreased numbers of NK cells are observed in HCC patients (53, 54). NK cells also exhibit an exhausted phenotype with alteration of their cytotoxic activity and IFNγ production (53, 55). Several mechanisms are involved in the impairment of NK functions (**Figure 1A**). In one study, patients with severe HCC showed a correlation between the infiltration of monocytes to the tumor and NK cell exhaustion. Co-cultures of NK cells with these tumor-derived monocytes revealed that they can directly regulate NK cells through the CD48/2B4 (CD244) axis. Blockade of CD244 on NK cells prevented their dysfunction (55). In another study, myeloid-derived suppressor cells (MDSCs) present in the tumor of HCC patients were able to directly inhibit NK cells function through a cell-to-cell contact involving the cytotoxicity receptor NKp30 (15).

The role of ILC1s in HCC has been poorly studied. Contrary to most ILC1 subsets, mouse liver ILC1s express perforin and granzyme b. They are thus capable of lysing cells although they remain less efficient than NK cells (22). In a fibrosarcoma model where MCA1956 cell line was subcutaneously injected into mice, an intermediate $CD49a^+$ $CD49b^+$ population was detected in the tumor and named intermediate (int)ILC1s (56). These cells came from the conversion of NK cells into ILC1-like cells, a process which was mediated by TGF-β *in vitro* and *in vivo* (**Figure 1A**). While NK cells were able to control tumor growth and metastasis, ILC1s and intILC1s upregulated tumor progression and resistance-related pathways with a higher expression of inhibitory receptors (NKG2A, KLRG1) and the production of PDGF-AB, a pro-angiogenic molecule. Moreover, NK-derived intILC1s and ILC1s produce high amounts of TNFα while NK cells mainly produce IFNγ. Although TNFα was first discovered as a rapid inducer of necrosis in tumor cells, it was later shown to have contextual roles in cancer (57). In HCC, TNFα is known to promote carcinogenesis *via* the activation of hepatic progenitor cells (58). Thus, the conversion of NK cells into ILC1-like cells likely favors the development of HCC.

Although, this demonstration was made in a fibrosarcoma model, Gao et al. were able to reconstitute NK, intILC1s and ILC1s in the livers of MCA1956-injected Rag γc-/- mice after intravenous injection of TGF-β-responsive splenic NK cells. In the liver, TGF-β is known to promote fibrosis and higher concentrations are found in HCC patients-derived supernatants (59). This suggests that NK conversion to intILC1s might take place in HCC and impact its progression.

ILC2s

ILC2s represent less than 5% of all ILCs in the liver at homeostasis and have been poorly studied in the context of liver diseases. However, it was demonstrated that they play a major role in liver fibrosis (60, 61). Their frequency is increased in fibrotic livers from mice and humans. In mice, the accumulation and activation of liver ILC2s was mediated through the IL-33/ST2 axis. Activated ILC2s start producing IL-13 which binds to its receptor IL-13Rα on hepatic stellate cells (HSCs) and triggers their differentiation from "quiescent" to "activated" cells leading to collagen deposition (60) (**Figure 1B**). In patients, the number of ILC2s is correlated to the severity of the fibrosis (61). Given that liver fibrotic patients are at high risk of developing cancer, this suggests a detrimental role for ILC2s in the HCC development.

This was confirmed in a recent study by Xu et al. where the abundance of ILC2s in the tumor area of HCC patients liver is correlated with poor prognosis (62). When looking closely at the phenotype, they identified a subset of ILC2s, enriched in the tumor tissue but absent in the non-tumoral area, lacking the expression of killer cell lectin-like receptor subfamily G member 1 (KLRG1), a known marker for mature and activated ILC2s. KLRG1 interacts with cadherins and particularly E-cadherin. Co-cultures of murine hepatic ILC2s with Hepa1-6 cells resulted in significant decrease in KLRG1 levels. Hepa1-6 cells do not express E-cadherin but overexpression of its *Cdh1* gene maintained KLRG1 expression in ILC2s (62). These experiments suggest that the loss of KLRG1 on ILC2s in HCC can be accounted for by a decrease in E-cadherin expression at the surface of hepatocytes. $KLRG1^-$ ILC2s isolated from HCC patients produced significant higher levels of IL-13 and CXCL2 and CXCL8, two chemokines known to recruit neutrophils. All those observations were confirmed in a c-Myc/NRas-induced murine HCC model where hepatic $KLRG1^-$ ILC2s produced CXCL2 and IL-13 at higher levels. *Klrg1* knockout (ILC2-CRISPR-KLRG1) and *Klrg1*-overexpressing (ILC2-PCDH-KLRG1) murine ILC2s were generated to assess its specific role in HCC. *Klrg1* overexpression in ILC2s led to a decrease in *Cxcl2* and *Il-13* mRNA levels whereas *Klrg1* knockout enhanced their expression. Neutrophils recruitment was also increased with the use of conditioned medium of *Klrg1*-deficient ILC2s in a chemotaxis assay (62). An immunosuppressive profile was induced in recruited neutrophils *via* the upregulation of *Arg1*, coding for Arginase 1. This increase was likely mediated by IL-13 produced by ILC2s as CXCL2 deficiency did not affect *Arg1* expression. Altogether, these results showed that ILC2s, which downregulate KLRG1 in the HCC tumor microenvironment, promote HCC *via* CXCL2-dependent neutrophil recruitment and IL-13-driven immunosuppression (**Figure 1B**).

ILC3s

Although rare in the liver, ILC3s might have substantial effects on the development of HCC. ILC3s are subdivided into NCR^+ ILC3s that represent the main source of intrahepatic IL-22 and NCR^- ILC3s that largely produce IL-17A (29, 30). The pro-tumorigenic role of IL-17A was studied. To note, IL-17A can also be secreted by Th17 T cells and γδ T cells. A significant reduction in tumor growth was observed in IL-17A-deficient mice in heterotopic models of HCC. Conversely, intravenous injection of recombinant IL-17A led to an increase in tumor volume. Mechanistically, IL17-A suppresses the cytolytic activity and cytokine production of $CD8^+$ T cells and promotes the recruitment of MDSCs through the CXCL5/CCR2 axis (63). Although the Vγ4 γδ T cells were responsible for the IL-17A secretion, this study marked the importance of this cytokine in HCC development. In another study, the same team showed that in mice, IL-17A-producing NCR^- ILC3s also participate to the progression of HCC in an IL-23-dependent manner by directly regulating $CD8^+$ T cell proliferation and enhancing their

apoptosis (**Figure 1C**). However, Vγ4 γδ T cells and ILC3s do not operate at the same stage of the disease. In IL-23-stimulated mice, ILC3s are the main producers of IL-17A at 1 week after intravenous injection of Hepa1.6 cells while $CD4^+$, $CD8^+$ and γδ T cells become the main IL-17A-producing populations from the second week after injection (64). These results were confirmed in an orthotopic surgical HCC model. Thus, hepatic ILC3s are early responders through the IL-23/IL-17A axis in the context of HCC and seem to promote the progression of the disease.

Interestingly, in a study on human fibrotic livers, a decrease in $NKp44^-$ ILC3s was observed at the most severe stages of the disease. Since cirrhotic patients have the greatest risk at developing HCC, this observation questions the actual impact NCR^- ILC3s might have on HCC (65).

NCR^+ ILC3s are even rarer in the liver than their NCR^- counterparts. Their implication in HCC has not been studied yet even though IL-22 has been presented with controversial roles in hepatocytes, either promoting their regeneration (model of Con-A induced hepatitis) (66) or their proliferation in the diethylnitrosamine (DEN)-induced mouse HCC model (66–68). In both cases, the effect is mediated by the activation of the STAT3 pathway. Thus, NCR^+ ILC3s could potentially participate to HCC aggravation *via* their secretion of IL-22.

SIGNALING PATHWAYS INVOLVED IN HCC: IMPACT ON ILCs

During HCC, there is remodeling and reactivation of numerous signaling pathways. We selected the signaling pathways that are the most conserved in development and could then occur both on hepatocytes and immune subsets such as ILCs.

Notch Pathway

The Notch signaling is one of the most evolutionary conserved pathways. It controls cell fate decision, development and function of numerous cell types including immune cells, and it enables direct cell-to-cell communication (69). First studied in *Drosophila melanogaster*, the Notch pathway displays more complexity in mammals with four heterodimeric receptors (Notch 1-4) that can all bind to five ligands (Jagged 1 and 2, and Delta like ligands 1, 3, 4) with variables affinities (70). When a receptor engages one of its ligands, the extracellular domain is cleaved by ADAM metalloproteases while the Notch intracellular domain (NICD) undergoes serial proteolytic cleavages by the gamma secretase which leads to its translocation to the nucleus of the responding cell (69–71). There, it binds to the transcription factor like recombination signal binding protein for immunoglobulin Jk region (RBP-Jk). Histone acetyltransferases (HAc) among other members of the MAML family are recruited leading to the formation of the NICD/MAML/RBP-Jk activation complex. The latter is responsible for the signaling cascade that enables the transcription of Notch target genes (69–71).

Although Notch is mostly known for its roles in embryonic development and adult tissue homeostasis, it has also been shown to be involved in cancer with pro- or anti-tumorigeneric effects. In HCC, numerous studies have underlined its carcinogenic action. Overexpression of Notch correlated with a decreased overall survival and aberrant expression is found in 30% of HCC patients (72). Notch 3 can regulate the activation of HSCs in the context of fibrosis and its overexpression in HSC leads to an increase in the expression of α-SMA and collagen I (73). Notch signaling can also be activated through IL-6/STAT3 axis leading to the acquisition of stem-like characteristics by HCC cells (74). However, Notch impact on HCC through its activation of immune cells has been poorly studied.

Notch signaling is not directly implicated in NK development as its abrogation does not prevent the formation of mature NK cells in the bone marrow (75, 76). Its role in ILC1 remains unknown although Notch is not required for early ILC commitment (77). However, Notch can influence NK and ILC1 functions by modulating the expression of the transcription factor T-bet, necessary for both NK and ILC1 maturation and activation (**Figure 2**). Indeed, it was shown that around half the hepatic ILC1 and NK cells express Notch1 and/or Notch2 (78). Using models such as the $IL7R^{Cre}$ $RBP\text{-}Jk^{flox}$ mouse strain, where the Notch pathway is defective in lymphoid cells and their progenitors, the deficiency in RBP-Jk was directly correlated to a shift in the T-bet/Eomes expression balance with the first being decreased and the second increased. Moreover, the levels of CD49a were decreased in RBP-Jk-deficient ILC1s and this was specifically linked to Notch1 signaling as hepatic ILC1s from $Il7R^{Cre}$ $Notch2^{flox}$ mice were not affected (78). Liver NK and ILC1s were also shown to have enhanced cytokine production and cytolytic activity with better control of initial stage of hepatic tumor in the absence of Notch signaling in these heterotopic models (78) (**Figure 2**). By regulating their functions, Notch signaling pathway could thus be considered as one of the factors influencing NK cell exhaustion and tumor progression in HCC.

Notch was proposed as essential for adult ILC2 development from both murine bone marrow CLPs and human progenitors (79). But Notch is also involved in ILC2 plasticity towards ILC3-like cells. ILC2s can be divided into resident ILC2s and inflammatory ILC2s (iILC2s), defined as the $KLRG1^{high}$ circulating subset. ILC2s produce Th2 cytokines while iILC2s can express low amount of RORγt, in addition to high levels of GATA-3, to produce IL-17A along with Th2 cytokines (80). iILC2s have been shown to be major inducers in airway inflammation after challenging mice with house dust mite (81). *In vivo* injections of antibodies targeting Notch1 and/or Notch2 showed that Notch could favor the plasticity towards RORγt-expressing ILC2s. Furthermore, the NICD/MAML/RBP-Jk activation complex can directly bind to the *Rorc* locus which drives the ILC3 differentiation (81) (**Figure 2**). Thus, Notch signaling is possibly required for iILC2 generation. Since $KLRG1^-$ ILC2s were implicated in HCC progression, the generation of $KLRG1^{high}$ ILC2s could appear as beneficial. However, iILC2 role has not been clearly defined in HCC making it difficult to conclude.

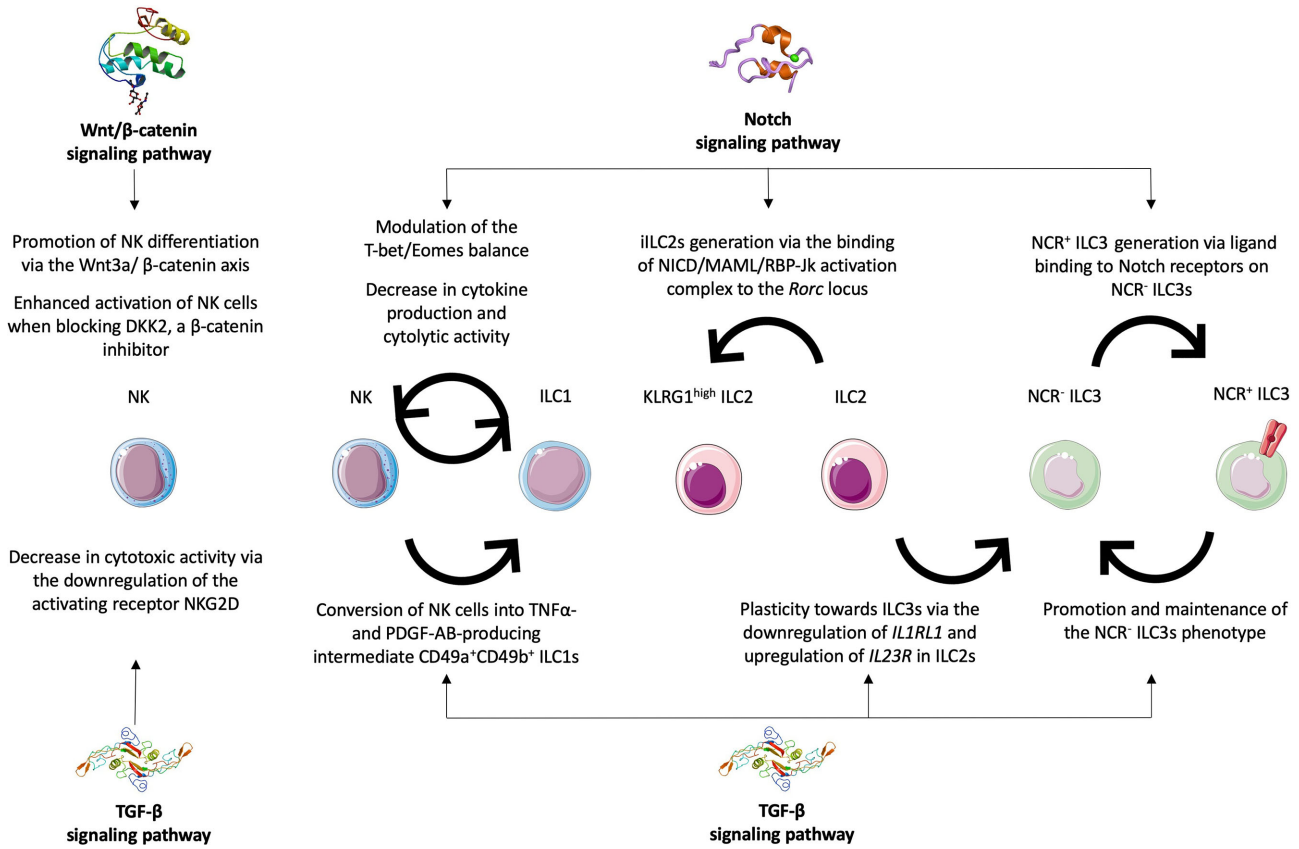

FIGURE 2 | Roles of Notch, TGF-β and Wnt/β-catenin signaling pathways in ILC plasticity and functions. The Notch signaling pathway was implicated in balancing the T-bet/Eomes expression as well as decreasing cytokine production and cytolytic activities in NK cells and ILC1s thus impacting their phenotypes and functions. The Notch signaling is required for the generation of iILC2s, the KLRG1^{high} subset, *via* the binding of the NICD/MAML/RBP-Jk activation complex to the *Rorc* locus. It is also required for the transdifferentiation of NCR$^-$ ILC3s into their NCR$^+$ counterparts. The TGF-β signaling acts antagonistically and favors the production of NCR$^-$ ILC3s either from NCR$^+$ ILC3s or from ILC2s *via* the downregulation of *IL1RL1* and the upregulation of *IL23R*. It was also shown to promote the transdifferentiation of NK cells into intermediate CD49a$^+$ CD49b$^+$ ILC1s in different tumor models. The TGF-β signaling is also involved in decreased NK cytotoxicity by downregulating the activating receptor NKG2D. The Wnt/β-catenin signaling pathway was mostly shown to have an impact on NK cells by promoting their differentiation and functional activation.

In the case of the ILC3s, Notch was shown as essential for the differentiation of NCR$^-$ ILC3s into NCR$^+$ ILC3s (**Figure 2**). This process of maturation is driven by T-bet and Notch2 signaling (36). Indeed, in mice lacking RBP-Jk or Notch2 expression, the numbers of intestinal NKp46$^+$ ILC3s were dramatically decreased (36, 82). Moreover, *in vitro* culture of NKp46$^-$ ILC3s on Notch ligand expressing OP9-DL1 stromal cells led to the generation of NKp46$^+$ ILC3s. However, when cultured on OP9 stromal cells alone, the same cells were not able to differentiate (83). T-bet deficiency also prevented NKp46$^-$ ILC3s from giving rise to NKp46$^+$ ILC3s regardless of the stromal cells used (83). Notch is thus required for the generation of IL-22 producing NCR$^+$ ILC3s. Use of *Ncr1* fate mapping mice revealed a heterogeneity among NCR$^-$ ILC3 precursors with some of them having transitionally expressed *Ncr1*. *In vitro* culture for 9 days of NCR$^+$ ILC3s on OP9 stromal cells did lead to 40% of the cells losing their expression of Ncr1 (83). Thus, they concluded that Notch is not only necessary for the production of NCR$^+$ ILC3s but also to maintain the expression of *Ncr1* and consequently maintain their identity.

TGF-β Pathway

Transforming growth factor (TGF)-β is a pleiotropic cytokine involved in many biological processes from cell fate and differentiation to proliferation, migration and apoptosis. In the canonical TGF-β signaling pathway, a molecule of active TGF-β engages the monomeric type II receptor (TGF-βRII) which in turn recruits the serine/threonine kinase type I receptor (TGF-βRI) and triggers its cross-phosphorylation (84). The heteromeric complex that is formed is able to phosphorylate SMAD2 and SMAD3 proteins. This enables them to form a transcriptional complex with SMAD4 that regulates the expression of target genes by binding to their regulatory regions. Non-canonical signaling pathways involving other factors exist and participate to the diversity of TGF-β biological roles. Notably, the TGF-β receptor complex can also

transduce its signal through mitogen-activated protein kinases (MAPKs), phosphatidylinositide-3 (PI-3) kinase, Rho family GTPases or TNF receptor-associated factor 4/6 (TRAF4/6). Some of their downstream molecules can however interact with the SMAD proteins such as the collaboration of JNK/p38/ERK with SMADs in the regulation of proliferation and cell death (85, 86). TGF-β-mediated activation of TRAF proteins can induce the NF-KB pathway, involved in many inflammatory responses (87, 88). Thus, TGF-β can signal through a great diversity of pathways enabling it to have a substantial variety of roles.

However, TGF-β is most known for its immunosuppressive properties which in cancers can lead to tumor immune-evasion and disease aggravation. In HCC, TGF-β is highly expressed in the liver but its role in tumor development and progression is stage-dependent (89). In the early onset of the disease, TGF-β tends to have an anti-tumor role with the restriction of hepatocyte proliferation. But TGF-β is also strongly associated with liver fibrosis/cirrhosis (90, 91). It is massively produced by HSCs and liver sinusoidal endothelial cells and induces the activation of hepatic Treg cells (92, 93). TGF-β also promotes the generation of pro-inflammatory Th17 cells through the activation of SMAD2/3 in naïve $CD4^+$ T cells (94). Moreover, it was shown to induce a shift from M1 towards M2 macrophages and to inhibit the cytotoxic activity of $CD8^+$ T cells *via* the suppression of their IFNγ secretion (95, 96). However, its impact on ILC populations in HCC is still not known.

For NK cells to display anti-tumoral role, recognition and targeting of tumor cells are essential. One of the main mechanisms by which NK cells recognize cancerous cells is the engagement of their activating receptors with the ligands expressed at the surface of target cells. The most studied activating receptor is NKG2D that can bind to several ligands (MICA, MICB, ULBP1-6), usually highly upregulated by tumor cells (97, 98). The role of TGF-β in decreasing NKG2D expression on NK cells has been widely studied. Some studies showed that in cancer patients, plasma levels of TGF-β were negatively correlated with the level of expression of NKG2D on circulating NK cells (99, 100). *In vitro* incubation of NK cells with plasma from patients led to a downregulation of NKG2D while the expression was restored with the addition of neutralizing anti-TGF-β antibody (99). Incubation with recombinant TGF-β1 also specifically reduced NKG2D surface expression impairing NK cell cytotoxicity (101). Other studies showed that TGF-β was also responsible for a decrease in NKp30, DNAM-1, granzyme A and perforin expressions and that this was mediated by SMAD2/3 signaling (102, 103). Treatment with the TGFβRI kinase inhibitor Galunisertib (104) or neutralizing anti-TGF-β1 antibody (105) restored the expression of these specific transcripts. Altogether, these results show that TGF-β-mediated NKG2D downregulation participates to the inhibition of NK cytotoxicity in cancer (**Figure 2**).

However, TGF-β has recently emerged as a driver in ILC plasticity. Several studies revealed the implication of TGF-β in multiple cancer diseases into generating a pro-angiogenic NK population (106–108). $CD56^+$ NK cells from patients with non–small cell lung cancer (NSCLC) or squamous cell carcinoma (SCC) showed enhanced production of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) (108). *In vitro* culture of peripheral blood $CD56^+$ NK cells from healthy donors with TGF-β resulted in the upregulation of VEGF and PIGF highlighting the essential role of this cytokine in polarizing NK cells towards a pro-angiogenic phenotype (108).

In 2017, Gao et al. went further in describing how TGF-β mediated the conversion of NK cells into intILC1s and ILC1-like cells in several cancer models (56) (**Figure 2**). Given that the tumor-infiltrating NK cells produce large amounts of IFNγ which is one of the key cytokines to inhibit tumor growth and that intILC1s and ILC1s mainly produce the pro-angiogenic molecule PDGF-AB and pro-tumorigenic cytokine TNFα (58), TGF-β signaling is considered as prone to favor tumor immune evasion. Additionally, using single-cell RNA sequencing and flow cytometry analysis of liver ILCs from HCC patients, another study revealed the presence in the tumor area of an NK-like population with a mixed NK/ILC1 phenotype. TGF-β mRNA levels were found to be significantly increased in the tumor area (109). These results suggest that TGF-β signaling could be involved in HCC progression by promoting NK conversion into ILC1-like cells. In another study, liver-derived TGF-β was shown to sustain the $Eomes^{hi}$ $T\text{-}bet^{low}$ phenotype in human liver NK cells (110). Given that the $CD49a^+$ $CD49b^-$ cells that might be considered as human counterparts of ILC1s are $T\text{-}bet^+Eomes^-$, the role of TGF-β in NK conversion to ILC1-like cells remains to be confirmed in patients.

TGF-β was also found to drive the plasticity of ILC2s and ILC3s. Recent studies revealed that human ILC2s cultured in presence of TGF-β, IL-1β and IL-23 can transdifferentiate into IL-17A-producting ILC3-like cells (111, 112). TGF-β is not necessary as *in vitro* culture of ILC2s with IL-1β and IL-23 can lead to IL-17A production. However, when TGF-β is added to the medium, the secretion of IL-17A is dramatically increased while that of IL-5 is decreased. This was explained by the substantial upregulation of *IL23R* in the presence of TGF-β as well as the reduced mRNA expression of *IL1RL1*, the gene coding for the IL-33 receptor ST2 (**Figure 2**). By increasing the response to IL-23 and decreasing the one to IL-33, TGF-β promotes the conversion of ILC2s into ILC3s that produce the pro-inflammatory IL-17A cytokine.

TGF-β was also shown to have a direct action on ILC3s to promote the NCR^- phenotype (**Figure 2**). In the same study that revealed an essential role for Notch signaling in NCR^+ ILC3 generation, TGF-β was shown to act in opposition (83). TGF-β signaling impairs the differentiation of NCR^- ILC3s in NCR^+ ILC3s *in vitro* and *in vivo*. But it can also drive the reverse conversion of NCR^+ ILC3s into NCR^- ILC3s as *in vitro* culture of NCR^+ ILC3s that express a constitutive active form of TGF-βRI leads to a decreased expression of *Ncr1* and decreased numbers of NCR^+ cells (83). Given that NCR^- ILC3s secrete higher amounts of IL-17A and were implicated in HCC progression, we can speculate that TGF-β may participate to liver tumorigenesis by mediating ILC2 and ILC3 plasticity.

Wnt/β-catenin Pathway

The Wnt/β catenin signaling pathway is a highly conserved pathway that controls embryonic development, cell proliferation, differentiation and fate determination (113). In mammals, 19 genes coding for different Wnt proteins are expressed (114, 115). The latter are lipid-modified in the endoplasmic reticulum and are then transported from the Golgi to the cell membrane thanks to the chaperone Wntless (116). Once secreted, the Wnt proteins can interact with the Frizzled (FZD) receptor at the surface of the responding cell. The FZD are associated with coreceptors, either LRP5/6 or the ROR/RYK complex. Binding to the FZD/ROR/RYK usually leads to the activation of the Wnt/β-catenin-independent pathway while interaction with FZD/LRP5/6 usually results in the activation of the canonical Wnt/β-catenin signaling pathway (117). The β-catenin is a protein found in the cytoplasm of cells. In the absence of interaction between Wnt and FZD, it is ubiquitinated by the β-catenin destruction complex, composed of the casein kinase 1 (CK1), the GSK-3β, the adenomatous polyposis coli (APC) and AXIN1, leading to its subsequent degradation by the proteasome (113, 114, 118). However, upon binding of Wnt to FZD, the scaffolding DVL protein is recruited to the FZD intracellular domain which leads to the inhibition of GSK-3β (119). The β-catenin is thus stabilized and can translocate to the nucleus where it promotes the transcription of target genes (120).

Although Wnt/β-catenin signaling is known to take part in many essential biological processes, its dysregulation was shown to be involved in HCC development (121, 122). One study also revealed how in HCC, tumor-derived Wnt ligands polarize tumor-associated macrophages (TAM) towards an M2 phenotype contributing to tumor progression (123).

The Wnt/β-catenin pathway was shown to be involved in NK cell differentiation. Exposure of human thymic $CD34^+CD1a^-$ progenitors to Wnt3a that signals through the β-catenin led to an increased production of NK cells compared to untreated samples (124) (**Figure 2**). Moreover, the blockade of Wnt signaling *via* DKK1, a known inhibitor of Wnt/β-catenin, in human $CD34^+$ hematopoietic progenitors led to a significant decrease in NK cell generation (125). Another study showed that β-catenin-deficient mice have decreased NK cell numbers with an action of β-catenin on the expression of the antiapoptotic protein Bcl2 (126). However, the Wnt/β-catenin pathway could also be implicated in NK cell function and cytotoxic activity. In one study, blockade of DKK2, another inhibitor of the β-catenin pathway, led to an enhanced activation of NK cells (**Figure 2**). Using a colorectal cancer mouse model, they injected 5F8, a molecule specifically preventing the binding of DKK2 to LRP5. It resulted into a decrease in the numbers of cancerous intestinal polyps. This suggests that DKK2 promotes tumor progression in this model. And this effect could be partly mediated by NK cells whose cytotoxic activity is enhanced in the absence of DKK2. Indeed, administration of 5F8 led to significant increases in *Gzmb*, *CD69*, *IFNγ* and *NKp46* gene expression (127). Although set in the colorectal cancer model, this study underlines the importance of Wnt/β-catenin signaling pathway in NK-driven anti-tumoral response. It can thus be hypothesized that Wtn-β-catenin could also regulate NK cell activation in HCC.

Implication of Wnt proteins in the differentiation and/or function of helper ILCs has not been studied yet. Several studies underpinned the role of the transcription factor TCF-1 in ILC2 and ILC3 differentiation. However, TCF-1 is not only a Wnt/β-catenin signaling target gene but is also a downstream target gene of the Notch pathway. One study showed that β-catenin-deficient hematopoietic progenitors, $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells, can develop normally *in vivo* into ILC2 while Notch-inhibited LSK cells fail to produce ILC2s. This suggests that Wnt-β-catenin pathway might not be involved in ILC2 differentiation as the Notch signaling (128).

CONCLUSION

ILCs are emergent actors in the field of cancer research. Here, we have summarized their roles in HCC and underlined the potential impact the Notch, TGF-β and Wnt/β-catenin signaling pathways might have on their response. The cytotoxic NK cells remain to this day the most studied cells however, helper liver ILCs are being increasingly scrutinized as their tissue residency characteristics position them as chronically exposed to this specific tolerogenic environment.

Another highlight of ILCs is their ability to transdifferentiate from one subset to another. Although this plasticity is mostly driven by the presence of specific interleukins, Notch signaling and TGF-β pathways are major actors in this process and can even harbor antagonist roles. Wnt/β-catenin signaling pathway impact has been poorly investigated but one can expect future research to uncover its role in ILCs in cancer and most specifically in HCC.

Further studies are needed to clarify the roles of these signaling pathways in immune cells, and in particular ILCs, in HCC as many studies have been performed in other cancer models. This will provide new insights into the molecular mechanistic underlying the response of ILCs and subsequently allowing new immunotherapeutic strategies to emerge in order to specifically target these signaling pathways in ILCs.

AUTHOR CONTRIBUTIONS

EB designed and prepared the manuscript and the figures. RG gave guidance on the outline and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Role of ILC2s in Solid Tumors: Facilitate or Inhibit?

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Group 2 innate lymphoid cells (ILC2s) are important mediators of type 2 immunity and play an important role in allergic diseases, helminth infections, and tissue fibrosis. However, the role of ILC2s in tumor immunity requires further elucidation. Studies over the past decade have reported that ILC2s play a promoting or suppressing role in different tumors. Here we reviewed the role of ILC2s in solid tumors demonstrating that ILC2s act as a crucial regulator in tumor immunity. We proposed that ILC2s could be an important predictor for tumor prognosis and a new therapeutic target after immunotherapy resistance. In conclusion, our study shed new light on modifying and targeting ILC2s for anti-tumor immunotherapy.

Keywords: group 2 innate lymphoid cell, tumor, inflammation, immunity, cytokine

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INTRODUCTION

Innate lymphoid cells (ILCs) are the counterparts of T cells in innate immunity. According to the development and function, ILCs are divided into five subsets—NK cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue-inducer cells (LTi cells). Human ILC2s were discovered in the fetal gut in 2011. Mjosberg et al. initially defined this cell population as Lineage negative CRTH2$^+$ CD127$^+$ CD161$^+$ cells; these cells expressed considerable amounts of IL-13 after *in vitro* stimulation with IL-25 and IL-33 (1). ILC2s have since been found in myriad healthy and diseased human tissues, such as lung, nasal polyps, kidney, intestine, skin, and tumors (2, 3). Recent discovery of CRTH2$^-$ ILC2s suggests that CRTH2 is no longer suitable as a common marker for all human ILC2s (hILC2s) (4).

ILC2 DIFFERENTIATION AND MIGRATION

Although ILCs are morphologically similar to lymphocytes and can produce high levels of T-helper cytokines, ILCs lack antigen receptor and lineage (Lin) markers and can expand and function normally in T-cell-deficient nude mice (5, 6). Therefore, ILC and T-cell differentiation and development are traditionally believed to be independent of each other (7). However, recent studies have shown that ILCs are a branch of neonatal T-cell progenitors that colonize peripheral tissues in the third trimester (8, 9). The CD4$^-$ CD8$^-$ double-negative (DN) T cells may differentiate into ILCs at DN1/ETP and DN2-DN3 transition stage, and this change is influenced by the intensity of Notch signaling and by E-ID protein and Bcl11b activity (10). In addition, Shin et al. reported in particular that the differentiation of tissue-resident ILC2s may be because of DN2 ineffectively rearranging their γ/δ loci (10). The exact source of ILC2s that colonize in peripheral tissues and organs remains unknown. In mouse lung tissue, IL-18R$^-$ ST2$^+$ ILC2s are differentiated *in situ* from

immature IL-18R1$^+$ ST2$^-$ innate lymphoid cell precursors (ILCPs) (11). Many studies have reported that tissue-resident human ILCs (hILCs) expand and maintain activity through self-renewal during inflammation and homeostasis (12–14). It can be inferred that initially colonized hILCs possess partial stem cell properties. Although human ILCPs have been detected in adult and neonatal lungs by single-cell RNAseq of RORα tracer (15), the origin of ILC2s in human tissue needs to be confirmed by further studies.

In mice, ILC2s are divided into tissue-resident natural ILC2s (nILC2s) and circulating inflammatory ILC2s (iILC2s). In mice, pulmonary nILC2s are defined as Lin$^-$ ST2$^+$ KLRG1^{int} cells which respond to IL-33 and pulmonary iILC2s are defined as Lin$^-$ ST2$^-$ KLRG1^{hi} cells which respond to IL-25 (16). However, the murine small intestinal lamina propria iILC2s are defined as Lin$^-$ CD25$^-$ ST2$^-$ cells (17). In a parabiotic mice model of inhibiting commensal bacteria with antibiotics, resting ILC2s residing in intestinal lamina propria are activated by IL-33 or helminths into iILC2s, which migrate to various extraintestinal tissues through sphingosine 1-phosphate (S1P)-mediated chemotaxis to participate in anti-helminth defense and tissue repair (18). hILC2s were previously considered to be exclusively tissue-resident, but this couldn't explain the increased abundance of ILC2s and other ILC subsets in peripheral blood of American cutaneous leishmaniasis patients compared with healthy volunteers (19). The latest research shows that, CD45RO$^+$ ILC2s are derived from resting CD45RA$^+$ ILC2s in airway inflammation and are regarded as human equivalent of the iILC2 subset (20). However, whether human intestinal ILC2s can be activated into iILC2s and migrate requires further investigation. Collectively, the pool of peripheral ILC2s formed in the early stage of innate immunity may consist of both tissue-resident ILC2s and migratory iILC2s.

ILC2 FUNCTION AND REGULATION

ILC2s are known to mirror the function of CD4$^+$ Th2 and are crucial mediator of type 2 immunity. IL-25 and IL-33 can strongly activate human and murine ILC2s *via* NF-κB and MAPK pathways. Activated ILC2s secrete a variety of cytokines to regulate effector cell function. For instance, murine ILC2-derived IL-4 stimulates Th2 differentiation and B cells to secrete IgG1 and IgE (21–23); murine ILC2-derived IL-5 facilitates eosinophil accumulation and eotaxin production (24); murine ILC2-derived IL-9 drives the recruitment of mast cells and enhances the secretion of IL-5 and IL-13 through the autocrine loop (25, 26); human ILC2-derived IL-10 reduces Th responses and maintains epithelial integrity in regulating grass-pollen allergy (27); human and murine ILC2-derived IL-13 induces mucus secretion in epithelial cells, smooth muscle cell spasm, and tissue fibrosis (28–30); In mouse bone marrow, ILC2-derived GM-CSF promotes the recovery of HSPCs from 5-FU-induced stress (31). In addition to secreting cytokines, ILC2s can modulate T cell function through direct interactions. In mouse lung, ILC2s directly activate T cell through Ag presentation by MHCII, and blocking MHCII interactions completely prevents the ability of ILC2s to stimulate DO11.10 T cell proliferation (32). This MHCII-mediated CD4$^+$ T cell activation was also observed in *Nippostrongylus brasiliensis*-infected mouse models, and co-stimulatory molecule CD80/86 was also detected in part of ILC2s (33). Activated T cells produce IL-2 to promote murine ILC2 proliferation and function, which in turn facilitate parasitic helminth expulsion (33). ILC2s in mice are kind of antigen-presenting cells and its expression of MHCII appeared to be enhanced by STAT6 signaling (34). Human and murine ILC2s express both ICOS and ICOS-L (35). In mice, ICOS-L on ILC2s can bind with ICOS on the same or separate ILC2s in cis or trans formation to promote ILC2 function and homeostasis; alternatively, ICOS-L on ILC2s can interact with ICOS on Tregs, which leads to Treg accumulation and cytokine production (36). Murine ILC2s also express the co-stimulatory molecule OX40L, which interacts with OX40 on Th2 to maintain Th2-mediated type 2 immunity and interacts with OX40 on Tregs to promote Treg survival and proliferation (37).

In addition to activating cytokines (IL25 and IL-33), ILC2s are regulated by a variety of co-stimulatory and suppressive cytokines. In some cases, co-stimulatory cytokines help activating cytokines optimally activate ILC2s by upregulating the expression of GATA3; co-stimulatory cytokines include γc family cytokines and TNF superfamily (38). In mice, γc family cytokines (IL-2, IL-4, IL-7, IL-9, and TSLP) upregulate GATA3 *via* JAK/STAT pathways; while TNF superfamily (TNFSF15 and TNFSF18) acts *via* NF-κB and MAPK pathways (38). In both human and mice, suppressive cytokines (Type 1 IFNs, IFN-γ, and IL-27) downregulate GATA3 expression *via* IFN-stimulated gene factor 3 and STAT1, thereby inhibiting ILC2 function and activity; in human, IL-10 and TGF-β suppress the production of IL-4, IL-5, and IL-13 only when ILC2s response to IL-33 or IL-33 + TSLP (38–40). Interestingly, TGF-β1 deficiency has shown to suppress murine ILC2 proliferation and IL-13 secretion (41).

PD-1 is a metabolic and suppressive immune checkpoint for ILC2s. In a high-fat diet mice model, TNF-induced PD-L1^{hi} M1 macrophages inhibit the function of PD-1$^+$ ILC2s *via* PD-1/PD-L1 interaction, leading to impaired glucose tolerance (42). In a mouse model of airway hyperreactivity, PD-1 deficiency shifts ILC2 metabolism to an anaerobic type (glycolysis, glutaminolysis and methionine catabolism) and enhances the activation and proliferation of ILC2s (43). The latest metabolomic and nutrient receptor analysis showed that hILC2s consumed amino acids to maintain high level of oxidative phosphorylation in a steady status, and the functional status of hILC2s relied on glycolysis and the mammalian target of rapamycin (44). These results suggest that activated ILC2s are low in oxygen dependence and mainly rely on glycolysis to maintain function; this metabolic profile of ILC2s makes it likely to adapt to the hypoxic tumor microenvironment (TME) and function in the TME. In addition, PD-1 negatively regulates the proliferation and function of human and murine KLRG1$^+$ ILC2s through inhibiting STAT5 phosphorylation (45). In mice, *Pdcd1* knockdown significantly increases nuclear STAT5 in KLRG1$^+$ ILC2s; in human, anti-PD-1 antibody along with rhIL-33 treatment can significantly

increase the secretion of type 2 cytokines by KLRG1$^+$ ILC2s (45). PD-1 upregulation was also detected in kinds of tumor-infiltrating ILC2s (TILC2s), which will be discussed below.

ILC2 PLASTICITY AND HETEROGENEITY

ILC2s demonstrate functional plasticity and can be converted into ILC1/ILC3-like cells in specific conditions. In human and mice, IL-12, IL-1β and IL-18 can switch the ILC2 phenotype to IFN-γ-producing ILC1-like cells by upregulating T-bet (46, 47); conversely, IL-4 can reverse this effect and maintain the ILC2 phenotype *via* GATA3 upregulation (48, 49). Recent study has classified hILC2s into more detailed subsets. According to the expression of CCR10, c-Kit (CD117) and CCR6, hILC2s was divided into 3 subgroups: CCR10$^+$ ILC2s, c-Kit$^+$ ILC2s and c-Kit$^-$ ILC2s (50). The phenotype of c-Kit$^+$ hILC2s can switch to ILC3-like cells when exposed to IL-1β and IL-23 in some pathological conditions. TGF-β in the microenvironment can upregulate the expression of IL-23R in c-Kit$^-$ hILC2s in the presence of IL-1β, and the response to IL-23 can lead to c-Kit$^-$ hILC2s differentiating into ILC3-like cells that produce IL-17 (51). In mice, under the regulation of Notch signaling, pulmonary nILC2s can switch to iILC2s, which produce IL-13 and IL-17; pulmonary iILC2s express high levels of GATA3 and also low amounts of RORγt, which is a key regulator for ILC3 differentiation and function (52, 53). The Notch transcriptional complex directly binds to the *Rorc* gene (encode RORγt) site and promotes its expression, so that iILC2s have the characteristics of both ILC2 and ILC3 (52). Moreover, murine iILC2s can switch to nILC2-like cells or ILC3-like cells which contribute to helminth defense and anti-fungi immunity (16).

ILC2s possess obvious heterogeneity. Mass cytometry and full-length single-cell RNAseq have shown that hILC2s exhibit distinct phenotypic and transcriptional signatures in different tissues (54, 55). For instance, hILC2s in peripheral blood and tonsil highly express *TNFSF10*, *TNFRSF19* and *CD200R1*, whereas hILC2s in lung highly express *IL1RL1* and *IL17RB* (55). CD69 on hILC2s was detected restrictively to skin, mucosa and spleen, whereas ICOS was mainly restricted to mucosal hILC2s (54). Collectively, ILC2s are highly regulated by the local tissue microenvironment and cytokine milieu.

ILC2s IN SOLID TUMORS

The role of ILC2s in solid tumors is currently unclear. In this review, we compared all relevant original studies and summarized some of the properties of ILC2s in solid tumors.

GASTRIC CARCINOMA (GC)

GC is one of the most common malignancies worldwide. *H. pylori* (Hp) infection is considered a class I carcinogen of GC, and the gastric body predominant type of chronic atrophic gastritis (CAG) caused by Hp infection often develops into GC (56). Chronic inflammation can damage gastric glands and lead to gastric metaplasia, a kind of precancerous lesion (57). Here we describe this pathogenic process as the "CAG-GC chain". ILC2s are the major ILC subset in the murine stomach and highly express IL-33 receptor (ST2) at steady state (58). The frequency of ILC2s is increased in peripheral blood mononuclear cells (PBMCs) of patients with GC (59). Li et al. (60) measured the ratio between type 1 (IFN-γ) and type 2 (IL-4/IL-5/IL-13) cytokines in clinical blood samples, and found that type 1 immunity was impaired in patients with CAG and GC, whereas type 2 immunity was induced. This effect was more pronounced in Hp$^+$ patients and was enhanced with the development of the "CAG-GC chain" caused by Hp infection. When inoculating mice with Hp *via* intragastric gavages, the researchers subsequently demonstrated that Hp infection significantly increased ILC2 and Th2 levels in gastric homogenate, accompanied by GATA3 upregulation (60). These results suggest that ILC2s play a facilitating role in the Hp-mediated "CAG-GC chain", but the specific molecular mechanism still needs to be further studied. In mice, tuft cell-derived IL-25 stimulates ILC2s to release IL-13, a growth factor for tuft cells, and this circle drives early metaplastic remodeling and gastric tumorigenesis (61). Genetic ablation of murine ILC2s, tuft cells or antibody neutralization of ILC2-derived type 2 cytokines suppresses gastric tumor growth (61). Single-cell RNAseq of gastric leukocytes have revealed that murine ILC2s highly express the glucocorticoid and androgen receptors; glucocorticoids and androgens synergistically inhibit the transcription of ILC2-derived IL-13, and simultaneous deficiency of glucocorticoids and androgens in mice results in the development of gastric inflammation and metaplasia (62). These murine experiments suggest that ILC2 blockade could inhibit the occurrence and development of gastric cancer, and glucocorticoid or androgen treatment also has some therapeutic potential. However, it is still controversial whether ILC2s play a major role in the process of GC tumorigenesis. In a mouse model of GC, mast cells were proved to be the major effector cells of IL-33 and could promote GC by recruiting macrophages; the frequencies of ILC2s and Tregs were comparable between tumor and normal tissues regardless of ST2 deficiency in mice (63). More studies on IL-33/ST2 pathways in gastric TME need to be conducted to identify the most decisive factors.

COLORECTAL CANCER (CRC)

In the past few years, IL-33 had been identified as an important cytokine in CRC tumorigenesis. A recent study has shown that IL-33 directly promotes murine CRC proliferation by upregulating COX2/PGE2 (64). In mice, epithelium-derived CRC cells generate IL-33 during polyposis and that IL-33 activates at least two cell types, subepithelial myofibroblasts and mast cells, to form a tissue microenvironment favorable to polyposis (65). sST2, a soluble form of the IL-33 receptor, can neutralize IL-33 in TME, thereby suppressing tumor growth,

metastasis and tumor angiogenesis (66). Moreover, immunohistochemical analysis of tumor tissue samples from a large number of CRC patients showed that the upregulation of IL-33/ST2 was significantly correlated with an early tumor stage, but not with the prognosis of patients (67). This suggests that IL-33 and its effector cells may play a more important role in tumorigenesis, compared with tumor progression. Colonic lamina propria ILC2s also express ST2 in steady state and TME (68, 69), and play a considerable role in the construction of TME.

ILC2s are not typically found in the gut of healthy individuals, whereas they are found in the gut of patients with CRC (70). The PD-1 expression level of TILC2s varies in different stages of CRC. In human, $PD1^{low}$ ILC2s are dominant in early CRC tumors, whereas $PD1^{high}$ ILC2s are dominant in late CRC tumors (71). In mice, ILC2s in advanced CRC highly express *Hs3st1* (encodes the effector product that catalyzes heparan sulfate biosynthesis) and *Pdcd1* (encodes immune checkpoint PD-1); deficiency of PD1 or HS3ST1 in murine ILC2s can considerably inhibit CRC tumor proliferation (71). Peroxisome proliferator-activated receptor γ (PPARγ) had been proved to directly regulate PD-1 expression on ILC2s (72). Recent study has found that ILC2s in human and mouse CRC express PPARγ, which maintains ILC2 secretion of IL-5 and IL-13 and the pro-tumor effect of ILC2s (73). These results suggest that $PD\text{-}1^+$ ILC2s may play a facilitating role in CRC. However, two recently published studies contradict these findings. One of the studies found that both human and mouse CRC tissue had considerably higher ILC2 levels than paracancerous tissue; in a mouse model, ILC2-derived IL-9 could activate $CD8^+$ T cells to inhibit CRC tumor growth and using anti-CD90.2 to block ILC2s in nude mouse (lacking T cells) could promote tumor growth, whereas intravenously injecting IL-9 inhibited tumor growth (74). Another study found that ILC2s were markedly absent in RORα-deficient mice, and this loss was accompanied by an increase in tumor burden; in human, the high ILC2 gene signature in tumor was an independent predictor of better outcome in CRC patients (75). The reason why ILC2s act in opposite ways in the same cancer requires further study.

HEPATOCELLULAR CARCINOMA (HCC)

The proportion and distribution of hILC2 in HCC may predict the prognosis of patients. Recent study has found that higher ratio of ILC2 to ILC1 in PBMCs of HCC patients is associated with prolonged survival (76). However, the higher the ratio of hILC2 abundance in tumor tissue to that in paracancerous tissue, the poorer the prognosis (77). In addition, patient clinical data have shown that microvascular invasion, HBV infection, and tumor recurrence are positively correlated with the abundance of TILC2s in patients with HCC (77). These results suggest that a tumor prognostic model may be established centered on ILC2s. In both steady state and HCC, hepatic ILC2s express ST2 and can be activated by IL-33 (77, 78). hILC2s in HCC do not express KLRG1, but highly express CD69 and core residency signature when compared with that of normal blood and tissue hILC2s; in mice, $KLRG1^-$ ILC2s in HCC can induce immunosuppressive neutrophils to accumulate in tumor tissues by releasing CXCL2, thereby promoting HCC progression (77). Collectively, ILC2s promote the progression of HCC, and blocking ILC2s may have a therapeutic effect. These results also indicate that the phenotype of main TILC2 subset may vary in different experiments. To better distinguish the ILC2 subgroups, we recommend that all studies about ILC2 targeting should include a detailed phenotypic identification of major ILC2 subsets that influence experimental outcomes.

PANCREATIC DUCTAL ADENOCARCINOMAS (PDAC)

In murine pancreas, resting and activated TILC2s express ST2 and PD-1 (79). In murine PDAC, IL-33 activates TILC2s to secrete CCL5, which subsequently recruits $CD103^+$ DCs to TME; DCs then activate $CD8^+$ T cells through antigen presentation, thereby enhancing the anti-tumor immunity (79). As previously mentioned, PD-1 is a suppressive immune checkpoint on the cell surface of ILC2s and blocking PD-1 can preserve the immune activity of ILC2s. Therapeutic anti-PD-1 antibodies can not only block PD-1 on $CD8^+$ T cells to maintain the anti-tumor effect of T cells, but also block PD-1 on ILC2s to indirectly enhance the anti-tumor immunity. In murine PDAC, IL-33-mediated TILC2 expansion is accompanied by enhanced intratumoral $CD8^+$ T cell infiltration and PD-1 upregulation in TILC2s, and IL-33 combined with anti-PD-1 treatment can significantly increase TILC2 abundance and reduce tumor volume in both PD-1-sensitive and PD-1-resistant tumors (79). This suggests that IL-33 combined with anti-PD-1 antibodies may break immunotherapy resistance in patients with advanced cancer. However, the role of ILC2s in PDAC is also complex and paradoxical. Another study found that PDAC tumor cells releasing IL-33 depended on the intratumoral fungal mycobiome, and genetic deletion of IL-33 or anti-fungal treatment in murine PDAC could reduce Th2 and ILC2 recruitment and improve survival (80). But whether the intratumoral fungal mycobiome affects the phenotype and function of TILC2s remains unknown. Collectively, the role of IL-33/ILC2 axis in PDAC requires further study.

BREAST CANCER

Breast cancer is the most prevalent malignancy worldwide. The frequency of hILC2s is significantly increased in surgically resected breast cancer tissue samples (3). In a 4T1 breast cancer mouse model, IL-33/ST2 axis could not only reduce NK cell cytotoxicity, but also recruit immunosuppressive cells (MDSCs and Tregs) and $ST2^+$ ILC2s to accumulate in tumors (81). The abundance of MDSCs and TILC2s is associated with distant metastasis of breast cancer. In murine breast cancer, adoptive transfer of pulmonary ILC2s promotes the infiltration

of IL-13Ra1$^+$ MDSCs in lung metastatic nodules through IL-13/IL-13Ra1; recruited MDSCs increase the number of lung metastatic nodules and reduce the survival of tumor-bearing mice by inhibiting CD4$^+$ T cells and CD8$^+$ T cells and inducing Treg (82). These results indicate that ILC2s are involved in the regulation of immunosuppressive TME in breast cancer. Although anti-PD-(L)1 antibodies have shown some efficacy in the treatment of triple-negative breast cancer, the role of PD-1 in breast ILC2s has not been reported.

LUNG CANCER

Lung cancer has the highest mortality and the second morbidity among the world malignancies. The abundance of ILC2s in PBMCs and tumor tissues of patients with non-small cell lung cancer (NSCLC) is significantly higher than that of healthy donors (83). Lung ILC2s stably express ST2 in both NSCLC patient and healthy people (83). And PD-1 is highly expressed in ILC2s obtained from NSCLC patients, both at the mRNA and protein levels; PD1^{high} hILC2s isolated from tumor tissue enhance the polarization of M2 macrophages (M2-TAMs) *in vitro* by secreting IL-4 and IL-13 (83). M2-TAMs are one of the predominant tumor-infiltrating immune cell population and promote tumor growth and metastasis (84). In a lung cancer mouse model, vitamin A deficiency diet can increase the abundance of ILC2s and M2 macrophages in tumor tissue and associates with higher tumor burden and lower survival (85). A small sample analysis showed that circulating ILC2s and MDSCs were up-regulated simultaneously in patients with lung cancer (86); another study found that Treg-induced immunosuppression in mice was associated with both CD8$^+$ T cell depletion and ILC2 augmentation (87). Furthermore, ILC2s are associated with lung metastasis of malignant tumors. Investigation of numerous lung metastasis mouse models has shown that ILC2-induced eosinophil can locally antagonize lung NK cell function *via* restraining NK cell glucose metabolism (glucose restriction and enhanced glycolysis), and consequently promote tumor metastasis and dissemination in the airways (88). Collectively, TILC2s in lung are associated with multiple immunosuppressive cells and are crucial regulator of immunosuppressive TME; ILC2 blockade in lung cancer may break immune resistance and become a new treatment option for patients with anti-PD-1 antibody tolerance.

BLADDER AND PROSTATE CANCERS

Bladder cancer is one of the most common tumors of urinary system, and intravesical instillation with bacillus Calmette-Guérin (BCG) is the standard treatment for patients at moderate to high risk of recurrence. The antitumor effect of BCG is mediated by induction of delayed hypersensitivity in the host. In a prospective study of non-muscle-invasive bladder cancer (NMIBC) (89), the total amount of immune cells in the urine of patients receiving BCG treatment is increased (mainly neutrophils), and urine CD14$^+$ cells mainly show the phenotype of monocytic myeloid-derived suppressor cells (M-MDSCs), which play a suppressive role in anti-tumor immunity (90). Even though ILC frequency is very low in patient's urine, the proportion of ILC2s in total urine ILCs remains elevated after patients receiving BCG treatment, and this elevation correlates with IL-13 and M-MDSC levels in urine (89). Using BCG to stimulate PBMCs isolated from healthy donor (HD) *in vitro*, it was found that amplified ILC2 population could produce large amounts of IL-13 and induce the expansion of IL-13Rα1$^+$ M-MDSCs, which significantly inhibited the proliferation of both CD8$^+$ and CD4$^+$ T cells; and anti-IL-13 antibody could partially restore T cell proliferation (89). This BCG-induced ILC2 expansion is more enhanced in MIBC patients compared to HD (89). These results suggest that the ILC2/IL-13/M-MDSCs axis is likely to be one of the pathways mediating BCG treatment failure. Enhanced ILC2s and M-MDSCs are also observed in human prostate cancer tissue samples, and this enhancement is not evident in samples from patients with benign prostatic hyperplasia (91). In a prostate cancer mouse model, the frequency of M-MDSCs in tumor was elevated and positively correlated with TILC2s in the same tumor, whereas NKT cells were reduced (91). This suggests that functional crosstalk occurs between ILC2s and M-MDSCs. ST2 expression has been detected in murine prostate ILC2s (91), but whether prostate ILC2s express PD-1 has not been reported yet. And it remains unknown whether bladder ILC2s express ST2 and PD-1. Together, studies on bladder and prostate ILC2s are still lacking.

MELANOMA

In a melanoma mouse model, IL-33-activated TILC2s recruit eosinophils by producing GM-CSF (92). RNA-seq in CRC demonstrated that eosinophils were critical for tumor rejection and displayed an IFN-dependent profile and cytotoxic machinery (93). In murine melanoma, eosinophils inhibit primary tumor by normalizing tumor vessels and enhancing CD8$^+$ T cells infiltration (94), in contrast to promote pulmonary metastasis of the melanoma cell line B16-F10 by restraining NK cell glucose metabolism in the lungs as outlined above (88). These dual effects of eosinophils on melanoma may be related to local TME in different tissues. In mice, the anti-tumor effects of IL-33/ILC2/eosinophil axis can be impaired by lactic acid produced by melanoma (95). This indicates that the pH of TME may affect ILC2 function; however, it is still unclear whether lactate molecules directly regulate cell function. ILC2s in mice melanoma express high levels of ST2 and PD-1, and co-administration of IL-33 with PD-1 blockade therapy can significantly improve the antitumor effect mediated by ILC2s (92).. PD-1 blockade on murine ILC2s in melanoma lung metastases can upregulate the production of ILC2-derived TNF-α, and TNF-α induces tumor hemorrhagic necrosis (96, 97). Furthermore, a potential interaction between ILC2s and NK cells was found in the melanoma microenvironment. In a nude mouse model of melanoma, IL-33 could respectively activate ST2$^+$ NK cells and ST2$^+$ ILC2s, and ILC2s suppress NK cell infiltration and function *via* CD73 (98). In murine melanoma,

ILC2s promote T cell infiltration but inhibit NK cell function; research on ILC2s in human melanoma is still lacking.

DISCUSSION

ILC2s are important mediators of type 2 immune response and are closely associated with allergic inflammation, helminth infection, and tissue fibrosis. However, the role of ILC2s in tumors remains unclear. In the present review, we examined prior studies on ILC2s in solid tumors and found that ILC2s act as a crucial regulator in tumor immunity (**Figure 1**).

ILC2s AND CANCER PROGNOSIS

Increased abundance of ILC2s has been observed in a variety of tumors. The phenotype of ILC2s is tissue-specific, and the role of ILC2s varies in different tumors. For instance, ILC2s promote lung cancer by recruiting M2-TAMs; ILC2s recruit MDSCs to facilitate breast, bladder and prostate cancer; but ILC2s inhibit melanoma by recruiting eosinophils, which enhance the infiltration of effector T cells. Interestingly, ILC2s have been found to exert both suppressive and promoting roles in the same tumor. In CRC, ILC2s suppress tumor by producing IL-9, but maintain pro-tumor effect by expressing PPARγ. In PDAC, ILC2s recruit DCs to promote antitumor immunity, but decreasing ILC2s by genetic deletion of IL-33 improves the survival of tumor-bearing mice. In melanoma, ILC2s enhance the infiltration of effector T cells by recruiting eosinophils, but ILC2s suppress NK cell function *via* CD73. The role of ILC2s also differs in primary tumor and metastases. In melanoma, ILC2-recruited eosinophils suppress primary tumors by promoting $CD8^+$ T cells but promote lung metastases by suppressing NK cells. The role of ILC2s in solid tumors is extremely complex. To better understand ILC2 function and enhance comparability between different experiments, we recommend that all studies on ILC2s should be supplemented with phenotypic identification of the main ILC2 subsets which affect experimental outcomes. In addition, type 2 immunity is enhanced while type 1 immunity is suppressed during GC tumorigenesis; the abundance and proportion of ILC2s in PBMC, tumor tissue and paracancerous tissue of HCC patients are related to the prognosis.

Current evidences suggest that ILCs are likely to be an important predictor of tumor prognosis. Here we propose a hypothesis that a tumor prognosis prediction system based on ILCs can be constructed in the following aspects: (1) the levels and proportions of type 1 and type 2 cytokines; (2) the levels and proportions of anti-tumor cytokines (mainly IL-9) and pro-tumor cytokines (IL-4, IL-13 and amphiregulin); (3) the levels and proportions of ILC and ILC2 subsets in peripheral blood and lesions. Considering the different roles of ILC2s in distinct cancer, we can construct different mathematical models based on the above aspects. Currently, the biggest limitation is that little is known about the changes in both ILC2 surface markers and ILC subset proportion during tumorigenesis and disease progression; and we still lack a general tissue-specific ILC2 test kit to overcome the detected deviation between different laboratories.

PROSPECTS FOR ILC2-BASED TARGETED THERAPY

For precancerous lesions promoted by ILC2s (e.g. CAG-GC chain), IL-33/ST2 inhibition therapy may be used as a

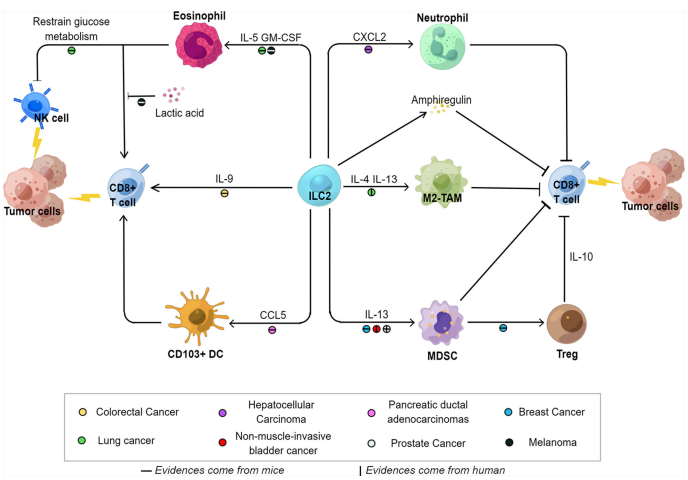

FIGURE 1 | The regulatory network of ILC2s in the tumor microenvironment. Influenced by the local microenvironment, ILC2s exist the signal and functional cross-talk with other immune cells and exhibit inhibitory (left) or facilitating (right) effects on tumors. A regulatory network is formed centered on ILC2s, which act as a crucial regulator in tumor immunity. Specific tumor types associated with each pathway have been marked with colorful spots. *M2-TAM* M2 tumor-associated macrophages, *MDSC* myeloid-derived suppressor cells, *GM-CSF* granulocyte macrophage colony-stimulating factor, *CXCL2* C-X-C motif chemokine ligand 2, *CCL5* C-C motif chemokine ligand 5.

complement measure to surgery to lower the risk of recurrence. For tumors in which ILC2s mainly play a facilitating role, one way is to block the transcription of ILC2 cytokines or neutralize related pro-tumor cytokines; another way is to block ILC2 activation. In mice, the majority of tumor ILC2s are regulated by IL-33/ST2. For IL-33/ST2-regulated pro-tumor TILC2s, sST2 may also be a good choice in addition to traditional antibodies. For tumors in which ILC2s mainly play an inhibitory role, IL-33-combined treatment may enhance the therapeutic effect of the standard regimen.

Recently, anti-PD-1/PD-L1 antibodies have become a standard treatment for many tumors, but most patients still fail to benefit from it or relapse after treatment. Understanding the mechanism of immune resistance is the current need for clinical application. In CRC, PDAC, LC and melanoma, TILC2s have shown high PD-1 expression and may amplify the anti-PD-1 efficacy. The ILC2-MDSC axis has been identified in breast, bladder and prostate cancers, respectively. The ILC2-M2-TAM axis has been identified in lung cancer. For tumors in which ILC2s recruit immunosuppressive cells, the application of anti-PD-1 antibodies may accelerate the formation of ILC2-mediated immunosuppressive TME. These results suggest that ILC2s may predict the efficacy of PD-1 blockade therapy and may become a new therapeutic target after immunotherapy resistance. For PD-1^{hi} anti-tumor TILC2s, IL-33 combined with anti-PD-1 treatment may break through the dilemma of immunotherapy resistance in current clinical practice.

CONCLUSION

In conclusion, we reviewed the original studies of ILC2s in solid tumors and found that ILC2s act as a crucial regulator in tumor immunity. Current evidences suggest that ILC2s are tissue-specific and play different roles in various tumors. It is our understanding that all studies on ILC2s should be supplemented with phenotypic identification of the main ILC2 subsets in the experiment. We also proposed the potential application value of ILC2s for tumor prognosis and analyzed the prospects of ILC2-based targeted therapies.

AUTHOR CONTRIBUTIONS

LW wrote the original draft and drew the illustration. MJ and XY contributed to the conceptualization. All authors participated in the revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Differential Engraftment of Parental A20 PD-L1 WT and PD-L1 KO Leukemia Cells in Semiallogeneic Recipients in the Context of PD-L1/PD-1 Interaction and NK Cell-Mediated Hybrid Resistance

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The contribution of natural killer (NK) cells to tumor rejection in the context of programmed death-ligand 1/programmed death 1 (PD-L1/PD-1) blockade is a matter of intense debate. To elucidate the role of PD-L1 expression on tumor cells and the functional consequences of engaging PD-1 receptor on cytotoxic cells, PD-L1 expression was genetically inactivated and WT or PD-L1-deficient parental tumor cells were adoptively transferred intravenously into F1 recipients. The engraftment of PD-L1-deficient A20 tumor cells in the spleen and liver of F1 recipients was impaired compared with A20 PD-L1 WT tumor counterparts. To elucidate the mechanism responsible for this differential tumor engraftment and determine the relevance of the role of the PD-L1/PD-1 pathway in the interplay of tumor cells/NK cells, a short-term competitive tumor implantation assay in the peritoneal cavity of semiallogeneic F1 recipients was designed. The results presented herein showed that NK cells killed target tumor cells with similar efficiency regardless of PD-L1 expression, whereas PD-L1 expression on A20 tumor cells conferred significant tumor protection against rejection by CD8 T cells confirming the role of the co-inhibitory receptor PD-1 in the modulation of their cytotoxic activity. In summary, PD-L1 expression on A20 leukemia tumor cells modulates CD8 T-cell-mediated responses to tumor-specific antigens but does not contribute to inhibit NK cell-mediated hybrid resistance, which correlates with the inability to detect PD-1 expression on NK cells neither under steady-state conditions nor under inflammatory conditions.

Keywords: PD-1, CD80, PD-L1, PD-L2, NK cells, hybrid resistance, A20 leukemia cells

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INTRODUCTION

Bone marrow-derived natural killer (NK) cells are a population of innate type I lymphoid cells (ILC-1) essential during the early phase of antiviral responses for the contention of viral spread. NK cells efficiently kill tumor cells and eliminate stressed cells without relying on major histocompatibility complex (MHC) specificity. Kärre and Ljunggren introduced the theoretical framework of the *missing self-concept* that accounted for the observations of hybrid resistance and the rejection of tumor cells that had low or had lost expression of MHC class I molecules (1). While T-cell cytotoxic responses depend on MHC restriction, NK cell recognition of non-self, as it lacks TcR, depends on a balance of positive (activating) and negative (inhibitory) receptor signals received from co-stimulatory and co-inhibitory ligands expressed in stressed cells or tumor cells when exposed to proinflammatory cytokines (2). A misbalance of these dominant co-inhibitory ligands on the target cells may occur for instance due to modifications of self-MHC class I molecule expression that would trigger NK cell cytotoxicity. Killer cell immunoglobulin-like receptors (KIRs) in humans and Ly49 in mice and NKG2A in both species are the most relevant dominant MHC class I-dependent co-inhibitory pathways. Apart from these classical regulators of NK cell function, NK cells may also depend on the recognition of other non-MHC class I inhibitory receptors, also known as immune inhibitory checkpoints [programmed death 1 (PD-1), BTLA, CD160, TIGIT, etc.], which are poorly characterized so far (3, 4).

NK cell function was first described in the 1960s, as the effector cells responsible for mediating *hybrid resistance* to parental bone marrow transplantation in lethally irradiated semiallogeneic F1 recipients (5–9). In addition to NK cells, CD8 T cells can also recognize hematopoietic antigens and tumor-specific antigens in parental tumor cells and contribute to resist the engraftment of parental cells, although to a lesser extent (10, 11).

The discovery of PD-1 as a receptor capable of conveying negative signals to T cells (12, 13) and, soon after, the therapeutic implications of the programmed death-ligand 1 (PD-L1)/PD-1 immune checkpoint blockade brought great excitement to the field of cancer immunotherapy (12, 14). Most of the antitumor cytotoxic activity achieved after PD-L1/PD-1 blockade has been assigned to enhance CTL responses (15–17; PD-L1 et al., 2018). Despite this claim, some authors have attributed the antitumor properties to NK cells or even macrophages in the context of PD-L1/PD-1 therapeutic blockade (18–26).

The motivation of this study was to elucidate whether or not the co-inhibitory receptor PD-1 was involved in the functional activity of NK cells. The aim was to bring some insight into the controversy derived from the difficulty of detecting PD-1 expression in human and mouse NK cells under homeostatic or inflammatory conditions (21–26). Bearing that in mind, we designed an experimental approach in which parental PD-L1 WT or PD-L1-deficient A20 leukemia cells were injected intravenously or intraperitoneally into semiallogeneic F1 recipients to study the role of PD-L1 expression on tumor cells in NK cell-mediated rejection and to assess the putative involvement of PD-1 co-inhibition in hybrid resistance to tumor implantation. We confirmed that NK cells and to lesser extent host CD8 T cells contributed to the phenomenon of hybrid resistance in the context of parental tumor cell engraftment into semiallogeneic F1 recipients. The expression of PD-L1 on tumor cells diminished tumor rejection by CD8 T cells but did not influence NK cell-mediated rejection, as they were capable of eliminating PD-L1 WT and KO tumor cells with similar efficiency, arguing against the claim that the co-inhibitory receptor PD-1 would play an inhibitory role in NK cell-mediated antitumor responses.

Abbreviations: CD, Cluster of differentiation; NK, Natural killer; NKT, Natural killer T cells; TCR, T cell receptor; MHC, Major histocompatibility complex; WT, Wild type; KO, Knock-out; MFI, Mean fluorescence intensity; BMCs, Bone marrow cells; mAb, Monoclonal antibody; PI, Propidium Iodide; FCS, Fetal calf serum; SD, Standard deviation; SEM, Standard error of the mean; F1 (Balb/c × B6): Balb/c (female) x B6 (male) F1 hybrid; IFN-g, gamma interferon; PD-1, Programmed death 1; CRISPR, Clustered regularly interspaced short palindromic repeats; i.p., intraperitoneal; PBS, Phosphate buffer saline; ILC, Innate lymphoid cells; SFM, Serum free medium.

MATERIAL AND METHODS

Animal Source

Mice

C57BL/6J mice were purchased from Janvier (France). Eight- to 12-week-old female F1 hybrid mice (Balb/c AnN × C57BL/6J) (H-$2^{d/b}$) were bred at the animal facility of the University of Leon for internal use in our experiments. All animals were maintained with a 12-h dark–light cycle at 22°C temperature and received *ad libitum* food and water.

The Animal Welfare Committee of the University of Alcala de Henares (Madrid) in accordance with the European Guidelines for Animal Care and Use of Laboratory Animals approved all experiments with rodents (authorization # OH-UAH-2016/015).

Hybridoma Cell Lines and Purification of Depleting Antibodies for *In-Vivo* Use

Hybridoma cell lines secreting anti-mouse NK1.1 antibody (clone PK136, mouse IgG_{2a}, kappa light chain) and anti-mouse CD8 antibody (clone 2.43, rat IgG_{2b}, kappa light chain) or purified isotype-matched controls (anti-CD45.1, clone A20, mouse IgG_{2a}, k, in-house made and clone RTK4530, rat IgG_{2b}, k Biolegend, San Diego, California) were initially grown in Petri dishes to permit their expansion. Cell lines were gradually adapted to grow in serum-free medium (SFM) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) supplemented with 0.25% of IgG-depleted fetal calf serum (FCS) and then scaled up to spinner flasks of 3-L volume. The cell culture supernatants were centrifuged, prefiltered, and purified by protein G Sepharose affinity chromatography. The eluted fraction of the purified antibodies was dialyzed against phosphate-buffered saline (PBS), and finally, the purified antibodies were passed through a 0.22-µm filter. The purified antibodies for *in-vivo* use were stored frozen at −80°C in

endotoxin-free PBS at a concentration of 1–5 mg/ml containing less than 2 EU/ml of endotoxin [Pierce (Thermofisher brand company, Waltham, Massachusetts, USA)].

A20 Lymphoma Tumor Cell Line

The A20 transplantable leukemia cell line was derived from B lymphocytes of a naturally occurring reticulum cell sarcoma from an old Balb/c AnN mouse (H-2^d, TIB-208, ATCC, American Type Culture Collection, Manassas, VA, USA) (27, 28). Cells were grown in complete RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah, USA), 2 mM L-glutamine (Sigma), 1 mM pyruvate, (Sigma), non-essential amino acids, and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 5% CO_2. The A20 cell line and its derivatives were routinely tested by PCR to rule out the presence of mycoplasma contamination.

CRISPR–Cas9-Mediated Generation of PD-L1-Deficient A20 Leukemia Cells

PD-L1 expression in the A20 cell line was knocked out by CRISPR–Cas9 (Clustered, regularly interspaced, short palindromic repeats–associated nuclease Cas9) technology (29, 30). pLenti-CRISPR-V2 plasmid encoding Cas9 and a puromycin resistance cassette (Addgene #52961) was used to clone an oligo DNA guide that was previously validated for the introduction of indel mutations into the PD-L1 gene (15, 31).

The A20 tumor cell line was then transduced with lentiviral particles produced in HEK293T cells, co-transfected with second-generation packaging plasmid psPAX2 (Addgene #12260) and envelope pCMV-VSV-G (Addgene #8454) along with the targeting vector pLenti-CRISPR-V2 plasmid encoding Cas9, puromycin cassette, and oligo DNA guide for exon 3 (Addgene #52961). PD-L1-deficient clones or emptied plasmid-transduced A20 PD-L1 WT tumor cells were selected in the presence of 1 μg/ml puromycin and cloned by limiting dilution. Several PD-L1-deficient cell lines were derived and screened by flow cytometry using an anti-PD-L1 monoclonal antibody (clone MIH5, rat IgG_{2a}) (32). To characterize the mutation introduced within exon 3, a set of flanking primers was designed to amplify the mutated gene, and the PCR product was later sequenced at the core DNA sequencing facility of the University of Leon. The lack of protein expression on the surface of the tumor cells was checked by flow cytometry. The sequence of mouse PD-L1 mutation in A20 PD-L1-deficient tumor cells was deposited in GenBank under the accession number OM975989.

Systemic Parental A20 Tumor Implantation Into Semiallogeneic F1 Recipients

The optimal number of A20 tumor cells to achieve their engraftment in F1 recipients capable of overcoming hybrid resistance was titrated after intravenous injection of distinct cell numbers. The number of 5×10^6 tumor cells was chosen for the *in-vivo* experiments based on the kinetics of tumor implantation and dissemination in F1 recipients. This number of tumor cells reached a similar engraftment level to that of injection of 1×10^6 of A20 tumor cells in syngeneic Balb/c mice, 1 month after the adoptive transfer. However, as expected, the extent of tumor engraftment was reduced in the liver, spleen, and bone marrow of F1 recipients when compared to the syngeneic setting due to the hybrid resistance mechanisms active in the former and absent in the latter (data not shown).

A20 PD-L1 WT and A20 PD-L1-deficient leukemia tumor cells were grown and expanded in a culture medium at a cell density of 3×10^5 cells/ml, collected at the logarithmic phase of cell growth, washed and resuspended at 5×10^6 cells in 200 μl of PBS, and injected i.v. with a 25-G needle. Eight- to 12-week-old semiallogeneic F1 female mice were injected intravenously with either A20 WT or A20 PD-L1 KO tumor cell lines and were euthanized a month after the adoptive transfer of the tumor cells.

A20 Leukemia Mouse Model of Tumor Implantation Into the Peritoneal Cavity

We adapted a previously reported peritoneal cavity model of tumor implantation for the assessment of short-term A20 tumor survival (33). The cells (5×10^6) of each tumor cell line, either A20 PD-L1 WT or PD-L1 KO, were co-injected intraperitoneally, and 6 days later, the remaining tumor cells within the cavity were harvested by peritoneal lavage and stained with an antibody panel that allowed us to distinguish tumor cells (K^{d+}/K^{b-}) from host F1 cells (K^{d+}/K^{b+}). Within the gate of tumor cells, the use of anti-PD-L1 antibody staining differentiates PD-L1 WT from PD-L1 KO A20 tumor cells. To determine the contribution of NK cells or CD8 T cells to hybrid resistance of parental tumor implantation, these immune cells were exhaustively depleted by injection of anti-NK1.1 antibody (clone PK136) or anti-CD8 T cell antibody (clone 2.43), respectively. Two milligrams of antibody/mouse/dose was injected i.p. at day −5 and day −1 prior to the co-injection of A20 WT and A20 PD-L1 KO leukemia cell lines.

For the harvest of tumor cells remaining in the peritoneal cavity, mice were injected with 6 ml of macrophage buffer composed of Dulbecco's PBS (Ca/Mg free) (Gibco, Thermofisher company brand, Waltham, Massachusetts, USA 14200-067), tetrasodium EDTA (Sigma, E-6511) (0.02%, 0.53 mM), glucose (Sigma, G-7528) (0.1%), and gentamycin (50 μg/ml). The lavage solution was left inside the peritoneal cavity for 2 min and then was harvested with a Pasteur pipette. The volume collected from each mouse was variable and lower than the volume injected. Then, the values obtained were normalized to the volume injected in order to calculate the absolute cell number in the peritoneal cavity (tumor and non-tumor cells) (34).

Flow Cytometry for the Immunophenotyping of Immune Cells in Primary and Secondary Lymphoid Organs, Peritoneal Cavity, and Tumor-Infiltrating Leukocytes in Metastatic Hepatic Lesions

To distinguish tumor cells from non-tumor cells (host F1 cells) in different hematopoietic compartments and in tumor

metastasis of the liver, cellular suspensions were prepared and stained with specific antibodies against MHC class I allele K^b (clone AF6-88.5) and MHC class I allele K^d (clone SF1-1.1).

Table 1 shows the list of biotinylated- or fluorochrome-labeled antibodies against cell surface markers that were used to monitor protein expression on the surface of tumor cells and immune cells located in primary and secondary lymphoid organs and tumor-infiltrating cells of the liver metastases. Biotinylated antibodies were developed with streptavidin (SA)–PE, SA–PECy7, or SA–BV421. All these antibodies were purchased from Biolegend or were produced, labeled, and titrated in our own laboratory. Fc receptors were blocked by incubating cell suspensions with 2 μg/ml (0.2 μg/1 × 10^6 cells) of homemade blocking anti-FcγR mAb (2.4G2) to reduce non-specific binding before adding the abovementioned mAbs (35). Dead cells and debris were systematically excluded from the acquisition gate by adding propidium iodide (PI) at the end of the staining, prior to data acquisition. Living cells were gated as PI negative and aggregates were gated out based on the FSC-H/FSC-A dot plot profile. Flow cytometry acquisition was conducted on a Beckman Coulter CyAn 9 flow cytometer or on a Cytek® Aurora Spectral Cytometer and data analysis was performed using FlowJo software version 10.

Statistical Analysis

Unpaired Student's *t*-test and two-way ANOVA and a post-analysis based on Tukey's test were applied to compare the differences of means between the PD-L1 WT and PD-L1 KO tumor groups. These statistical analyses were performed under the conditions of independence of the data, normality test (Kolmogorov test), and equal variances among groups (Bartlett's test). The statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

CRISPR/Cas9 Gene Inactivation of PD-L1 Expression in A20 Leukemia Cells

To evaluate the *in-vivo* role of PD-L1 expression on tumor cells, the CRISPR/Cas9 approach was applied for the genetic introduction of indel mutations into the PD-L1 encoding gene expressed on the surface of the A20 leukemia cell line to abolish protein expression (30, 31). The sequence-encoding mouse PD-L1 was retrieved from the NCBI database with accession number NM_021893.3 to design the targeting strategy to functionally inactivate exon 3 that encodes 2 bp of the signal peptide and the complete Ig V-like domain. We took advantage of a sgRNA guide previously validated in a different tumor model (EG7-OVA cell line derived from T-cell lymphoma EL-4) (15). This sgRNA guide targeted a sequence located at the proximal exon 3 encoding the Ig V extracellular domain of the PD-L1 molecule. The indel mutations introduced into the PD-L1 gene were PCR-amplified and the amplicon was characterized by gene sequencing. The expected band for PD-L1 exon 3 in the A20 PD-L1 WT cell line was 342 bp, whereas in the PD-L1-deficient cell line, it was 326 bp. The indel mutation consisted of an insertion of 5 bp after the codon encoding amino acid Arg (R, position 84) and a deletion of 21 bp from Ala (A, position 85) to Gln (Q, position 91) within exon 3, leading to a frameshift mutation and the introduction of several stop codons (**Supplementary Figures 1A–C**).

TABLE 1 | List of antibodies describing the specificity, labeling, clone name and the provider.

Receptor	Label	Clone	Company
CD3	BV711	17A2	Biolegend, (#100241)
Kd	FITC	SF1-1.1	Biolegend, #116606
Kb	Alexa Fluor 647	AF6-88.5	Biolegend, # 116512
CD49b	APC	DX5	Biolegend, #108910
PD-1	BV421	29F.1A12	Biolegend, #135217
PD-L1	Bio	MIH5	Home-made, (74, 32)
NKp46	Bio	29A1.4	Biolegend, # 137616
CD8	PE	53-6.7	Biolegend, # 100708
CD11b	PerCP-Cy5.5	M1/70	BD, #561114
Ly6C	Bio	Monts-1	Home-made (72)
Ly6G	PE	1A8	BD, # 551461
CD4	PE-Cy7	GK1.5	Biolegend, # 100422
B220	Bio	RA3-6B2	Biolegend, # 103203
B220	FITC	RA3-6B2	Thermofisher, # 48-0452-82
PD-L2	Bio	TY25	Thermofisher, #13-5986-85
CD80	Bio	16-10A1	Thermofisher, # 13-0801-81
Ki-67	Bio	SolA15	eBioscience 13-5698-82
Isotype control mIgG2b	Bio	MPC-11	Home-made (71)
Isotype control rat IgG2a	Bio	AFRC MAC 157 (ECACC)	Home-made (70)

List of antibodies describing the specificity, labeling, clone name and the provider.

A20 Leukemia Tumor Cells Express *In-Vivo* PD-L1, Whereas CD80 Was Barely Expressed and PD-1 and PD-L2 Were Undetectable

PD-L1 is the ligand of two members of the immunoglobulin superfamily (PD-1 and CD80), and binding to these two receptors in *trans* delivers co-inhibitory signals that co-inhibit T-cell function (12, 36). The PD-L1/PD-1/CD80 and PD-L2/PD-1 pathways represent an example of multiple receptor–ligand interactions in which *trans* interplay with nearby cells is likely to be conditioned by co-expression of paired molecules on the same cell (*cis* interaction) (37). Thus, the co-expression of CD80 and PD-L1 in *cis* on tumor cells prevents PD-L1 from the tumor to deliver co-inhibitory signals in *trans* to T cells (38, 39). This occurs because PD-L1/CD80 *cis* heterodimerization inhibits both PD-L1/PD-1 and CD80/CTLA-4 interactions but maintains the ability of CD80 to activate T cells through the co-stimulatory receptor CD28 (40). The A20 leukemia transplantable cell line was chosen as the tumor model because PD-1 and PD-L2 cell surface receptors are completely absent and the expression of CD80 is barely detectable, whereas PD-L1 is clearly expressed (**Figure 1A**). The *in-vivo* expression of PD-L1 on A20 WT tumor cells (red dots) present in the metastatic nodules of the liver is higher than on either host B cells, CD4 T cells, or CD8 T cells (black dots) (**Figure 1B**). We postulated that in this tumor mouse model, PD-L1 co-inhibitory function would not be compromised by CD80 interaction in *cis* due to its weak expression, allowing PD-L1 freely to engage PD-1 inhibitory receptors that might be present on NK cells and modulate their functional responses.

In summary, the A20 leukemia transplantable cell line is a convenient tumor model for the assessment of the contribution of PD-L1 expression on hematopoietic tumor cells without the interference of CD80 co-expression, which permits the interplay of tumor PD-L1 with PD-1 expressed in immune cells to inhibit their function.

In-Vitro Tumor Cell Growth Rate Was Not Compromised in PD-L1-Deficient Tumor Cells

The accumulation of living cells in cell culture results from the balance of cell division, cell survival, and cell death. We then performed *in-vitro* studies of tumor cell proliferation to evaluate whether loss of PD-L1 in A20 leukemia cells affected their overall survival or delayed its growth rate *in vitro*. An equal number of WT (A20-WT) or PD-L1-deficient cell line (A20-PD-L1 KO) was seeded in 24-well plates under the same culture conditions, and cell counting was monitored every day from day 1 to day 6. The results shown in **Figure 2A** (left panel) demonstrated a similar and parallel growth rate for both the A20 WT control and the A20 PD-L1-deficient cell lines.

During the course of the cell cycle, cells go through a sequence of phases starting in the G1 phase, continuing to the S and G2 phases and finishing in the M phase. Ki-67, a proliferating cell nuclear antigen, is a measure of the fraction of cells entering the cell cycle and mitosis, often used to evaluate the proliferating fraction versus the non-proliferating fraction within a tumor. The fraction of non-dividing cells was similar in A20 PD-L1-

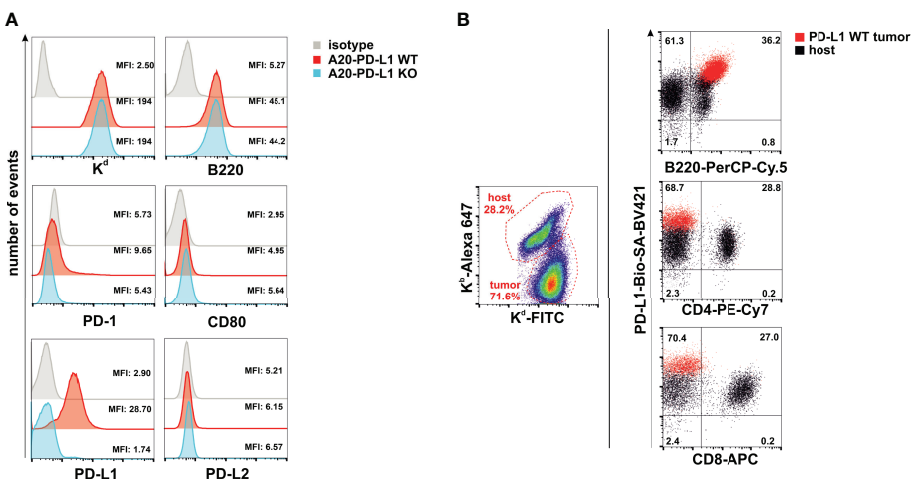

FIGURE 1 | A20 leukemia cell line is negative for programmed death 1 (PD-1) and programmed death-ligand 2 (PD-L2), but barely expressed CD80, leaving programmed death-ligand 1 (PD-L1) freely available to interact in *trans* with PD-1 expressed in immune cells. **(A)** Upper panel: Flow cytometry histograms displaying the staining with the isotype control and anti-K^d (H-2^d) and anti-B220 antibodies in A20 PD-L1 wild-type (WT) and A20 PD-L1 knockout (KO) leukemia cells. Middle and lower panels: Flow cytometry histograms showing the pattern of expression of PD-1, CD80, PD-L1, and PD-L2 in A20 PD-L1 WT compared to KO tumor cells. Notice that in A20 PD-L1-deficient cells, PD-L1 protein expression is absent on the cell surface, whereas the level of expression of the other molecules of the pathway is similar to that observed in A20 PD-L1 WT leukemia cells. This experiment was repeated three times with similar results. A representative histogram displaying the mean fluorescence intensity (MFI) value for each biomarker of the PD-L1/PD-L2/CD80/PD-1 pathway is shown along with the expression of MHC class I (K^D) and B220. **(B)** Overlapped dot plot illustrating simultaneously PD-L1 expression on host B cells (B220-positive cells), host CD4 T cells, and host CD8 T cells (black dots) infiltrating the tumor and on A20 tumor cells (red dots) in metastatic nodules of the liver of F1 recipients collected at day 30 after the adoptive transfer of tumor cells.

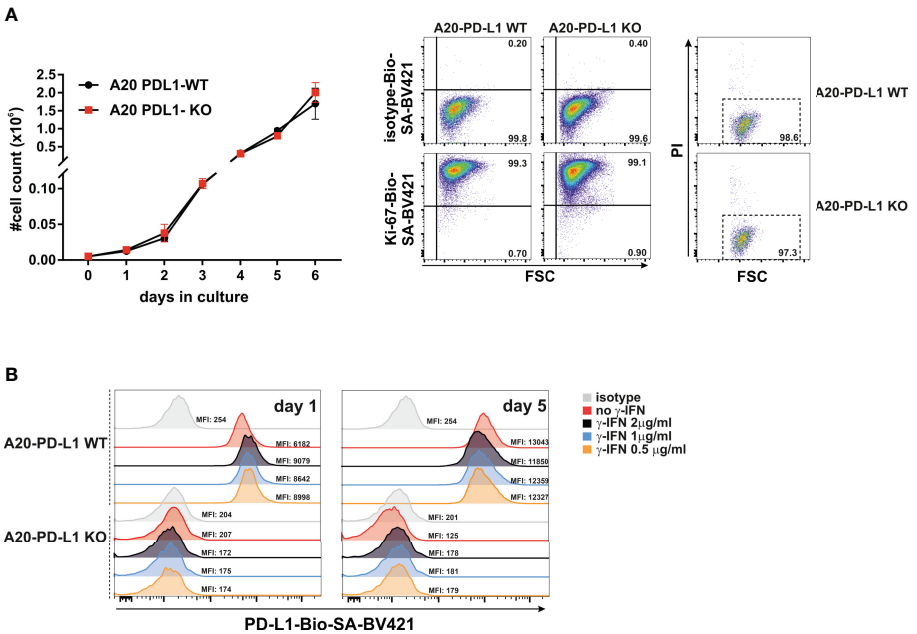

FIGURE 2 | Similar *in-vitro* proliferation rate of A20 PD-L1 WT and PD-L1 KO leukemia cells and *in-vitro* upregulation of PD-L1 expression in response to IFN-γ. **(A)** Left panel: Four replicates of A20 PD-L1 WT or PD-L1-deficient tumor cells were seeded in a 24-well plate at the rate of 5,000 cells per well, and cell counting was performed every day over a period of 6 days. The total number of cells ($\times 10^6$) in culture is plotted at different time points. Middle panel: A representative experiment of two showing intracellular Ki-67 staining was used to measure the *in-vitro* fraction of dividing cells (Ki-67 positive) versus non-dividing cells (Ki-67 negative) in WT and PD-L1-deficient cell lines 1 day after seeding them at 3×10^5 cells/ml. Right panel: A representative experiment of three illustrating cell death in cell culture measured at the exponential phase of cell growth by staining with propidium iodide. **(B)** A20 PD-L1 WT or PD-L1-deficient leukemia cells were left untreated or incubated for 24 h and 5 days with different concentrations of IFN-γ ranging from 0.5 to 2 μg/ml, and PD-L1 expression was monitored by flow cytometry. One experiment out of two with similar results. The mean fluorescence intensity (MFI) is indicated for each histogram.

deficient cells (0.90%) compared to their WT counterparts (0.73%) in the exponential phase of cell growth, suggesting that deficiency in PD-L1 did not impact cell proliferation in A20 PD-L1 tumor cells (**Figure 2A**, middle panel). Cell death at the exponential phase of cell growth was negligible as assessed by propidium iodide intake and similar in both tumor cell lines (**Figure 2A**, right panel).

These data suggest that the indel mutations introduced into the PD-L1 gene led to a successful inactivation of protein expression but did not perturb the overall tumor cell growth and survival *in vitro*.

Slight Upregulation of PD-L1 on A20 Tumor Cells in Response to Exposure of IFN-γ *In Vitro*

Most transplantable syngeneic tumor cell lines upregulate PD-L1 in response to IFN-γ to counterattack and evade cytolytic T cells through PD-1 co-inhibition of their functional activity (41). This emulates the behavior of naturally developed tumors *in vivo* that acquire adaptive mechanisms of resistance by augmenting PD-L1 expression, in response to IFN-γ released by antitumor CTLs (PD-L1 et al., 2018).

To prove that A20 leukemia cells behave just like other transplantable syngeneic tumor models, the PD-L1 WT and its counterpart PD-L1-deficient A20 tumor cells were exposed *in vitro* to IFN-γ (200 ng/ml for 24 h) or left untreated to determine whether PD-L1 expression was modulated in response to this cytokine. As seen in **Figure 2B**, the A20 PD-L1 WT cell line in response to IFN-γ augmented slightly the PD-L1 expression compared to the untreated control, but the increase in IFN-γ concentration did not lead to a concomitant increase in PD-L1 expression. A concentration as low as 200–500 ng was sufficient to achieve a modest upregulation of PD-L1 expression in A20 tumor cells. As expected, the PD-L1-deficient cell line expressed PD-L1 neither in resting conditions nor in response to the exposure to IFN-γ.

To sum up, the A20 transplantable leukemia tumor model, like many other syngeneic tumor cell lines, upregulates PD-L1 in response to IFN-γ.

A20 Leukemia Cells Expressing PD-L1 Engrafted More Efficiently Than PD-L1 KO Tumor Cells in the Spleen but not in the Bone Marrow

The spleen and the bone marrow were the two hematopoietic compartments where tumor colonization was monitored. The abundance of immune cells responsible for hybrid resistance in primary and secondary lymphoid organs is probably tissue-specific. This may reflect the distinct tissue distribution and engraftment pattern of the tumor cells in the spleen versus the bone marrow. The

PD-L1 expression on parental A20 WT tumor cells that colonized the spleen exhibited significant protection against rejection compared to A20 PD-L1-deficient tumor cells (**Figure 3A**, $^{***}p < 0.0005$). In contrast, the engraftment of parental A20 WT or A20 PD-L1 KO tumor cells in the bone marrow of F1 recipients did not follow the same pattern as that of the spleen. The implantation of tumor cells in the bone marrow of F1 recipients was residual and nearly undetectable regardless of whether PD-L1 was expressed or not. PD-L1 expression on tumor cells did not confer any advantage to the tumor for the colonization of this primary lymphoid organ, suggesting that PD-L1 expression on tumor cells may not provide sufficient protection against rejection by the host immune cells involved in hybrid resistance in this hematopoietic compartment (**Figure 3B**).

In summary, the data indicate that hybrid resistance to parental tumor engraftment follows a distinct rejection pattern in different hematopoietic compartments.

The Increase in Liver Weight due to Clusters of Nodular Metastases Was Higher in F1 Recipients of A20 PD-L1 WT Tumor Cells Than in Those Receiving PD-L1-Deficient Tumor Cells

A20 leukemia cells express the CXCR4 chemokine receptor that guides them toward a chemokine gradient of CXCL12 (stromal cell-derived factor-1, SDF-1) actively produced by the biliary epithelium and bone marrow stromal cells (42, 43), which may account for the preferential metastatic behavior of A20 leukemia cells for these tissues.

We compared PD-L1 WT and PD-L1 KO A20 tumor dissemination and the formation of metastases in the livers of F1 recipients 1 month after intravenous injection. The increase in liver weight due to metastatic nodules was higher in F1 recipients implanted with A20 PD-L1 WT leukemia cells than in those injected with A20 PD-L1 KO leukemia cells (**Figure 4A**, $^{***}p < 0.0005$) or naive F1 controls (**Figure 4A**, $^{*}p < 0.05$). This indicates that PD-L1 expression on A20 leukemia cells confers a survival advantage to the tumor probably by inhibiting either the NK cell-mediated response or the CD8 T-cell-mediated response, the two immune cell contributors to hybrid resistance against the tumor.

We then assessed the frequency of tumor-infiltrating leukocytes inside the metastatic nodules of the liver, but no significant differences were found in the tumor arising from either A20 PD-L1 WT or KO leukemia cells (**Figure 4B**). We went on to analyze by flow cytometry the frequency of the different subpopulations of the host immune cells inside the metastatic nodules of the liver. No significant differences were found when the frequencies of NK cells ($CD3^{-}/DX5^{+}$) (**Figure 4C**), CD4 and CD8 T cells (**Figure 4D**), $CD11b^{+}/Ly6C^{+}/Ly6G^{-}$ (monocytes) and $CD11b^{+}/Ly6C^{+}/Ly6G^{+}$ (granulocytes) (**Figure 4E**), or B cells ($B220^{+}$) (**Figure 4F**) were analyzed in F1 recipients of A20 PD-L1 WT or A20 PD-L1 KO tumor cells.

PD-L1 expression on parental A20 leukemia cells enhances tumor fitness and improves tumor survival in F1 recipients allowing efficient liver colonization by conferring them with a greater capacity to cope with the host resistance mechanisms of rejection.

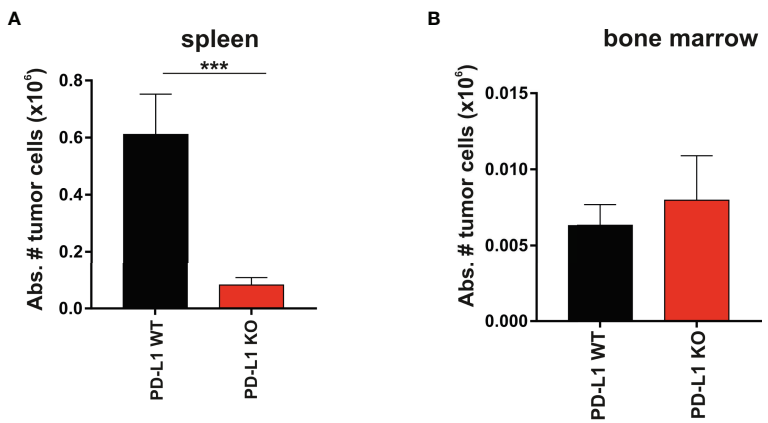

FIGURE 3 | Superior engraftment of A20 PD-L1 WT tumor cells compared to A20 PD-L1-deficient tumor cells in the spleen of F1 recipients contrasted with the bone marrow resistance to tumor implantation of either tumor cell line. **(A)** Bar plot shows the absolute number of PD-L1 WT and PD-L1 KO leukemia cells in the spleen calculated at the time of the euthanasia (day 30 after tumor implantation). Tumor cells (K^{b-}/K^{d+}) were distinguished from non-tumor cells (host cells, K^{b+}/K^{d+}) by flow cytometry. **(B)** The absolute number of PD-L1 WT and PD-L1 KO leukemia cells in the bone marrow of F1 recipients was also monitored by flow cytometry at the time of the euthanasia. The absolute number of tumor cells was calculated from the cell suspension obtained after fluxing one tibia with culture medium. The plotted data represent the mean ± SEM from 10 to 15 mice per group. *p*-values were considered statistically significant according to the following criteria: $^{***}p < 0.0005$. Unpaired Student's *t*-test was used to assess the statistical significance of the means. WT, wild type; KO, knockout.

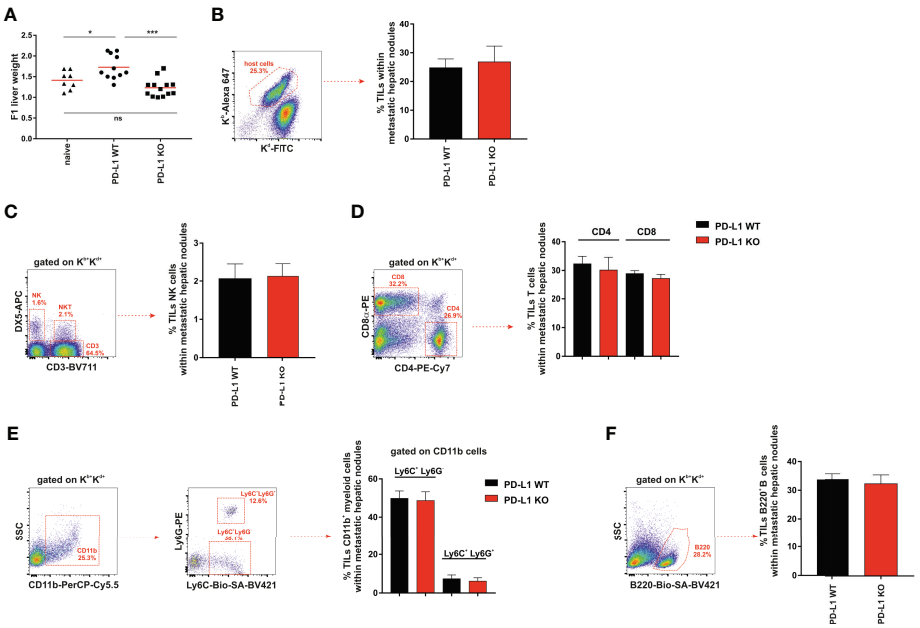

FIGURE 4 | Significant increase in liver weight in F1 mice receiving A20 PD-L1 WT tumor cells compared to those implanted with A20 PD-L1 KO tumor cells. No substantial changes in tumor leukocyte infiltration in metastatic liver nodules arose from either A20 PD-L1 WT or PD-L1-deficient tumor cells. **(A)** The livers of non-treated F1 naive control mice (triangles) and F1 mice injected with either A20 PD-L1 WT (circles) or KO tumor cells (squares) were weighted at the time of the necropsy, 1 month after tumor injection. **(B)** The frequency of tumor-infiltrating leukocytes (TILs) in metastatic nodules of the liver was calculated and represented. The percentages of NK cells ($CD3^-/DX5^+$) **(C)**, CD4 and CD8 T cells **(D)**, $CD11b^+/Ly6C^+/Ly6G^-$ (monocytes) and $CD11b^+/Ly6C^+/Ly6G^+$ (granulocytes) **(E)**, and B cells ($B220^+$) **(F)** were analyzed in metastatic liver nodules of F1 recipients engrafted with A20 PD-L1 WT or A20 PD-L1 KO tumor cells. Representative dot plots depicting the gating strategy and the subpopulations of each analysis are shown. The bar graph shows the mean ± SEM from 10 to 15 mice per group. *p*-values were considered statistically significant according to the following criteria: $*p < 0.05$; $***p < 0.0005$. WT, wild type; KO, knockout. Unpaired Student's *t*-test was used for the evaluation of the statistical significance of the means.

PD-L1 Expression on Parental Tumor Cells Does not Protect Against NK Cell-Mediated Hybrid Resistance

NK cells are particularly efficacious in the rejection of tumors lacking MHC class I expression or when MHC class I expression has been reduced (missing self-hypothesis) (1, 44). NK cells are also the main players involved in the rejection of parental bone marrow cells or parental hematopoietic tumors in F1 recipients (45–47).

We hypothesized that if the PD-1 receptor were expressed in NK cells, as claimed by some authors, then one would expect that PD-L1 WT tumor cells would exhibit a resistance advantage over PD-L1 KO tumor cells and, consequently, they would be less vulnerable to NK cell-mediated rejection than PD-L1 KO tumor cells. To test that hypothesis, a short-term experimental strategy was designed to elucidate the relative contribution of cytotoxic cells (host NK cells or host CD8 T cells) to tumor clearance in the peritoneal cavity of F1 recipients (**Figure 5A**). Parental A20 WT and PD-L1-deficient tumor cells were co-injected in equal numbers (5×10^6 of each cell type) in isotype control-treated, NK cell-depleted, or CD8 T-cell-depleted F1 recipient mice, and tumor survival was assessed 6 days after injection (**Figures 5B, C**).

We monitored the recruitment of leukocytes into the peritoneal cavity in response to co-injection of A20 PD-L1 WT and KO tumor cells, which mimics somehow a sterile inflammatory environment, promoted by tumor implantation and danger signals linked to damage-associated molecular patterns (DAMPs) detected by immune cells (48). A statistically significant increase in the total number of host immune cells in the peritoneal cavity of tumor-bearing F1 recipients was seen in response to the presence of tumor cells when compared to control naive F1 mice (**Figure 5D**, upper left, $**p < 0.005$). This increase in immune cells was mainly due to the recruitment of CD11b myeloid cells in tumor-bearing mice compared to naive F1 controls (**Figure 5D**, upper right, $**p < 0.005$).

The immune cell populations of the peritoneal cavity likely to participate in hybrid resistance were also monitored ($CD3^-/DX5^+$ NK cells, NKp46-positive subpopulation of NK cells and $CD3^+/DX5^+$ NKT cells) by flow cytometry in the different experimental groups. NK cell recruitment to the peritoneal cavity of tumor-bearing mice was significantly greater than in naive F1 mice (**Figure 5D**, lower left panel). Within the NK cell pool, the NKp46 cell subpopulation was the most vulnerable to NK cell depletion as this was significantly reduced in NK cell-depleted tumor-bearing F1 mice compared to the isotype control or the anti-CD8 T-cell-depleted group (**Figure 5D**, lower left panel). On the other hand, a significant increase of NKT cells was also observed in NK cell-depleted mice compared with the rest of the groups (**Figure 5D**, lower right panel). Regarding T cells,

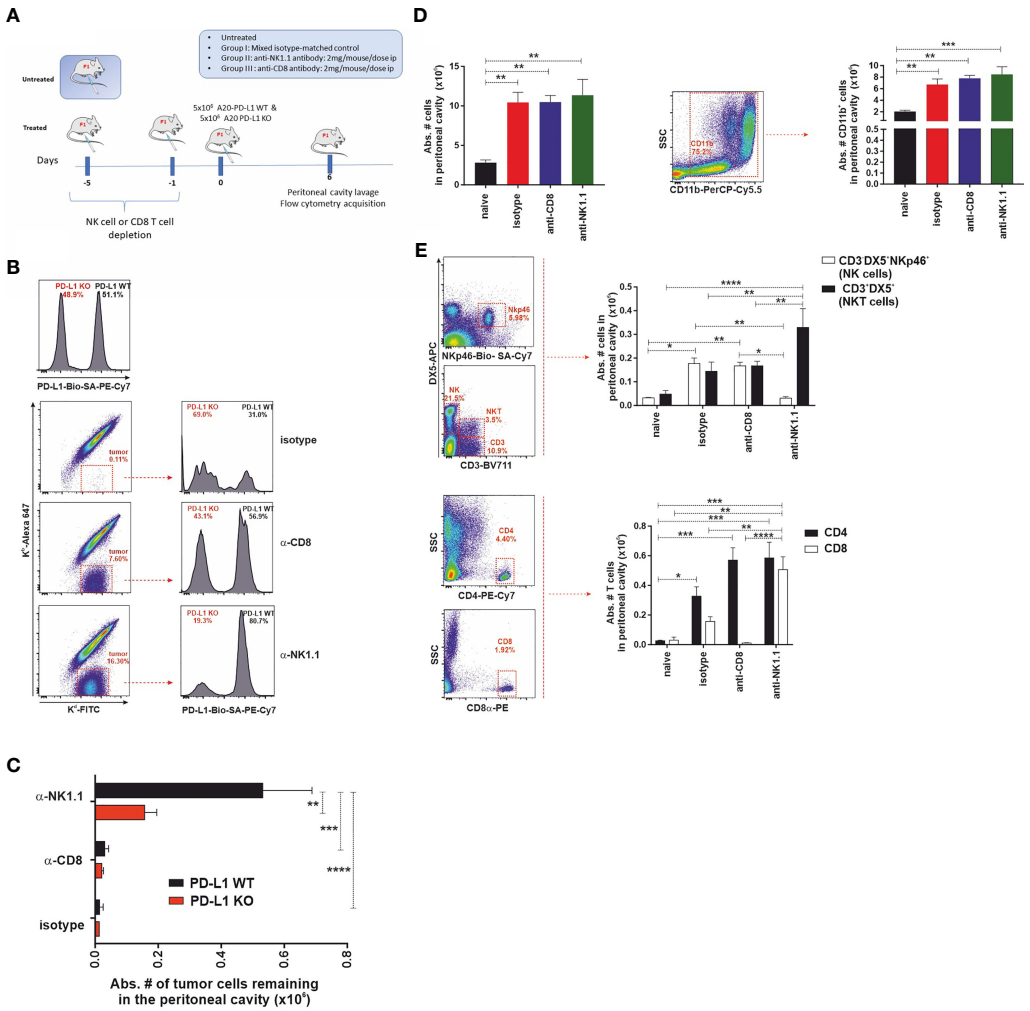

FIGURE 5 | The expression of PD-L1 in A20 tumor cells confers protection against CD8 T-cell-mediated antitumor response but does not affect the NK cell-mediated component of hybrid resistance. **(A)** Experimental design to assess the contribution of host CD8 T cells and NK cells to the phenomenon of hybrid resistance in F1 mice receiving A20 PD-L1 WT and PD-L1-deficient leukemia cells into the peritoneal cavity. F1 recipient mice were treated with isotype control or depleting antibodies against CD8 T cells or NK cells at days –5 and –1 prior to tumor implantation at day 0. An equal number of A20 PD-L1 WT and A20 PD-L1 KO tumor cells were intraperitoneally injected and mice were euthanized on day 6 after tumor implantation. Then, the cellular composition of the peritoneal lavage was analyzed by flow cytometry distinguishing tumor cells (positive for MHC class I, K^d) from non-tumor cells (host immune cells double-positive for MHC class I K^b and K^d). **(B)** Top panel: A representative histogram example showing the ratio of the A20 PD-L1 WT/A20 PD-L1 KO tumor cell mix (ratio 1:1) after staining with anti-PD-L1 antibody and prior to the injection into the peritoneal cavity of F1 recipient mice. Bottom panel: A representative dot plot showing host cells (K^{b+}/K^{d+}) and tumor cells (K^{b-}/K^{d+}) of each experimental group (isotype-matched control and anti-CD8α- or anti-NK1.1-depleted F1 recipients are depicted). The percentage of A20 PD-L1 WT and A20 PD-L1 KO tumor cells remaining in the peritoneal cavity 6 days after tumor injection was calculated by excluding residual red cells and gating on K^{b-}/K^{d+} tumor cells and the histogram plot shows PD-L1 staining to differentiate A20 PD-L1 WT from PD-L1 KO leukemia cells. **(C)** The absolute number of A20 PD-L1 WT and KO tumor cells remaining in the peritoneal cavity 6 days after their injection is depicted for isotype matched control and anti-CD8α or anti-NK1.1-depleted F1 recipients. **(D)** Tumor inoculation into the peritoneum attracts immune cells toward this location. Upper panel: The bar graph shows the absolute number of immune cells in the peritoneal cavity of tumor-bearing F1 mice compared to the normal number of immune cells in the peritoneal cavity of naive F1 mice. Representative dot plot depicting the gating strategy and the subpopulation of CD11b cells in the peritoneal cavity. The bar chart represents the absolute number of host CD11b myeloid cells recruited into the peritoneal cavity in response to tumor implantation compared with that of naive F1 mice. Middle panel: Representative dot plot depicting the gating strategy for NK cells and NKT cells in the peritoneal cavity. The bar chart represents the absolute number of NK cells ($CD3^-/DX5^+/NKp46^+$ cells) and NKT cells ($CD3^+/DX5^+$) recruited into the peritoneal cavity of F1 mice in response to the inoculation of an equal number of A20 PD-L1 WT and KO tumor cells, 6 days after tumor injection. Lower panel: Representative dot plot depicting the gating strategy for CD4 T cells and CD8 T cells in the peritoneal cavity. The bar chart illustrates the absolute number of CD4 T cells and CD8 T cells in the peritoneal cavity of F1 mice co-injected with an equal number of A20 PD-L1 WT and KO tumor cells, 6 days after tumor injection. The plotted data represent the mean ± SEM from 5 to 8 mice per group. *p*-values were considered statistically significant according to the following criteria: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$; ****, $p < 0.00005$. Student's *t*-test and two-way ANOVA were used to assess the statistical significance of the means. WT, wild type; KO, knockout.

host CD4 T cells were significantly increased in tumor-bearing mice compared to naive F1 controls (**Figure 5D**, right lower panel). Host CD8 T cells also increased significantly in the NK cell-depleted group when compared to the rest of the experimental groups (**Figure 5D**, right lower panel).

In line with previous findings in the context of parental bone marrow transplantation into F1 recipients, depletion of the host NK cells was the major immune mechanism involved in hybrid resistance to parental A20 tumor cells (**Figure 5E**). Irrespective of whether PD-L1 was expressed or not on the cell membrane of the A20 leukemia cells, tumor cells were readily rejected with similar efficiency by NK cells in CD8 T-cell-depleted F1 mice (**Figure 5E**). A20 PD-L1 WT tumor cells survived significantly better in NK cell-depleted F1 recipients than in the anti-CD8 T-cell-depleted group or naive F1 controls (**Figure 5E**). Remarkably, apart from NK cells, the host CD8 T cells were also found to contribute to hybrid resistance, although to a much lower extent. Interestingly, the absolute number of A20 PD-L1 WT tumor cells remaining in the peritoneal cavity of F1 recipients was superior to that of A20 PD-L1 KO tumor cells in NK cell-depleted F1 recipients (**Figure 5E**). This means that PD-L1 expression on tumor cells protects them from CD8 T-cell rejection. These findings are in line with the current paradigm in cancer immunotherapy claiming that PD-L1 expression on tumor cells can effectively modulate CD8 T-cell-mediated antitumor responses.

In summary, PD-L1 expressed on tumor cells does not inhibit NK cell function ruling out the postulated claim that the PD-L1/PD-1 pathway contributes to modulating NK cell rejection of parental tumor cells.

Neither Homeostatic nor Inflammatory Conditions Led to the Expression of the Co-Inhibitory Receptor PD-1 on Host NK Cells

The expression of the PD-1 co-inhibitory receptor was monitored in F1 recipients under steady-state conditions (naive F1 mice, **Figure 6A**) and inflammatory conditions in the liver, spleen, metastatic nodules of the liver, and peritoneal cavity of A20 tumor-bearing F1 mice (**Figure 6B**). Whereas the expression of PD-1 is readily detectable on CD8 T cells and NKT cells, a complete lack of PD-1 expression was observed in NK cells irrespective of the tissue compartment analyzed (**Figures 6A, B**). PD-1 upregulates its expression upon CD8 T-cell and NKT cell activation in tumor-bearing mice in different hematopoietic compartments suggesting that A20 tumor cells are immunogenic and susceptible to be recognized by the immune system but are undetected in NK cells. The upregulation of PD-1 expression on CD8 T cells is dependent on the presence of the tumor in the peritoneal cavity and appears soon, declining later on gradually as tumor cells fade out due to the antitumor response (**Figure 6C**).

Our data support the hypothesis that in this mouse hematopoietic tumor model, the co-inhibitory receptor PD-1 is absent in NK cells suggesting that tumor cells bearing PD-L1 expression cannot directly co-inhibit NK cell function through PD-1.

DISCUSSION

Since the initial efforts involved in the generation of inbred strains of mice, researchers soon realized that tumor cell lines enjoy immune privilege features as they could engraft in histoincompatible hosts across some minor mismatch barriers while skin grafts were always consistently rejected. This suggests that tumors are endowed with the capacity to adapt and escape the antitumor response. Hematological tumors have even challenged the laws of transplantation formulated by Little, Gorer, and Snell (49, 50). Thus, parental skin grafts are accepted by F1 hybrids, whereas parental hematopoietic bone marrow cells, lymphoid cells, or tumor cells are eliminated by an NK cell-mediated mechanism of rejection (known as *hybrid resistance*) (6, 9, 51–55).

Today, it is universally accepted that tumors undergo genetic mutations that give rise to neoantigens that may become immunogenic and susceptible to recognition by the immune system. Tumors have evolved several direct and indirect mechanisms to evade recognition and resist the antitumor responses. Thus, for instance, tumor cells co-opt physiological regulatory mechanisms of tissues that have naturally evolved to prevent the development of immunopathology when they are exposed to long-lasting chronic inflammation. The upregulation of ligands for the co-inhibitory receptors, such as PD-L1, is one of the most relevant modulatory mechanisms to dampen inflammation and thus defend the living organism against the immune attack of cytotoxic T lymphocytes (15–17; PD-L1 et al., 2018). Accumulating evidence suggests that multiple immune evasion mechanisms may simultaneously operate in patients with advanced tumors. However, the contribution of each of these mechanisms to immune evasion and their temporal cross-regulation during tumor progression remain to be defined.

The A20 leukemia cell line was used as the hematopoietic tumor model because it lacks PD-1 expression *in vitro* or *in vivo*; therefore, trogocytosis (a phenomenon that permits immune cells to acquire relevant molecules from the cell surface of tumors or cells of the tumor microenvironment) is unlikely to occur and this scenario can be discarded. In addition to this intrinsic feature, A20 leukemia cells barely express CD80 allowing PD-L1 to interact in *trans* with PD-1 expressed on immune cells. Moreover, as shown for other transplantable tumor cell lines, the exposure to IFN-γ upregulates PD-L1, allowing the tumor to acquire a competitive advantage to co-inhibit the cytotoxic function of T cells (41).

Parental hematopoietic tumors engraft into F1 recipients as do hematopoietic bone marrow transplants due to their ability to overcome the barrier of hybrid resistance (6, 47, 51). In this scenario, parental tumor cells manage to escape NK cell-mediated attack and disseminate throughout the hematopoietic system. As in dysfunctional T cells, NK cells driven by the continuous presence of the tumor cells can also become

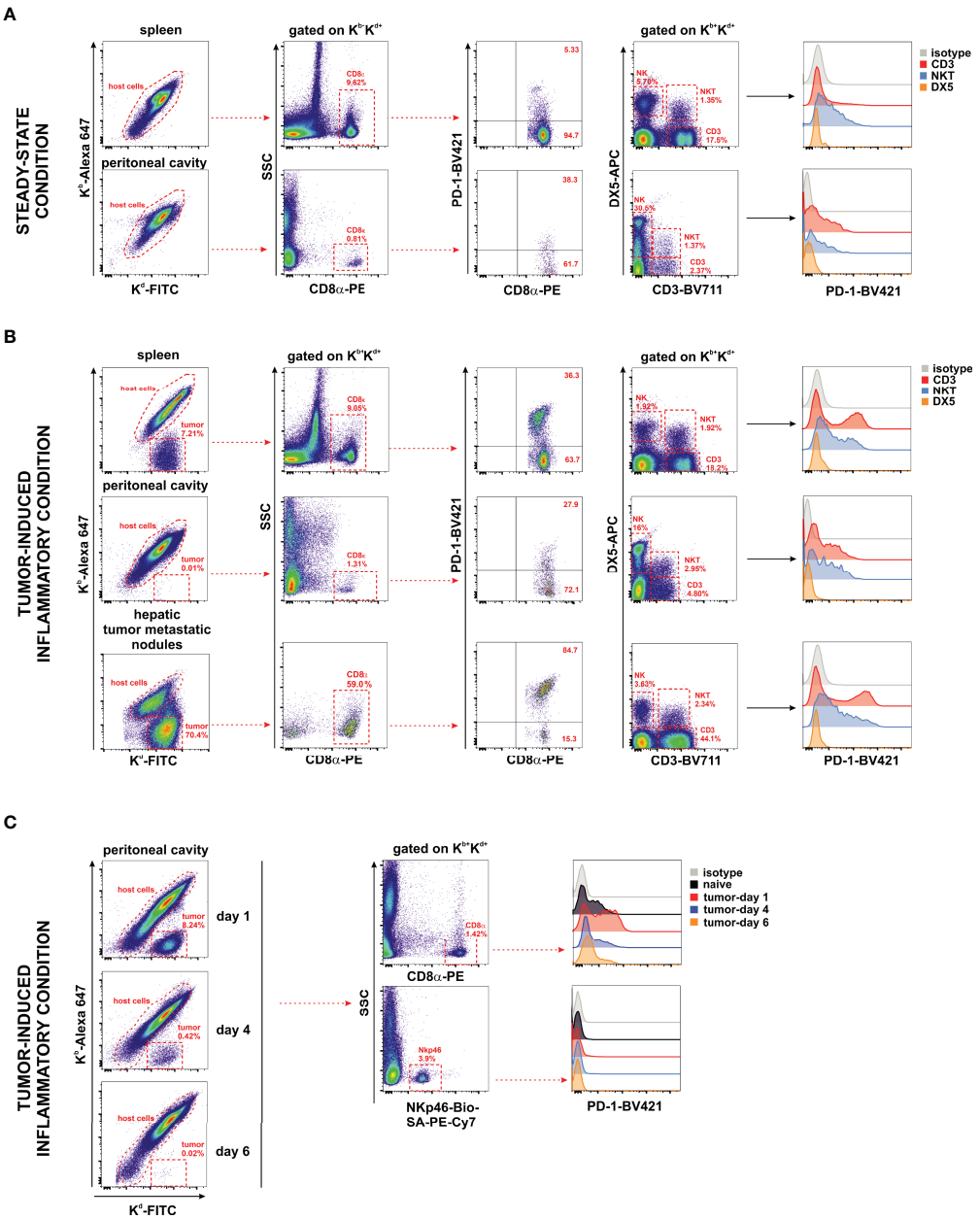

FIGURE 6 | Undetectable PD-1 expression on NK cells under homeostatic or inflammatory conditions. Naive F1 mice **(A)** or A20 WT tumor-bearing F1 mice **(B)** were analyzed for the expression of PD-1 on NK cells ($CD3^-/DX5^+$), NKT cells ($CD3^+/DX5^+$), CD3 T cells, and CD8 T cells collected from the peritoneal cavity and spleen of naive mice (steady-state conditions) and from the spleen, peritoneal cavity, and liver metastases of tumor-bearing mice. Host immune cells co-expressing K^{b+}/K^{d+} were differentiated from K^{b-}/K^{d+} tumor cells in F1 mice injected with tumor cells. Representative dot plots of the analysis strategy in the spleen and metastatic nodules of the liver of F1 mice euthanized at day 30 after tumor implantation. One representative experiment out of three with similar results is depicted. **(C)** Time course expression of PD-1 on CD8 T cells and NK cells ($NKp46^+$ cells) of the peritoneal cavity exposed to A20 WT tumor cells. Left panel: Representative dot plot showing host cells versus tumor cells remaining over the period of 6 days of follow-up depicting the kinetics of tumor rejection at days 1, 4, and 6 after tumor implantation. Right panel: Representative dot plot illustrating the gating strategy of CD8 T cells and $NKp46^+$ NK cells for the analysis of PD-1 expression.

functionally impaired (exhausted), allowing tumor cells to thrive and move to different locations, giving rise to metastases (56). Despite the fact that F1 recipients resist tumor engraftment at the initial phase after their implantation, tumors manage to survive. Tumor cells are selected for variants that escape the antitumor response or the antitumor response becomes exhausted and dysfunctional due to their inability to cope with the tumor burden accumulated during the course of tumor progression.

An intense controversy has been set around the role of NK cells in the field of tumor immunotherapy in the context of PD-

1/PD-L1 blockade. It is an open question whether PD-L1/PD-1 blockade could enhance NK cell functional activity, as the expression of PD-1 in this immune cell population has been difficult to demonstrate. Despite the proponents' claim that PD-1 can be detected in human NK cells of healthy individuals and in the context of different diseases (25, 25, 57–59), emerging evidence supports that the PD-1 receptor is either non-detectable or at best minimally present and restricted to activated NK cells within the tumor under inflammatory conditions in humans and mice (21, 23–25, 60). As opposed to those predominant tenets, others claim that PD-1 is completely absent on the cell surface of NK cells (22, 61). Our data support the notion that NK cells lack the PD-1 co-inhibitory receptor on their cell surface independently of their location (outside or inside the tumor) or their degree of activation (steady-state conditions or inflammatory conditions). This correlates with the finding that the absence of PD-L1 on A20 leukemia cells does not increase susceptibility to tumor rejection by NK cells compared to PD-L1 WT tumor cells while cytolytic response mediated by CD8 T cells was sensitive to expression of PD-L1 on tumor cells.

To reconcile our data with previous reports attributing a critical role to NK cells in the context of PD-L1/PD-1 immune checkpoint blockade and to account for the findings observed in a set of patients who responded to PD-L1/PD-1 blockade therapy despite bearing a PD-L1-negative tumor, two hypotheses have been put forward (62). One possible scenario is that PD-L1 expressed on NK cells would cross-regulate antitumor CD8 T-cell responses by inhibiting DC activation through PD-1 that would ultimately result in a reduced ability to support CD8 T-cell priming (63). Moreover, in some tumors, PD-L1 expression appears to be upregulated in NK cells, and antibodies against PD-L1 would enhance their function and revert exhaustion (64).

In most tumor models of hematopoietic origin, parental tumor cells can engraft in F1 recipients despite host hybrid resistance to their implantation, unless poly I:C is administered to enhance host NK cell cytotoxic function (46, 65). This innate resistance of the host depends largely on the age of the recipient F1 mice as the aging process declines the function of NK cells (66). Similarly, parental bone marrow transplantation into F1 recipients in the absence of pharmacological NK cell activation leads to successful engraftment and long-term multilineage donor chimerism in low-dose-irradiated (1–3 Gy) F1 recipients or even in non-irradiated recipients. The chimerism levels in F1 recipients were, however, lower than those achieved after transplantation of syngeneic bone marrow, supporting the idea that host NK cells of F1 recipients resist the engraftment of parental bone marrow cells (47, 52, 67). As expected and in agreement with these previous antecedents, the intravenous injection of parental A20 leukemia tumor cells into F1 recipients led to the systemic dissemination of tumor cells. Tumor cell distribution within hematopoietic and non-hematopoietic niches likely obeys a balance of preferential tropism and tissue-specific forces of resistance that in turn is the result of the relative composition and abundance of innate cells and CD8 T cells on those tissue compartments. This may account for the finding that F1 recipients are refractory to tumor implantation in the bone marrow regardless of PD-L1 expression. Neither PD-L1 WT A20 leukemia cells nor PD-L1 KO A20 leukemia cells were able to settle in great numbers in the bone marrow compartment of F1 recipients, although this hematopoietic site represents a niche for metastases in syngeneic Balb/c recipients. This was an unexpected finding as the bone marrow stromal niche is enriched with a chemokine gradient of CXCL12 (SDF-1) that may potentially attract A20 leukemia cells expressing the CXCR4 chemokine receptor (42, 43). The impossibility for the tumor to engraft successfully into the bone marrow of F1 recipients likely reflects the local hybrid resistance in this hematopoietic compartment, which is considered by many authors as a secondary lymphoid organ with immunological function of defense against foreign entities and not uniquely devoted to the maturation of immune cells (68). In this respect, a recent work has pointed out that bone marrow macrophages are important players in resisting the engraftment of syngeneic tumor cells and allogeneic bone marrow cells (18). Moreover, PD-L1/PD-1 interplay is a relevant pathway modulating the phagocytosis of tumor cells by a subset of macrophages expressing PD-1, extending the modulatory function of this co-inhibitory ligand to the regulation of phagocytic cells (18). The poor implantation of A20 PD-L1-deficient tumor cells compared with A20 PD-L1 WT cells in the spleen and liver may reflect their greater vulnerability to be eliminated by the host immune system.

To account for this differential behavior in tumor tropism and based on the data gathered from the A20 tumor model implanted into the peritoneal cavity, we observed that A20 PD-L1 WT tumor cells are protected to some extent from rejection by the host F1 CD8 T cells, likely due to PD-1-mediated inhibition of their cytotoxic function. As opposed to CD8 T cells, NK cell-mediated rejection of tumor cells was similarly efficient regardless of PD-L1 expression on tumor cells. This finding argues against the notion that PD-L1 expression on tumor cells inhibits NK cell function, and therefore, it is highly unlikely that PD-L1/PD-1 blockade would enhance NK cell-mediated tumor rejection or reverse NK cell functional impairment due to exhaustion.

CD8 T cells can also contribute to resist parental tumor engraftment in F1 recipients. This is likely due to the fact that parental tumor cells are seen as foreign entities in F1 recipients as they may express tumor-specific antigens that can be cross-presented by the host DCs to host CD8 T cells stimulating their cytotoxic response (69). Indeed, it is known that the A20 leukemia cell line bears a high tumor mutational burden being classified in the ranking of immunogenicity close to the MC38 colon carcinoma cell line. This antigenic tumor burden often correlates with a good response to treatment with anti-PD-L1 blocking antibody with the percentage of tumor growth inhibition of 71% (A20 leukemia) and 69% (MC38 tumor), respectively (69).

The initial purpose of this research was first to assess the role of PD-L1 expressed on tumor cells on NK cell function and second to determine the impact of PD-L1/PD-1 interaction in NK cell-mediated mechanisms of rejection. However, this

experimental approach can also recreate a more general preclinical platform to study how to modulate the cytotoxic activity of NK cells and T cells against tumor cells and the reversal of their exhausted phenotype. Tumor cells in this mouse model evade the immune response and this could be reversed by therapeutic interventions aimed at blocking the co-inhibitory immune checkpoints to reinvigorate the functional activity of exhausted cytotoxic cells before they become fully dysfunctional.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data can be found here: https://www.ncbi.nlm.nih.gov/ under the accession number OM975989.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Alcala de Henares (authorization # OH-UAH-2016/015).

AUTHOR CONTRIBUTIONS

M-LR and J-IR-B designed the study, performed and analyzed the experiments, and wrote the manuscript. JP-S provided the reagents and shared his expertise in the field. All authors contributed to the final version of the manuscript.

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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.2022.887348/full#supplementary-material

Supplementary Figure 1 | Generation of a PD-L1-deficient A20 tumor cell line using a CRISPR-Cas9 approach. **(A)** The indel mutation located at exon 3 of PD-L1 gene consisted of 5 bp insertion and 21 bp deletion (Genebank accession # OM975989). **(B)** PCR amplification of PD-L1 exon 3 from genomic DNA of A20 PD-L1 WT and A20 PD-L1 KO cell line. The expected band for PD-L1 exon 3 in A20 cell line was 342 bp whereas in PD-L1 deficient cell line was 326 bp. The following set of primers were used for PCR amplification: Primer forward exon3-F: 5' CGTTTACTATCACGGCTCC 3' and primer reverse exon 3-R: 5' CATTGACTTTCAGCGTGA 3'. A 2.5% agarose gel was run to resolved the WT and KO PCR amplicons of PD-L1 exon 3. **(C)** Amino acid sequence alignment between exon 3 of A20-PD-L1 WT vs PD-L1 KO cells showing the frameshift mutation and the formation of several stop codons. The amino acid sequence alignment of the PD-L1 protein WT versus PD-L1 mutated protein was performed with Clustal Omega (http://www.ebi.ac.uk/tools/clustalo/). An asterisk displays identical amino acids indicating perfect alignment (*). Amino acid strong similarity or weak similarity are represented by (): or (.), respectively.

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REVIEW
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The Multifaceted Role of STAT3 in NK-Cell Tumor Surveillance

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Signal transducer and activator of transcription 3 (STAT3) is a member of the Janus kinase (JAK)-STAT pathway, which is one of the key pathways contributing to cancer. STAT3 regulates transcription downstream of many cytokines including interleukin (IL)-6 and IL-10. In cancer, STAT3 is mainly described as a tumor promoter driving tumor cell proliferation, resistance to apoptosis, angiogenesis and metastasis and aberrant activation of STAT3 is associated with poor prognosis. STAT3 is also an important driver of immune evasion. Among many other immunosuppressive mechanisms, STAT3 aids tumor cells to escape natural killer (NK) cell-mediated immune surveillance. NK cells are innate lymphocytes, which can directly kill malignant cells but also regulate adaptive immune responses and contribute to the composition of the tumor microenvironment. The inborn ability to lyse transformed cells renders NK cells an attractive tool for cancer immunotherapy. Here, we provide an overview of the role of STAT3 in the dynamic interplay between NK cells and tumor cells. On the one hand, we summarize the current knowledge on how tumor cell-intrinsic STAT3 drives the evasion from NK cells. On the other hand, we describe the multiple functions of STAT3 in regulating NK-cell cytotoxicity, cytokine production and their anti-tumor responses *in vivo*. In light of the ongoing research on STAT3 inhibitors, we also discuss how targeting STAT3 would affect the two arms of STAT3-dependent regulation of NK cell-mediated anti-tumor immunity. Understanding the complexity of this interplay in the tumor microenvironment is crucial for future implementation of NK cell-based immunotherapies.

Keywords: STAT3, NK cells, tumor immune surveillance, JAK-STAT, immunotherapy

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INTRODUCTION

Natural killer (NK) cells belong to the group 1 innate lymphoid cells and are characterized by the ability to kill virally infected and malignant cells. In contrast to T cells, NK cells do not require major histocompatibility complex (MHC)-dependent priming by antigen presenting cells. The activity of NK cells is regulated by a delicate balance of germ-line encoded activating and inhibitory receptors. Upon recognition of the target cell, an NK cell releases cytotoxic granules for direct cell lysis as well as produces immunomodulatory cytokines (1, 2). In humans, these tasks are fulfilled by different subtypes of NK cells: $CD56^{bright}CD16^{lo/-}$ NK cells are main producers of cytokines such as interferon (IFN) γ. In contrast, $CD56^{dim}CD16^{+}$ NK cells are highly cytotoxic, but do not produce substantial

amounts of IFNγ (3). The inborn ability to lyse transformed cells renders NK cells an attractive tool for cancer immunotherapy with a potentially better safety profile compared to T cells (4, 5). Different approaches to exploit NK cells in immunotherapy are being investigated. These include adoptive transfer of cytokine-induced memory-like NK cells or chimeric antigen receptor NK (CAR-NK) cells. Currently, numerous clinical trials using such approaches are ongoing, but the efficacy of these treatments still needs to be evaluated (5, 6). In this context, understanding the complex processes employed by tumor cells to evade NK-cell immunity is crucial. These escape mechanisms include transcriptional downregulation and shedding of ligands for NK-cell activating receptors, upregulation of inhibitory ligands, as well as immune suppressive signals derived from the microenvironment (7–11). Signal transducer and activator of transcription 3 (STAT3) is constitutively activated in various cancers and plays a pivotal role in regulating all of these processes and thereby mediates the crosstalk between the tumor microenvironment and immune cells (12).

STAT3 is a member of the Janus kinase (JAK)-STAT signaling pathway, which coordinates central cellular mechanisms including differentiation, development, proliferation, immune function, or apoptosis (13, 14). The alternatively spliced STAT3 isoforms, full-length STAT3α and C-terminally-truncated STAT3β, have opposing function during tumor development. While STAT3α promotes tumor growth, STAT3β was identified as a tumor suppressor and favorable prognostic marker in cancers of different origin (15, 16). Mechanistically, JAK-STAT3 signaling is activated by diverse growth factors, peptide hormones and all interleukin (IL)-6-type cytokines including IL-6, IL-11, IL-27, IL-31, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) neuropoietin (NP), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC) (17–19). IL-6 family cytokines, except for IL-31 which exerts its effects through IL-31 receptor α, induce signaling *via* binding to either a glycoprotein 130 (gp130) receptor β- subunit hetero- or homodimer (19, 20). Ligands bind to their cognate receptors, which undergo a conformational change, and induce subsequent activation of receptor associated JAKs (JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2)) by autophosphorylation and/or transphosphorylation. The JAK-induced tyrosine phosphorylation of the receptor provides a docking-site for the SH2 domain of STAT3, which in turn gets phosphorylated on tyrosine 705 by JAKs (21). Activated STAT3 forms anti-parallel to parallel homo- or heterodimers with other STATs, is released from the receptor and translocates into the nucleus through interaction with importin-β1 (22, 23). To control gene expression, activated STATs target palindromic consensus sequences located in promoter and enhancer regions and in the first introns of target genes (24). Negative regulation of STAT3 occurs in the nucleus through antagonization by PIAS (protein inhibitor of activated STAT), an E3 SUMO-protein ligase, or at the receptor by SOCS (suppressor of cytokine signaling) E3 ubiquitin ligases (25, 26) (**Figure 1**). The transcriptional activity of STAT3 can be further regulated by phosphorylation at serine 727 (Ser727) mediated by mTOR, p38, ERK and other serine/threonine kinases. However, the exact effects of Ser727 phosphorylation have to be put in a cellular and/or promoter dependent context. Phosphorylated Ser727 promotes association with different transcription co-factors and thus activates or diminishes transcriptional responses of STAT3 (27). Moreover, it can also drive the mitochondrial metabolic activity of STAT3 and augment the electron transport chain (28). Activated STAT3 in cancer cells contributes to oxidative and glycolytic phosphorylation, survival, epithelial-to-mesenchymal transition, proliferation, metastasis, and radiation- and chemotherapy resistance (29, 30). Due to its central contribution to several hallmarks of cancer and its association with poor clinical prognosis, STAT3 represents a promising therapeutic target for cancer therapy (14, 31, 32).

Inhibition of STAT3 signaling is currently explored in many clinical trials for solid and hematopoietic tumors. The direct approaches to specifically inhibit STAT3 include small molecules and decoy oligonucleotides (33). The most successful small molecule STAT3 inhibitor Napabucasin (BBI-608), which selectively binds to the DNA-binding domain of STAT3, has reached phase III trials for advanced colorectal cancer and provided excellent results as monotherapy (34). Another small molecule, TTI-101, targets the receptor binding site within the SH2 domain of STAT3 to block its recruitment and activation

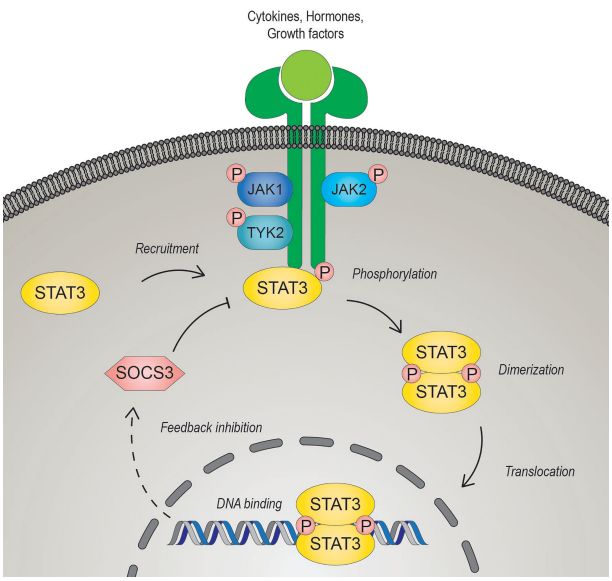

FIGURE 1 | The JAK-STAT3 signaling pathway. Signal transducer and activator of transcription 3 (STAT3) is activated upon binding of diverse cytokines, hormones, or growth factors to their cognate receptors. Ligand-bound receptors undergo conformational changes leading to the activation of the Janus kinases (JAK). Activated JAKs trans- and/or auto- phosphorylate each other and the cytoplasmic domain of the receptor, enabling STAT3 to bind *via* its SRC homology 2 (SH2) domain. JAK-mediated phosphorylation of a conserved C-terminal tyrosine residue of STAT3 induces dimerization of phosphorylated STAT3 and the subsequent translocation to the nucleus to regulate gene transcription. STAT3 induces transcription of suppressor of cytokine signaling 3 (*SOCS3*), which can act as a negative regulator by interacting competitively with the receptor.

(35). Phase I clinical trials in advanced solid cancers including breast cancer are ongoing (NCT03195699, NCT05384119). The SH2 domain is also targeted by other small molecules, OPB-51602, OPB-31121, OPB-111077, which are undergoing phase I/II clinical trials for solid and in case of OPB-51602 also hematopoietic tumors (reviewed in (36)). The antisense oligonucleotide, AZD9150, which is designed to target *STAT3* mRNA (37), has until now reached phase I/II trials for different advanced solid cancers (e.g. NCT01839604; NCT01839604) (33). Although the specificity and potency of such antisense oligonucleotides is very promising, they face problems of efficient penetration of solid tumors and fast degradation (33). To the best of our knowledge, the STAT3 inhibitors currently tested in clinical trials have not been thoroughly studied in the context of NK cell anti-tumor responses. Here, we summarize the current knowledge on how STAT3 contributes to NK-cell fitness and tumor cell evasion from NK cells, and speculate on how targeting STAT3 may affect NK-cell tumor surveillance.

STAT3 IN TUMOR CELLS – THE DRIVER OF IMMUNE EVASION FROM NK CELLS

Regulation of NK-Cell Receptor Ligands

NK cells exhibit cytolytic activity towards cells that overexpress ligands for activating receptors and/or lack the expression of MHC class I and other ligands, recognized by inhibitory receptors. In healthy cells, the ligands for activating receptors are absent or expressed at very low levels. Transformed cells upregulate these ligands and become sensed by NK cells as 'danger' (1, 38). The activating NK-cell receptor natural killer group 2D (NKG2D) served as a paradigm in studying this mechanism. In humans, NKG2D binds to MHC class I polypeptide-related sequence A (MICA) and B (MICB) and UL16-binding proteins (ULBP1-6) (39–42). In mice, NKG2D ligands comprise of the RAE1 family (α-ϵ), H60 (a -c), and MULT1 (43–45). Upon binding of a ligand to NKG2D, the co-stimulatory molecule DAP10 is activated and the release of cytotoxic granules is induced (40). Natural cytotoxicity receptor (NCR) NKp30 recognizes the B7-H6 molecule on transformed cells and induces NK-cell activation (46). Other receptors of this family are NKp44 and NKp46, which recognize heterogenous ligands including viral and bacterial proteins (47). Further activating NK-cell receptors function more as amplifiers of NK-cell activation triggered by NKG2D or NCRs (48). An important example is DNAM-1 and its corresponding ligands CD112 and CD155 often overexpressed on tumor cells (49, 50).

NKG2D is crucially involved in NK cell-mediated tumor surveillance and is one of the best studied receptors in this context. Mice deficient in NKG2D show strong defects in immune surveillance of epithelial and lymphoid tumors (51). In line, high expression of NKG2D ligands in leukemic patients correlates with better survival (52). Absence of NKG2D ligands is also a feature of leukemic stem cells, which allows them to escape NK-cell surveillance in acute myeloid leukemia (AML) *in vivo* models (53). Tumor cells evade NKG2D-mediated recognition by downregulation or shedding of the ligands. Not only does it allow to hide from NK-cell cytotoxicity, but also leads to desensitization of NKG2D-mediated NK-cell activation. High levels of shed NKG2D ligands result in downregulation of NKG2D-mediated signaling (54, 55).

STAT3 has been implicated in direct transcriptional repression of NKG2D ligands. Bedel et al., revealed that inhibition or knockdown of STAT3 in the colorectal cancer cell line HT29 leads to stronger activation of NK cells and therefore killing of tumor cells in an NKG2D-dependent manner. Further, they could show that STAT3 directly binds to the *MICA* promoter, repressing its transcription (56). A similar mechanism has been described in multiple myeloma (MM) cell lines. Upon treatment with glycogen synthase kinase 3 (GSK-3) inhibitor, MM cell lines showed decreased STAT3 activation and reduced STAT3 binding to the *MICA* promoter. The effects corresponded to enhanced sensitivity of treated cell lines to NK cell-mediated lysis. Importantly, GSK-3 inhibition had no effect on *MICA* expression in cell lines with constitutively active STAT3. In line, the GSK-3 inhibitor was not able to reduce the activation level of constitutively active STAT3. This strongly suggests that GSK-3-induced susceptibility of MM cells to NK cells is greatly dependent on inhibiting STAT3 activation (57). In another study using colorectal cancer cells, GSK-3 inhibition significantly upregulated NKG2D ligands and increased their sensitivity to NK cells. However, in this context the dependence on STAT3 remains to be elucidated (58).

The correlative observations of low STAT3 activity and/or expression and high NKG2D ligands surface levels have also been made in other cancer entities. The adriamycin-resistant chronic myeloid leukemia (CML) cell line K562 is killed more efficiently by NK cells upon treatment with a STAT3 inhibitor and shows an upregulation of MICA and ULBP2 (59). STAT3 inhibition or silencing also enhances ULBP2 expression in parental K562 cells (60). In AML cell lines, an inverse correlation between phosphorylated STAT3 (pSTAT3) levels and MICA expression was observed after rapamycin (61) or decitabine treatment (62) but the mechanism behind it remains unclear. In hepatocellular carcinoma cell lines, a STAT3 decoy resulted in upregulation of NKG2D ligands and increase of NK cell-mediated killing (63). A layer of complexity is added by the fact that in human gastric adenocarcinoma cell lines inhibition of STAT3 results in upregulation of MICB on the cell surface as well as of the soluble ligands. This implies a potential desensitization of NK cells driven by inhibition of STAT3 (64). Although all the mentioned studies point towards a similar effect of STAT3 inhibition or silencing, the interpretation is limited, as all of the experiments where only performed *in vitro*. A robust *in vivo* xenograft model of STAT3-deficient tumor cell lines with adoptive transfer of human primary NK cells would be necessary to further elucidate the impact of STAT3 on NKG2D-mediated NK-cell surveillance.

The missing-self hypothesis, formulated in the 1980s, states that NK cells kill those cells that do not express sufficient levels of MHC I. In line, several classes of inhibitory receptors were discovered, which unleash NK-cell cytotoxicity upon downregulation of MHC

I on the target cells (65, 66). These include the Ly49 family in mice and the KIR family in humans (67–69). Ly49 and KIRs sense the levels of conventional MHC I, therefore, tumor cells that downregulate MHC I molecules to escape T cell responses become targets for NK cells (70, 71). In a mouse model of carcinogen-induced Non-Small Cell Lung Cancer (NSCLC), epithelial cell-specific knockout of *Stat3* led to downregulation of MHC I on transformed epithelial cells. This rendered the emerging cancer cells more susceptible to NK cell-mediated lysis (72).

Regulation of Tumor Microenvironment

STAT3 is considered as a driver of an immune suppressive tumor microenvironment. STAT3 activation is associated with high expression of tumor promoting cytokines and growth factors such as IL-10, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF)-A. The majority of STAT3-dependent effects in the tumor microenvironment are described in the context of T cells, macrophages or dendritic cells that have been extensively reviewed by others (12, 73). However, the soluble immune suppressive modulators present in tumor microenvironment not only suppress the function of NK cells, but also impair their infiltration into the tumor (13) or even confer a switch towards pro-tumorigenic, VEGF-A-producing NK cells (74–77). The IL-10/STAT3 axis directly drives VEGF-A expression in these cells (77). TGF-β in the microenvironment also drives the conversion of cytotoxic NK cells ($CD49a^-CD49b^+EOMES^+$) into ILC1 ($CD49a^+CD49b^-EOMES^{int}$), which lose the ability to control the tumor growth (78).

In the previously mentioned, carcinogen-induced NSCLC model with epithelial cell-specific *Stat3* knockout, the tumor microenvironment is enriched for proinflammatory cytokines which might contribute to enhanced NK-cell responses against the tumor (72). In support of this finding, hepatocellular carcinoma cells treated with STAT3 decoy secrete higher levels of IFNs and lower levels of immune suppressive TGF-β. Upon culture in conditioned medium derived from hepatocellular carcinoma cells pre-treated with STAT3 decoy, NK cells showed a more activated phenotype with higher expression of IFNγ, granzyme and perforin (63). In line, STAT3 in a murine melanoma cell line was shown to inhibit the expression of proinflammatory cytokines such as TNFα and IL-12 and the chemokine CCL5. Inhibition of STAT3 signaling was associated with increased levels of CCL5 and thereby enhanced lymphocyte infiltration into the tumor (79). Importantly, the conclusions were made in melanoma model overexpressing the alternatively spliced, truncated isoform of STAT3 – STAT3β, which was believed to have dominant negative functions over the full length isoform (80). However, several studies have shown that STAT3β is transcriptionally active and drives expression of its unique target genes (81–83). The potential of STAT3β to drive cytokines and chemokines that support the immune system is in accordance with the tumor suppressive potential of STAT3β-overexpressing macrophages in breast cancer (84).

Interestingly, in BCR-ABL-driven lymphoma, the deletion of STAT3 has opposite effects to those described above. STAT3-deficient B cell lymphoma shows decreased expression of proinflammatory cytokines, e.g. TNFα and chemokine CCL5. This is paralleled by a lower abundance of NK cells in the tumors. Transplantation of the lymphoma cells lacking STAT3 into mice harboring NK cells results in accelerated tumor growth, but the difference is lost in immune-deficient mice. The study postulates that targeting STAT3 in BCR-ABL-driven malignancies might impair NK-cell surveillance (85).

In summary, STAT3 activity is critically implicated in determining the outcome of cancer immunity by orchestrating the release of immunomodulating cytokines. In the majority of cases, inhibition of STAT3 signaling switches the tumor microenvironment towards immune activation (**Figure 2**, right).

STAT3 IN NK CELLS – THE VERSATILE MODULATOR OF NK CELL RESPONSES

STAT3 is activated in NK cells by a variety of cytokines, including type I IFNs, IL-2, IL-6, IL-10, IL-12, IL-15, IL-21

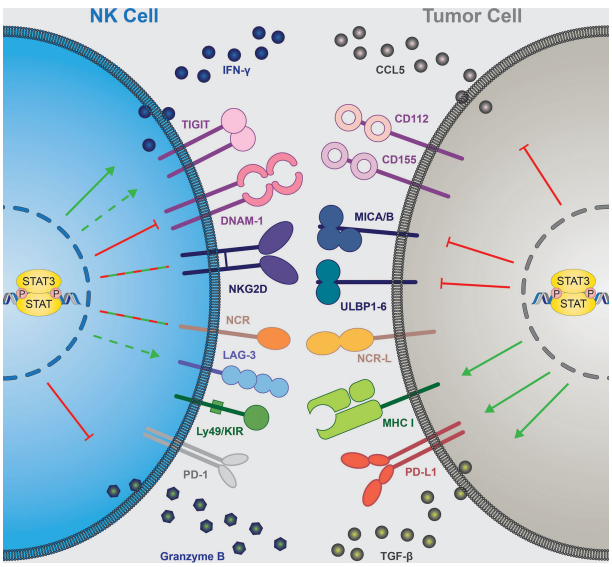

FIGURE 2 | STAT3 contribution to NK cell-mediated tumor immune surveillance. NK cell-intrinsic STAT3 (**left**) inhibits expression of granzyme B and DNAM-1 (⟞), increases IFNγ secretion (⟶) and seems to upregulate TIGIT and LAG-3 (- ->), while the effect on NCRs and NKG2D expression remain context dependent (⟷). Tumor cell-intrinsic STAT3 (**right**) inhibits expression of NKG2D ligands (MICA/B, ULBPs) and NK-cell attracting chemokine CCL5 (⟞). STAT3 in tumor cells upregulates surface expression of MHC I and PD-L1 molecules and secretion of immune suppressive TGF-β (⟶). NK, natural killer; IFN, interferon; DNAM-1, DNAX accessory molecule; NKG2D, NK-cell receptor natural killer group 2D; NCR, natural cytotoxicity receptor; KIR, killer-cell immunoglobulin-like receptor; CCL5, C-C motif chemokine ligand 5; CD, cluster of differentiation; MICA/B, major histocompatibility complex class I-related sequence A/B; ULBP, UL16-binding protein; MHC I, major histocompatibility complex I; TGF-β, transforming growth factor β, STAT, signal transducer and activator of transcription. TIGIT, T cell immunoreceptor with Ig and ITIM domains; LAG-3, lymphocyte-activation gene 3; PD-(L)1, programmed cell death ligand/protein 1.

and IL-27, with diverse effects on NK-cell activation (86–91). NK cell-intrinsic roles of STAT3 have been analyzed in *Stat3*$^{fl/fl}$ *Ncr1-iCre* mice lacking STAT3 in NKp46+ cells. *Stat3* deletion does not affect NK-cell development, numbers and maturation. Furthermore, NK cell-intrinsic loss of STAT3 does not impact on proliferation of NK cells in *Stat3*$^{fl/fl}$ *Ncr1*-iCre mice (87). However, knockdown of STAT3 in the human NK-cell line NK-92 is associated with a decreased proliferation rate, correlating with reduced *cyclin D1* expression, and overexpression of STAT3 enhances human NK-cell expansion (92). In line, IL-21, which primarily activates STAT3 in NK cells, promotes human NK-cell expansion associated with an increased telomere length (93–95).

Effects of cancer cell-extrinsic STAT3 deficiency on anti-tumor immunity have initially been reported in an inducible STAT3 knockout mouse model (*Stat3*$^{fl/fl}$ *Mx1*-Cre mice) (96). Both *Stat3*$^{fl/fl}$ *Mx1*-Cre and *Stat3*$^{fl/fl}$ *Ncr1*-iCre mice demonstrated that lack of STAT3 enhances NK cell-mediated surveillance in different transplantable tumor models (87, 96). Treatment with the small-molecule STAT3 inhibitor CPA7 boosts anti-tumor responses against the subcutaneously injected urothelial carcinoma cell line MB49, which is largely dependent on T cells with a partial involvement of NK cells (96). NK cell-intrinsic STAT3 deficiency is sufficient to increase surveillance of melanoma and leukemia cell lines (87). Overall, these data provide evidence that STAT3 suppresses the anti-tumor activity of NK cells (13, 87, 96). The enhanced cytotoxicity of NK cells upon loss of STAT3 goes along with increased levels of the cytotoxic effector molecules perforin and granzyme B (87, 88).

STAT3 is also involved in regulating expression of activating NK-cell receptors. STAT3 decreases DNAM-1 expression on NK cells. Elevated DNAM-1 levels upon *Stat3* deletion contribute to enhanced killing of DNAM-1 ligand-expressing tumor cells, e.g. B16F10 melanoma cells (87). NK cells show a STAT3-dependent upregulation of NKG2D surface levels in response to IL-10 and IL-21 stimulation, associated with enhanced NK-cell degranulation (88, 95, 97). While NK cell-intrinsic loss of STAT3 does not suffice to impact on NKG2D expression, STAT3-deficiency in the entire hematopoietic system (*Stat3*$^{fl/fl}$ *Tie2*-Cre mice) causes a reduction of NKG2D levels (87, 95). In contrast to a potential negative regulatory role of STAT3 on DNAM-1 expression in murine NK cells (87), these results indicate that STAT3 enhances NKG2D expression (95). However, another report demonstrated that IL-2-activated human NK cells display decreased NKG2D levels upon IL-21 stimulation (98). In addition, STAT3 activation by IL-6 and IL-8 produced by tumor cells has been reported to decrease levels of NKG2D and NKp30 on NK cells (91). Therefore, the impact of STAT3 on the regulation of NKG2D might vary depending on the specific upstream stimuli, signaling pathways and additional STAT proteins involved (13, 88). Similar to NKG2D, STAT3 has also been reported to bind to the promoter and drive the transcription of N*CR1* gene encoding NKp46 (92, 95).

In line with the suppressive effect of STAT3 on NK-cell functionality reported in the murine system (87), tumor-derived cytokines, such as IL-6 and IL-8, impair human NK-cell function in a STAT3-dependent manner (91). On the contrary, another study found a positive correlation between STAT3 levels and expression of cytotoxic effector molecules and cytokines in human NK cells (92). STAT3 levels are reduced in NK cells from chronic hepatitis B virus (HBV) patients, which is associated with lower degranulation and IFNγ production (92). Early cytokine production, including IFNγ, in IL-15 primed human NK cells also requires STAT3 (99). An involvement of STAT3 in the regulation of IFNγ production has also been described in murine NK cells, where STAT3 directly binds to the *Ifng* promoter and contributes to cytokine-induced IFNγ production (87). Besides cytotoxic activity and production of proinflammatory cytokines, NK cells also produce immunosuppressive cytokines and have immunoregulatory functions (100, 101). STAT3 also impacts on these functions. For example, the immunosuppressive cytokines IL-10 and TGF-β are upregulated in a STAT3-dependent manner in tumor-infiltrating NK cells that are positive for the immune checkpoint CD73 (12, 102).

Hypoxia is an important feature of solid tumors that is associated with immune suppression and escape (103). Indeed, hypoxia represses NK-cell cytotoxicity by induction of SHP-1 expression, which in turn reduces STAT3 activation (104). STAT3 also interacts with one of the main players driving hypoxic response - hypoxia inducible factor 1α (HIF-1α) (105). In IL-15 primed human NK cells, HIF-1α responses rely on STAT3 (99). In line, STAT3 induces HIF-1α-mediated upregulation of miR-224, which is paralleled by reduced NKp46 expression and a dampened NK cell-mediated killing of prostate cancer cells (12, 106, 107). The exact underlying mechanisms how STAT3 activity contributes to hypoxia-driven effects on NK-cell cytotoxicity remain to be determined.

Altogether, STAT3 has complex effects on NK-cell activity, including the expression of cytotoxic granule proteins, cytokines and NK-cell receptors (**Figure 2**, left). Whether STAT3 has an overall beneficial or detrimental effect on NK-cell function appears to be context-dependent (13, 87, 92, 95). STAT3 induces its own negative feedback regulation, including the upregulation of SOCS3 (**Figure 1**). SOCS3 suppresses NK cells, as loss of SOCS3 enhances NK-cell proliferation and cytotoxicity (108). The effects of SOCS3 on STAT3 activation might depend on the upstream stimuli, which are likely to be differently susceptible to SOCS3-mediated inhibition (109, 110). This could contribute to the context-dependent effects of STAT3 in NK cells. As mentioned above, cancer cell-intrinsic alternatively spliced STAT3 isoforms have opposite roles in driving tumor progression. Since STAT1 isoforms differentially affect NK-cell functionality (111), it is attractive to speculate that also STAT3α and β have non-redundant functions in regulating NK cells. Analyzing the consequences of STAT3α or β deficiency in NK cells might improve our understanding of STAT3-dependent effects.

STAT3 MUTATIONS IN NK CELLS – INSIGHTS FROM PATIENTS

Another level of evidence for a crucial role of STAT3 in NK-cell biology stems from studies analyzing NK cells in patients with STAT3 mutations. Heterozygous germline STAT3 loss-of-

function (LOF) mutations are found in autosomal dominant hyper IgE syndrome (HIES) patients, which display immunological deficiencies with increased susceptibility to infections linked to impaired STAT3-regulated T helper 17 (Th17)-mediated immune responses and B cell function (112–116). NK cells from HIES patients harboring STAT3 LOF mutations have decreased NKG2D levels (95). This might be associated with impaired NK cell function, however a thorough functional characterization of NK cells from HIES patients has not been published. Apart from LOF mutations, germline and somatic activating mutations of STAT3 have been described in humans with different disease characteristics (112–115, 117). Germline STAT3 gain-of-function (GOF) mutations are associated with diverse clinical manifestations, including immunodeficiencies and autoimmune diseases (112, 118–122). Haapaniemi et al. reported that NK-cell numbers are reduced in patients with germline STAT3 GOF mutations, while maturation and functionality of NK cells are unaffected (120). However, another study did not find reduced NK-cell numbers (123), indicating that the impact of STAT3 GOF mutations on immune cells, including NK cells, varies between patients (122).

An oncogenic potential of STAT3 in NK cells has been indicated by the discovery of somatic STAT3 GOF mutations, predominantly within the SH2 domain, in a subset of patients with different NK-cell malignancies, including chronic lymphoproliferative disorder of NK cells (CLPD-NK), aggressive NK-cell leukemia (ANKL) and extranodal NK/T-cell lymphomas of nasal type (NKTCL) (124–135). Apart from STAT3 GOF mutations, enhanced STAT3 phosphorylation can also be observed in NK-cell malignancies by other means, including activation of upstream JAKs or reduced expression of negative regulators of STAT3 (124, 127, 131–133, 136–139). Pro-tumorigenic effects of STAT3 on NK cells are linked to its role in proliferation and survival (133, 136, 140–142). IL-10 mediated STAT3 activation as well as somatic STAT3 GOF mutations increase expression of MYC and thereby drive metabolic activation of ANKL cells, fueling leukemia cell survival and proliferation (131). To the best of our knowledge, the effects of somatic STAT3 GOF mutations on the functionality of malignant NK cells have not been directly tested. Interestingly, STAT3 GOF mutations in CLPD-NK patients correlate with a cytotoxic $CD16^{hi}CD57$-phenotype and a more symptomatic disease, characterized by anemia and severe neutropenia (127, 134, 135, 143).

STAT3 – A POTENTIAL TARGET TO ENHANCE IMMUNE CHECKPOINT INHIBITOR THERAPY?

Immune checkpoint inhibitors are one of the most successful approaches of immunotherapy. By targeting the inhibitory ligands (programmed cell death ligand 1 - PD-L1) or inhibitory receptors (programmed cell death protein 1 - PD-1, cytotoxic T-lymphocyte-associated protein 4 - CTLA-4) with monoclonal antibodies, the immune response against tumors can be unleashed (144). It is clear that T cells are the main drivers of immune checkpoint inhibitor responses, but a role of NK cells herein has been proposed (145). Several reports find PD-1-dependent effects of NK cells in specific tumors including MM (146), Kaposi sarcoma (147), Hodgkin lymphoma (148) and head and neck cancer (149). Other studies indicate that the expression of PD-1 in NK cells is minor or neglectable (150) and the exact function of NK cells in anti-PD-1/PD-L1 therapy remains a matter of debate (151). Importantly, vast evidence indicates the direct involvement of STAT3 in driving PD-L1 and PD-L2 expression in tumor cells (132, 152–154). For example, in T cell lymphoma STAT3 is required for induction of *PD-L1* transcription by directly binding its promoter (153). Therefore, combinatorial inhibition of STAT3 and PD-L1/PD-1 axis has been explored as an attractive approach. Encouraging results from preclinical studies (33, 155, 156) led to first clinical trials combining STAT3 inhibitor (BBI-608) with anti-PD-L1 therapies in metastatic colorectal carcinoma (NCT03647839, NCT02851004) or STAT3 targeting antisense oligonucleotide (AZD9150) with anti-PD-L1 therapy in NSCLC (NCT03334617) and other solid tumors. It remains unclear whether NK cells contribute to this combination therapy outcome. Xu et al. suggested that *in vitro* combination of PD-L1 and STAT3 inhibition enhances NK-cell cytotoxicity against prostate cancer cell lines, but the *in vivo* relevance still needs to be elucidated (157).

Novel immune checkpoint molecules are currently emerging and some have entered clinical trials or have recently been approved. In contrast to the above described PD-1 and CTLA-4, the expression of novel checkpoints: T cell immunoreceptor with Ig and ITIM domains (TIGIT), lymphocyte-activation gene 3 (LAG-3) and T cell immunoglobulin and mucin-domain-containing-3 (TIM-3) is clearly shared between T and NK cells (158–161). The exact role of STAT3 in the regulation of these checkpoints has not been addressed in NK cells. In T regulatory cells, TIM-3 is strongly downregulated upon STAT3 inhibition suggesting a potential dependency of TIM-3 expression on STAT3 in other lymphocytes (162). It is attractive to speculate that LAG-3 expression in NK cells might be enhanced by STAT3 signaling. IL-12, which predominantly signals *via* STAT4 and STAT3, was shown to upregulate LAG-3 expression in NK cells (163). In line, STAT3 inhibition together with blockage of the STAT3-activating cytokine IL-6, downregulated TIGIT expression in the human NK92 cell line. Combination of TIGIT checkpoint inhibitor with blockage of IL-6R and STAT3 enhanced cytotoxicity of NK92 cells towards prostate cancer cells (164). Although further investigations are essential, both studies suggest a potential synergism between STAT3 targeting molecules and novel checkpoint inhibitors in driving NK responses.

CONCLUSION AND OUTLOOK

STAT3 regulates immune evasion from NK cells on several different levels. It helps tumor cells to hide from NK cells by downregulating activating ligands, drives an immune suppressive environment, which in turn limits the chemoattraction and activity of NK cells, and intrinsically regulates NK-cell responses

(**Figure 2**). Somatic GOF mutations in STAT3 have an oncogenic potential in NK cells underlining its key role in NK-cell biology. It remains unclear why the effects of STAT3 on NK-cell biology are so complex and context dependent. The discrepancy between some findings in human and mouse NK cells might come from *in vitro* cultures of human NK cells that do not reflect the situation *in vivo* with different cytokines present in the tumor microenvironment in the mouse models. Moreover, one cannot exclude that the results obtained using mice with STAT3-deficient NK cells are influenced by compensatory mechanisms, including upregulation of STAT5, which is a key driver of NK-cell survival and functionality (77, 165, 166). Not only can the STATs compensate for each other (23, 167) but also crosstalk to other signaling pathways (168). This adds another layer of complexity in understanding the role of STAT3 in NK-cell anti-tumor responses.

Inhibition of STAT3 signaling is currently explored in many clinical trials for solid and hematopoietic tumors. The direct approaches have reached clinical trials but achieving specificity over other STAT family members remains challenging (33, 169, 170). Based on the current evidence, STAT3 inhibition might not only impair tumor cell survival but also enhance their recognition by NK cells. Moreover, targeting NK cell-intrinsic STAT3 could unleash their anti-tumor responses in some tumor models (87), while the consequences on other aspects of NK-cell functionality are difficult to predict. At the moment, there is an unmet need to understand the effects of STAT3 inhibitors on NK-cell anti-tumor responses *in vivo* to be able to foresee the clinically relevant consequences.

It is well appreciated that combination therapies enhance the efficacy and reduce resistance compared to monotherapies. This has triggered extensive attempts in combining STAT3 inhibitors with other drugs (33). A new avenue in immunotherapy is opened by combinations of STAT3 inhibitors explored with the emerging immune checkpoint inhibitors. Importantly, targeting STAT3 in the immune system might have complex systemic effects ranging from autoimmunity to immunodeficiency as indicated by phenotypes of patients with mutations in STAT3 (112–116). In summary, targeting STAT3 might be an attractive approach in restoring NK-cell anti-tumor immunity but needs to be carefully evaluated in different tumor types and biological contexts.

AUTHOR CONTRIBUTIONS

AW-S, KK and BZ designed the concept and wrote the manuscript. DS designed the concept and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Innate lymphoid cells in early tumor development

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Innate and adaptive immune cells monitor, recognize, and eliminate transformed cells. Innate lymphoid cells (ILCs) are innate counterparts of T cells that play a key role in many facets of the immune response and have a profound impact on disease states, including cancer. ILCs regulate immune responses by responding and integrating a wide range of signals within the local microenvironment. As primarily tissue-resident cells, ILCs are ideally suited to sense malignant transformation and initiate anti-tumor immunity. However, as ILCs have been associated with anti-tumor and pro-tumor activities in established tumors, they could potentially have dual functions during carcinogenesis by promoting or suppressing the malignant outgrowth of premalignant lesions. Here we discuss emerging evidence that shows that ILCs can impact early tumor development by regulating immune responses against transformed cells, as well as the environmental cues that potentially induce ILC activation in premalignant lesions.

KEYWORDS

innate lymphoid cell (ILC), tumor development, damage associate molecular pattern (DAMP), cytokines, carcinogenesis, immunosurveillance, tumor immunity

1 Introduction

Tumorigenesis is a complex, multistep process in which normal cells evolve progressively to a neoplastic state. Thus, tumors are often preceded by different stages of premalignant tissue changes, including hyperplasia, metaplasia, and dysplasia, which are linked to an increased cancer risk. The immune system can detect tissue changes and eliminate transformed cells in a process referred to as tumor immunosurveillance. The original cancer immunosurveillance hypothesis was formulated in the 1950s and described that adaptive lymphocytes reduce tumor growth in response to recognizing tumor antigens (1, 2). Since then, this theory has been refined and cancer immunosurveillance is now widely accepted as being part of the cancer immunoediting process, wherein the tumor-suppressive and tumor-promoting activities of the immune system shape tumor development. This process is divided into three different phases: elimination (cancer immunosurveillance), equilibrium (cancer persistence/dormancy), and escape (cancer progression) (3–5).

Our current understanding of cancer immunosurveillance is primarily based on studies in mice, which have shown that the immune system can prevent the outgrowth of many different types of primary and transplantable tumors (4). Evidence for the importance of the immune system in preventing tumor development in humans is found in studies showing increased incidences of malignancies in immunocompromised patients with AIDS and recipients of organ transplants using immunosuppressants, as well as spontaneously regressing benign and malignant melanocytic lesions accompanied by lymphocytic infiltrates (4). Despite the immune system's anti-tumorigenic activities, deregulated inflammatory responses have also been linked to carcinogenesis and often precede tumor development (6). Thus, the immune system does not only protect the host against tumor development but also promotes progression of premalignant to malignant cells.

A comprehensive view of tumor immunosurveillance would include not only adaptive immune cells but also innate immune cells since it is well known that they detect and destroy transformed cells (4, 5). Besides T cells, natural killer (NK) cells are known to play a key role in cancer immunosurveillance (7). NK cells are innate lymphoid cells (ILCs) that mirror $CD8^+$ T cytotoxic cells and secrete cytotoxic molecules such as granzymes and perforin to eliminate virus-infected cells and tumor cells. Increasing evidence suggests that other ILC family members also play an important role in the immune response against tumors (8) and their role in tumor development is starting to being explored. ILCs have been classified into NK cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue inducer (LTi) cells based on their cytokine and transcription factor expression profiles, and developmental pathways (9). ILC1s, ILC2s and ILC3s share features with CD4 T helper (h)1, Th2, and Th17/22 subsets, respectively. NK cells and ILC1s express the transcription factor T-box transcription factor 21 (T-BET) and secrete interferon (IFN)-γ. In addition, NK cells, but not ILC1s, require the transcription factor Eomesodermin (EOMES) for their development. However, a proportion of ILC1s can express EOMES (10). ILC1s are involved in the immune response against viruses and intracellular bacteria. They express multiple granzyme molecules, but at lower levels compared to NK cells. ILC2s are dependent on the transcription factors GATA-binding protein 3 (GATA3) and retinoic acid-related orphan receptor (ROR)α and produce classical type 2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 in response to parasite infection and allergen exposure. ILC3s and LTi cell subsets share a characteristic expression of the retinoic acid receptor-related orphan nuclear receptor γt (RORγt) and the cytokines IL-17A and IL-22 but follow different developmental pathways. ILC3s are immune effectors that contribute to host defense against extracellular bacteria and fungi, whereas LTi cells initiate the development of fetal lymphoid tissues (9).

NK cells circulate in the body, whereas the other ILC subsets are primarily tissue resident cells that preferentially reside in barrier tissues. In addition to providing immunity against infections, they also play critical roles in maintaining tissue homeostasis by responding rapidly to environmental cues, initiating effector responses in a tissue-specific manner and interacting with tissue-resident cells (11). This makes them ideally suited to sense malignant transformation and initiate anti-tumor immunity. However, ILCs have been associated with pro-tumor and anti-tumor activities in established tumors (8) and could therefore have a dual role during tumor development as well. In this review, we discuss the stress signals that could potentially activate ILCs during tumor development and recent advances supporting a role of ILCs in immune surveillance and carcinogenesis.

2 ILCs and tumor development

Premalignant lesions arise from various causes, including infection, inflammation, and environmental exposures. Innate immune cells are considered the first responders to cellular stress and mediate adaptive immune responses. This is supported by a study that profiled 122 bronchoscopy biopsies from 77 patients using gene-expression profiling and multispectral imaging, which included 9 morphological stages of invasive lung squamous cell carcinoma (SCC) development. During hyperplasia, the earliest stage of transformation, there was an increase of innate immune cells, such as neutrophils, activated mast cells and NK cells, and resting dendritic cells (DCs), as well as naïve CD4 T cells. This was followed by an increase of CD8 T cells and activated memory CD4 T cells in metaplastic and dysplastic tissues. Thus, NK cells are part of an early immune response against tissue changes associated with malignant transformation. Although this study did not assess other ILC populations in these tissues, it is likely that they respond to the same stress signals that activate other innate immune cells (section 3.).

Studies in mice have provided important evidence that ILCs play a role in mediating tumor immunosurveillance, either directly or indirectly through modulation of effector immune cell responses. Expression of IFN-γ and the effector molecule tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) by NK cells has been shown to prevent tumor initiation in mice (12, 13). In addition, low cytotoxic activity of NK cells is associated with an increased cancer risk in humans (14–16). Mice depleted of NK cells and ILC1s by anti-NK1.1 or anti-asialo-GM1 are more susceptible to the formation of chemically induced tumors (17). Furthermore, tumor incidence in $Rag1^{-/-}IL2R\gamma^{-/-}$ and $Rag2^{-/-}IL2R\gamma^{-/-}$ mice, which in addition to B and T cells also lack ILCs, was increased compared to $Rag1^{-/-}$ and $Rag2^{-/-}$ mice lacking only adaptive immune cells (18, 19).

With the exception of NK cells, the contribution of individual ILC subsets to immune responses during early tumor development is less well defined. Using the MMTV-

PyMT mammary tumor model, Dadi et al. demonstrated that a cytotoxic ILC1-like population accumulates in precancerous lesions (20). Importantly these cells were dependent on IL-15 and displayed toxicity against tumor cells (20) (Figure 1). Interestingly, unlike the ILC1-like population, NK cells did not expand in these precancerous lesions, suggesting that tissue-resident ILC1-like cells may play a more important role in early sensing of cellular transformation. ILC2 stimulation by epithelial and/or Th2-derived cytokines induces IL-5, GM-CSF and IL-13 expression, leading to eosinophil recruitment, activation and survival (21, 22). In a model for chemically-induced fibrosarcomas, IL-5 overexpression protected mice from tumor establishment through an increased recruitment of eosinophils to the tumor and surrounding connective tissue (27). Thus, ILC2s could potentially mediate tumor immunosurveillance by regulating eosinophil accumulation in premalignant tissues (Figure 1). A protective role of ILC2s during tumor development was also described in a chemically-induced colorectal cancer (CRC) mouse model as ILC2-deficient mice had an increased tumor burden compared to WT mice (28). This is further supported by a recent study showing that IL-33 mediated expansion of ILC2s was associated with reduced colonic inflammation in a colitis model (29). ILC2s may be involved in the immune response against developing CRC by activating eosinophils, as eosinophils have been shown to prevent the development of CRC in a colitis-associated cancer model independently of $CD8^+$ T cells (30). However, in an adenomatous polyposis coli (*Apc*)-mutation-driven model of spontaneous intestinal tumorigenesis, IL-25 activated ILC2s promoted CRC development by promoting myeloid-derived suppressor cell (MDSCs) function to suppress T cell responses (24). In addition, another study found that in response to gastric tissue damage in mice, IL-13-secreting ILC2s are recruited to the gut mucosa and drive metaplasia development (25). Together, these studies provide evidence that ILC2s might promote malignant transformation, depending on the environmental cues and tissues involved (Figure 1). Additional studies will be required to distinguish between pro- and anti-tumorigenic functions of ILC2s, similar to what is observed for their role in established tumors (8).

A recent study by Goc et al. identified a major histocompatibility complex class II positive (MHCII+) ILC3 population in precancerous adenomas in mice and humans (23). In a spontaneous CRC mouse model, deletion of ILC3-

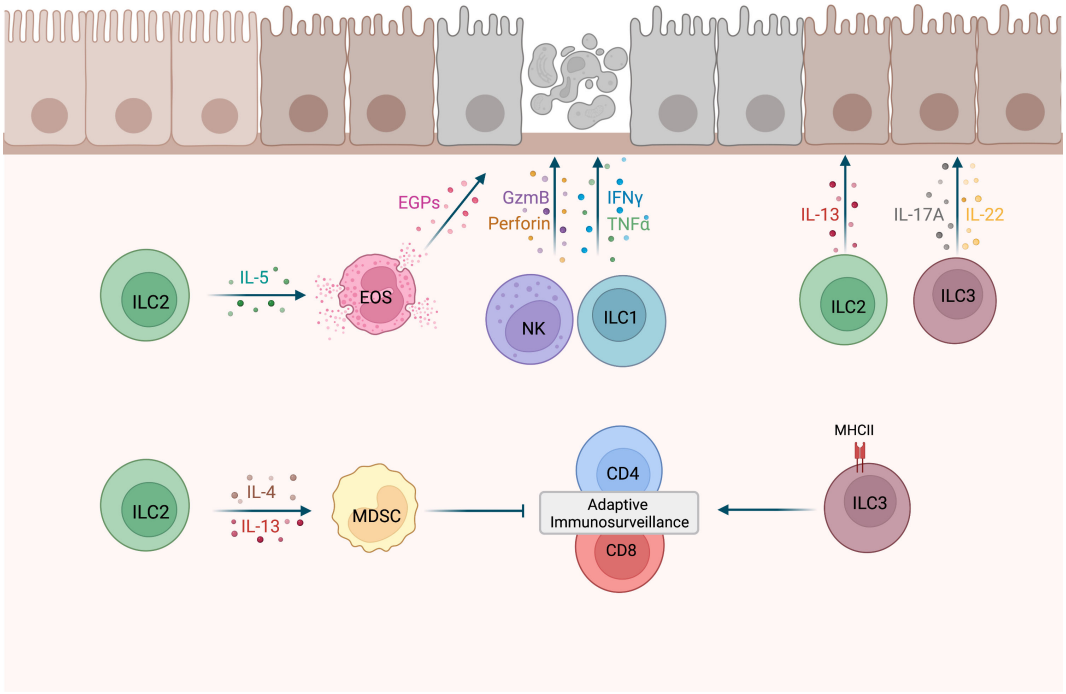

FIGURE 1
Potential roles of ILCs during tumor development. NK cells and ILC1s have shown cytotoxic activity against precancerous cells (12, 13, 20). IL-5-secreting ILC2s may recruit eosinophils (EOS) to precancerous tissues and activate cytotoxic effector functions in EOS, such as the release of eosinophilic granule proteins (EGPs) (21, 22). $MHCII^+$ ILC3s were shown to promote CD4 and CD8 cell responses to prevent tumor development (23). Conversely, ILC2s may drive MDSCs activation and subsequent T cell suppression *via* IL-4 and IL-13 secretion (24), as well as metaplasia development *via* the release of IL-13 (25). IL-17A and IL-22 expression by ILC3s may promote clonal expansion of precancerous cells (26). This figure has been created with BioRender.com.

specific MHCII resulted in an increased number of advanced tumors and a significant reduction in overall survival, suggesting that MHCII$^+$ ILC3 limit tumor development. Further analysis revealed that mice lacking MHCII expression in ILC3s were characterized by a significant reduction of Th1 and T-bet$^+$ CD8 T cells, thereby providing evidence that interactions between ILC3s and T cells promote type-1 immunity (Figure 1). Although this study supports a role of ILC3s in tumor immunosurveillance, there are other studies that implicate ILC3s in tumorigenesis. Dysregulated IL-23 mediated ILC3 activation and IL-17 and IL-22 production has been shown to promote gut inflammation and tumorigenesis (31, 32). In addition, in a hepatocellular carcinoma (HCC) mouse model established by a murine HCC cell line, IL-23 over-expression promoted HCC development in an IL-17-dependent manner (33). Interestingly, most IL-17-producing cells in early tumors were NCR$^-$ILC3s, suggesting that they are the initial responders to IL-23. Using a model for UV–induced cutaneous carcinogenesis, Lewis et al. also demonstrated that chronic UV exposure leads to an increase in IL-22 and IL-17A-producing ILC3s in the skin, which drive mutant keratinocytes clonal expansion in the absence of T cells (26) (Figure 1). Based on studies in established tumors, it is known that ILC3s can have conflicting functions (8, 34) and current evidence suggests that this might also be the case for their role during early tumor development.

3 Stress signals during cell transformation and their potential role in ILC activation

Stressed and dying cells in precancerous tissues express and/or release various endogenous danger molecules, such as damage-associated molecular patterns (DAMPs), cell-surface receptors, and cytokines, that activate the immune system (35). Early detection of stress signals is important for successful cancer immunosurveillance. However, some signals may activate inflammatory responses that contribute to malignant transformation instead of protecting against it. ILCs sense changes in the tissue microenvironment through a broad array of cell surface and intracellular receptors, including costimulatory receptors, cytokine and chemokine receptors (36–38). Binding of ligands to these receptors also drives ILC plasticity, thereby shaping their function and phenotype (39). Here, we will discuss how known stress signals released by precancerous cells could potentially activate ILCs and initiate ILC-mediated immune responses that impact tumor development. In particular, we will focus on signals associated with an inflammatory response in the absence of infection. ILCs express toll-like receptors that could potentially recognize pathogen-associated molecular patterns (PAMPs) during infection-associated tumor development (40, 41). However, it is likely that the immune responses to these infections and developing tumors in the same tissues overlap and the role of ILCs in response to intracellular pathogens has already been extensively reviewed elsewhere (40).

3.1 DAMPs

DAMPs are endogenous danger signals released by damaged or dying cells to induce an immune response during non-infectious inflammation. Although DAMPs have been proposed to activate local antigen-presenting cells (APCs), it is also possible that these signals promote inflammation by activating ILCs. However, the role of inflammatory signals during tumor development is often not clearly defined as they can also be associated with cancer growth. Here, we discuss

TABLE 1 Expression of DAMPs and cytokine receptors by ILCs.

DAMP/Cytokine	Receptor(s)	NK cells		ILC1		ILC2		ILC3		Reference(s)
		Ms	Hu	Ms	Hu	Ms	Hu	Ms	Hu	
HMGB1	RAGE	Yes	nd	nd	nd	Yes	nd	nd	nd	(42, 43)
	TLR2	Yes	Yes	nd	Yes	Yes	Yes	nd	Yes	(44–48)
	TLR4	Yes	Yes	nd	Yes	Yes	Yes	nd	Yes	(44, 47–49)
ATP	*P2Y$_{1/2/4/6/11-14}$	Yes	Yes	nd	nd	nd	Yes	nd	Yes	(50–53)
	*P2X1-7	Yes	Yes	Yes	nd	nd	nd	nd	Yes	(51, 54–56)
IL-33	ST2	Yes	Yes	nd	nd	Yes	Yes	nd	nd	(57–60)
IL-25	IL-25R	No	No	nd	No	Yes	Yes	No	No	(9, 61)
IL-12	IL-12R	Yes	Yes	Yes	Yes	Yes	Yes	Yes	nd	(36, 62–66)
IL-15	IL-2Rβ	Yes	Yes	Yes	nd	Yes	Yes	Yes	Yes	(20, 67–71)
IL-18	IL-18R	Yes	Yes	Yes	Yes	Yes	Yes	nd	Yes	(62, 71, 72)
IL-23	IL-23R	No	Yes	No	Yes	nd	No	Yes	Yes	(9, 36, 73)

Mouse, Ms; Human, Hu; nd, not determined. *Expression of 1 or more of indicated receptors has been reported.

DAMPs with receptors found on ILCs (Table 1) and their potential role during tumor initiation and activation of ILC responses.

3.1.1 High mobility group box 1

HMGB1 is a nuclear protein widely expressed in mammalian cells and is involved in various cellular processes, including the maintenance of chromosome structure and function, DNA damage repair and transcription. Cells undergoing necrosis passively release HMGB1, while various exogenous and endogenous stimuli can induce the active release of HMGB1 by immune cells, endothelial and epithelial cells (74). Notably, HMGB1 is one of the DAMPs released during immunogenic cell death, which is induced by infectious pathogens and anticancer chemotherapeutics (75). Extracellular HMGB1 acts as a danger signal that mediates inflammation and repair responses *via* binding to the inflammatory receptor advanced glycation end-products (RAGE) and Toll-like receptors (TLRs). These receptors are expressed by various immune cells, including NK cells and ILC2s (Table 1). In established tumors, conflicting roles have been described for HMGB1, including the activation of tumor-promoting inflammatory responses and immunosuppressive pathways, as well as the induction of anti-tumor responses (76). Current evidence supports a pro-tumorigenic role of HMGB1 during tumor development. Studies assessing serum levels in patients with normal tissue, premalignant lesions, early and advanced stages of cancer, showed that HMGB1 levels increase according to the progression of gastric and hepatocellular carcinogenesis (77, 78). This suggests that HMGB1 is released during cellular transformation. However, HMGB1 might promote tumor development rather than activating immune responses against premalignant cells as chemically-induced skin and inflammation-induced liver cancer development was inhibited in mice deficient for the HMGB1 receptor RAGE (79, 80). This is further supported by a study of premalignant and malignant lesions of the uterine cervix, which showed that HMGB1 inhibited maturation of plasmacytoid DCs to render them tolerogenic (81). More studies are needed to understand the complex role of HMGB1 during tumor development.

The impact of HMGB1 expression on ILCs in premalignant lesions has not been assessed yet. However, studies in established tumors support a role of HMGB1 in NK activation. In mice, HMGB1 released from chemotherapy-induced necrotic tumor cells induced NK cell activation and infiltration into the tumor (82). Mouse and human ILC2s were also shown to express RAGE and respond to HMGB1 activation (42, 83). Thus, ILC2s could potentially respond to HMGB1 *via* RAGE or its other receptors (Table 1) in premalignant and malignant lesions, however, if and what effect this has on tumor development is currently unknown.

3.1.2 Adenosine triphosphate

ATP is a multifunctional nucleotide best known for storing and transferring energy in cells. Extracellular ATP is actively secreted by stressed cells or passively released by dead cells, and acts on P2 purinergic receptors (Table 1). Released ATP is enzymatically converted into adenosine by the ectonucleotidases CD39 and CD73, which binds to P1 purinergic receptors. Purigeneric receptors are widely expressed by various immune and non-immune cells. Established tumors are characterized by high concentrations of ATP and adenosine. Adenosine and ectonucleotidases are predominantly associated with tumor-promoting and immunosuppressive activities (84). Extracellular ATP-binding can support or inhibit anti-tumor responses, depending on ATP concentration, the type of receptor, and the target cell (85). The role of extracellular ATP and adenosine in tumor development has not been extensively studied and related studies have provided contradictory results. For example, studies assessing the role of the ATP receptor P2X7R in inflammation-associated CRC models have described an increase as well a reduction of tumor incidence in mice deficient for P2X7R (86, 87). Evidence for the involvement of ATP and adenosine in activating ILC responses was provided in the context of tissue repair and inflammation. Blocking of the ATP receptor P2X1R abrogated cytokine secretion in NK cells and ILC1s and impaired liver regeneration in a model for partial hepatectomy (54). In a chemically induced intestinal injury model, IL-22-secreting ILC3s accumulated in the colon and were important for the control of colitis. Treatment with an ectonucleotidase inhibitor prevented ILC3 activation and IL-22 production by ILC3s. Thus, accumulation of ATP was associated with ILC3 inhibition, while conversion to adenosine lead to activation of ILC3s (88).

3.1.3 IL-33

IL-33 acts as a cytokine and a DAMP, as it's released by epithelial cells, endothelial cells, and fibroblasts in response to tissue damage, as well as actively secreted by APCs. The primary receptor for IL-33 is ST2, which exists in soluble form as a decoy receptor, and as part of a membrane-bound heterodimer together with the co-receptor IL-1 receptor accessory protein (IL1RAP) that initiates downstream signaling (89). IL-33 activates ST2-expressing mast cells, eosinophils, macrophages, ILC2s, NK cells, and T cell subsets, such as Th1, Th2, $CD8^+$ T cells, and Tregs (90) (Table 1), thereby modulating both innate and adaptive immune responses. IL-12-induced IFN-γ production by murine and human NK cells is enhanced by IL-33 (57, 58) and as a central regulator of type 2 immunity, IL-33 mediates ILC2 activation and proliferation (9). IL-33 has a dual role in established cancer and has been associated with both anti-tumor and pro-tumor immune responses (91). Anti-tumor functions are mostly attributed to the induction of type 1 immune responses and pro-tumor activities include the activation of Tregs and type 2 responses. The role of IL-33 in

tumor development has been mostly studied in the context of CRC and current data suggests that IL-33 can contribute to the pathogenesis (92, 93) as well as the suppression of CRC development (94). IHC analysis of precancerous colorectal lesions also revealed that precancerous epithelial cells, as well as stromal and endothelial cells can be a source of IL-33 (95). It remains to be elucidated how IL-33 contributes to the described pro- or anti-tumorigenic functions of ILC2s in CRC and other cancers, and if the cytokine milieu in these tissues allows for IL-33-mediated enhancement of NK cell responses.

3.1.4 IL-25

Like IL-33, IL-25, also known as IL-17E, functions as a cytokine and a DAMP. IL-25 signals through the IL-25R, a heterodimer complex composed of IL-17RB and IL-17RA, and is produced by epithelial cells and immune cells including activated Th2 cells, mast cells, and eosinophils. Expression of IL-25 is regulated by harmful environmental cues and plays an important role in activating Th2 immune responses. Dysregulated IL-25 expression has been linked to airway inflammation and severe asthma exacerbation (96). IL-25 has also been shown to promote inflammatory responses in the context of colitis (97), suggesting that it might favor tumor development. However, pro- and anti-tumorigenic functions have been described for IL-25. A study by Thelen et al. found that blocking of IL-25 in a colitis-driven colon cancer model, leads to increased tumor burden and a decrease of eosinophils in colon tissues (98). Conversely, Jou et al. found that IL-25 treatment of *Apc*$^{1322T/+}$ mice, an APC-mutation-driven CRC mouse model, resulted in an increased tumor burden, which was accompanied by increased ILC2 infiltration (24). In this model, ILC2 indirectly suppressed anti-tumor T cell responses by activating MDSCs *via* IL-4 and IL-13. Genetic ablation of ILC2s or IL-25, or treatment with IL-25 blocking antibodies in these mice led to reduced tumor growth and increased survival.

3.2 Cytokine-mediated ILC activation

Cytokines are small soluble proteins that are crucial for immune cell homeostasis and the regulation of innate and adaptive immune responses. Proinflammatory cytokines are released in response to cellular stress and infection to alert the immune system to the presence of potential danger. Transformed cells are known to secrete and promote production of diverse cytokines in different types and stages of cancers (99). Furthermore, DCs and macrophages that are activated in response to cellular stress also start expressing proinflammatory cytokines and contribute to the local cytokine milieu. Studies of murine and human tissues found that there is a reduction of proinflammatory cytokines when premalignant lesions or early tumors progress to clinically apparent tumors (100–102) and an increase of immunosuppressive cytokines (103). In addition, aberrant release of proinflammatory cytokines contributes to tumor progression and immune cell dysfunction. Thus, the cytokines present in premalignant tissues will shape local immune responses, including ILC activity. Common cytokines associated with ILC activation are IL-12, IL-15 and IL-18 for ILC1s and NK cells; IL-2, IL-18, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) for ILC2s; and IL-1ß and IL-23 for ILC3s (104). Here, we discuss the role of ILC-activating cytokines in the context of tumor development.

3.2.1 IL-12

The heterodimeric pro-inflammatory cytokine IL-12 is known for its role in activating anti-tumor immunity (105). IL-12 is produced by APCs, such as DCs and macrophages, and induces Th1 differentiation and the production of IFN-γ in T and NK cells (106). In addition, IL-12 negatively regulates Treg cell function and proliferation (107, 108), as well as Th2 and Th17 differentiation (106). The lack of IL-12 subunits p35 or p40 results in increased or earlier tumor development in mice (109–112). These studies highlight the importance of IL-12 in regulating early immune responses against transformed cells. In addition, various IL-12 gene polymorphisms leading to decreased IL-12 production are associated with increased susceptibility to cancer (113). Besides NK cells, ILC1 also respond to IL-12 stimulation and IL-12 promotes conversion of ILC2s and ILC3s to IFN-γ-producing ILC1s (114).

3.2.2 IL-15

IL-15 is a proinflammatory cytokine crucial for the proliferation and survival of T cells and NK cells (115). Lack of IL-15 in mice results in severe reduction of both cell types (116). IL-15 mainly exists as a heterodimeric complex with membrane bound or the soluble form (sIL-15) of IL-15Rα, and binds to the IL-2Rβγ heterodimer on nearby effector cells. Cellular sources of IL-15 include monocytes, macrophages, DCs, stromal cells, and epithelial cells (115). Various murine tumor cell lines have also been shown to express IL-15 (102). IL-15 enhances anti-tumor responses of murine and human CD8$^+$ T cells and NK cells (117–120), and is considered a promising agent for cancer immunotherapy (121). In NK cells, IL-15 treatment leads to upregulated expression of NKG2D and the cytotoxic effector molecules TRAIL and perforin (122). In transplanted and spontaneous tumor models, IL-15-deficiency and the subsequent reduction in T and NK cell numbers leads to accelerated tumor development (102, 123–125), suggesting that IL-15 plays a critical role during early anti-tumor responses. Moreover, deletion of IL-15 in CRC patients was associated with a higher risk of relapse and reduced disease-free survival (126). Besides NK cells, mouse and human helper ILC1s have also been shown to respond to IL-15 (20, 67, 127–129). Other ILC populations may also get activated in response to IL-15 in

early tumors as IL-15 has been shown to induce conversion of ILC3s into IFN-γ-producing ILC1s and cytotoxic NK cells (114, 130).

3.2.3 IL-18

IL-18, originally termed IFN-γ-inducing factor, is part of the IL-1 family. Binding of IL-18 to its receptor, which consists of IL-18Rα and IL-18Rβ, can be prevented by the soluble IL-18 binding protein (IL-18BP). IL-18 is expressed by various types of cells, including macrophages, DCs, and epithelial cells (131). Together with IL-12, IL-18 induces Th1 responses by acting on T cells and NK cells to induce IFN-γ production. Treatment with IL-18 also enhances Fas-L-expression and FAS-L-mediated cytotoxicity in NK cells and $CD8^+$ T cells (132, 133). In patients with cervical premalignant lesions, low expression of IL-18 was associated with an increased risk of progression of pre-neoplastic lesions to cancer (134), supporting its role in activating immune responses against transformed cells.

3.2.4 IL-23

IL-23 is an IL-12 family member and a heterodimer that consists of a p19 and a p40 subunit, which is shared with IL-12. The IL-23 receptor is made up by IL-23R and IL-12Rβ1 subunits. The main sources for IL-23 are macrophages and DCs, which release IL-23 in response to exogenous or endogenous signals associated with host defense and wound healing (135). IL-23 plays a crucial role in the differentiation and maintenance of Th17 cells, and promotes Th17 production of IL-17A, IL-17F, IL-6, IL-22, and TNF-α. IL-23 is also one of the main mediators of ILC3 activation, resulting in their constitutive secretion of IL-22, which in turn acts on mucosal epithelium to induce the expression of antimicrobial peptides, tight-junctions and promote the colonization of beneficial commensal bacteria protecting against intestinal inflammation (136). The role of IL-23 in cancer is complex and has been associated with tumor-promoting and tumor-suppressive activities (135). Its role in tumor development is not well understood. In a model for MCA-induced fibrosarcomas, tumor incidence was reduced in mice deficient for the IL-23 subunit p19 and depletion of NK cells, but not $CD8^+$ T cells, abrogated the protective effect of IL-23 depletion (137). Conflicting roles were described for IL-23 in the development of chemically-induced cutaneous tumors, as tumor growth was either inhibited (112) or enhanced (138) in p19-deficient mice, depending on the background strain. A study of murine and human premalignant lesions for head and neck squamous cell carcinoma reported elevated levels of IL-2, IFN-γ, TNF-α, IL-6, and IL-17 in premalignant lesions, which was dependent on IL-23 and accompanied by an increase in IFN-γ^+ $CD4^+$ T cells (100, 139). In IL-23R KO mice, production of these cytokines was reduced and the progression of premalignant oral lesions toward cancer accelerated (139), suggesting that IL-23 has a protective role during tumor development. Although IL-23–driven immune responses have been primarily linked to T cells, IL-23 could potentially activate ILC3 in premalignant lesions as well.

3.3 Cell surface receptors and molecules

Unlike T and B cells, ILCs do not express antigen receptors and therefore do not recognize specific tumor antigens. However, ILCs express other activating cell surface receptors that initiate anti-tumor responses (37). NK cell activity is regulated by a balance between various activating and inhibitory receptors that bind to cognate ligands on target cells (140, 141). Healthy cells express MHCI molecules on their surface that act as inhibitory ligands for inhibitory receptors on NK cells, such as killer cell immunoglobulin-like receptors (KIRs) and the CD94/NKG2A heterodimer, thereby contributing to tolerance from NK cell recognition (142). Other central activating and co-activating NK cell receptors include the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46, CD16, NKG2D, NKG2C, DNAX Accessory Molecule-1 (DNAM-1), and 2B4 (142, 143). NK cell activating ligands are often upregulated in response to cellular stress associated with infection and malignant transformation (144).

The NKG2D receptor recognizes several MHCI-like ligands, including MHCI-polypeptide-related sequence MICA, MICB, and UL16 binding proteins (ULBP1-6) in humans, and retinoic acid early inducible-1 family (RAE-1α-ϵ), H60a-c, and murine UL16 binding protein-like transcript (MULT-1) in mice (145). Homodimerization of NKG2D by membrane-expressed ligands recruits phosphatidylinositol 3-kinase (PI3K) and growth factor receptor-bound protein 2 (GRB2), resulting in a phosphorylation cascade. If then, the overall balance of signaling from both activating and inhibitory receptors favors NK cell activation, it can stimulate NK cell effector functions resulting in perforin/granzyme-mediated cytotoxicity and cytokine release. NKG2D is considered an important receptor in NK cell immune surveillance of cancer since spontaneous tumor development was shown to be more frequent in NKG2D-deficient mice compared to wild type mice (146). Cell surface expression of NKG2D ligands is low or not present on healthy tissues, but is upregulated on rapidly proliferating cells, virally infected cells, and cancer cells (147–150). Ectopic expression of NKG2D ligands in tumor cell lines results in tumor cell rejection in mice (151, 152). However, only a few studies have examined the expressionof these ligands in premalignant tissue. In mouse models for cutaneous carcinogenesis, exposure to carcinogens induces

the expression of NKG2D ligands in skin cells (153–155). NK cell depletion in one of these studies resulted in higher numbers of papillomas (153), suggesting that NK cells play an important role in the elimination of DNA-damaged skin cells. Notably, recruitment of NK cells to the epidermis was dependent on TNF-α-induced chemokines CCL2 and CXCL10. In humans, premalignant skin lesions lacked expression of MICA (156) and low expression of MICA, MICB, and ULBP1 is found on thymic hyperplasia (157). Further studies are needed to understand the role of NKG2D ligands during tumor development in humans.

The activating receptor DNAM-1 is expressed by many lymphocyte subsets, including NK cells and T cells. Binding of DNAM-1 (CD226) to its ligands PVR (CD155) and Nectin-2 (CD112) induces NK cell cytotoxicity (158). These ligands are highly expressed on tumor cells, but only low or no expression is found on healthy tissues. Lack of DNAM-1 expression results in reduced T and NK cell cytotoxicity against tumor cells and accelerated tumor outgrowth of chemically-induced fibrosarcomas (159) as well as spontaneous tumors (160, 161). PVR and Nectin-2 overexpression was observed in human premalignant lesions of CRC and pancreatic ductal adenocarcinoma, respectively (162, 163). Together, these studies provide evidence for a role of DNAM-1 in tumor immune surveillance, which likely not only involves NK cell but also T cell activation. Notably, DNAM-1 is also expressed by human peripheral blood ILC2s (164) and murine liver ILC1s (55). DNAM-1-mediated ILC1 activation was critical for their activation and production of IFN-γ. Thus, DNAM-1-ligands expressed on premalignant and malignant tissues may also activate DNAM-1-expressing non-NK cell ILCs.

NCR receptors were originally identified based on their ability to mediate cytotoxic functions of NK cells. The three known NCRs, NKp46, NKp44, and NKp30, comprise a family of type I transmembrane (TM) receptors and are encoded by the genes, *NCR1, NCR2*, and *NCR3*, respectively (165). Originally, these receptors were thought to be NK cell specific surface molecules, but many studies have provided evidence for expression on other cell types, including a subset of T cells, ILC1s and ILC3s (166–168). In the context of cancer, NCRs bind to a broad range of soluble, membrane-bound and nuclear ligands, including B7H6, platelet-derived growth factor (PDGF)-DD, and Galectin-3. However, the full spectrum of NCR ligands and their role in cancer remains to be fully characterized. Studies have shown that NKp46 is required for expression of the apoptosis-inducing ligand TRAIL on NK cells and ILC1s in mice, and genetic deficiency of NKp46 impairs tumor clearance (169–174), thereby implicating a role for NKp46-mediated activation of NK cells and ILC1s in tumor immunosurveillance. In addition, expression of the NKp30 ligands B7H6 and BAT3 by tumor cells was shown to trigger NK-cell cytotoxicity and cytokine secretion (175–177). The expression of NKp46 and NKp30 in human precancerous lesions is variable. NKp46 ligands were shown to be expressed on human benign and malignant melanocytic lesions (178), but NKp46 and NKp30 ligands were only found on primary human prostate tumors and not benign prostate hyperplasia (179). B7H6 was expressed in high-grade but not low-grade cervical lesions (180). Thus, it remains to be determined during which stage of tumor development NCR ligands mediate NK cell responses against tissue changes associated with malignant transformation.

In addition, expression of NKp30 and NKp44 was also reported on tumor-associated ILC2s and ILC3s, respectively (167, 181). In these studies, ILC3s were shown to interact with tumor cells and tumor-associated fibroblasts *via* NKp44, and were associated with a protective role against cancer, whereas $NKp30^{+}$ ILC2s interacted with tumors cells *via* B7H6 and promoted an immunosuppressive tumor microenvironment. Further studies are needed to decipher the role of NCRs on helper ILCs during early tumor immunosurveillance.

4 Perspectives

Despite the growing body of research on ILCs, there is still a lot we do not understand about how the responses of these primarily tissue-resident cells are shaped by disease- and tissue-specific signals. This incomplete knowledge is reflected by research studies describing conflicting roles for ILCs in inflammation, immunopathological conditions, and cancer. This review specifically highlights a gap in our understanding of the role of ILCs in immunosurveillance and carcinogenesis. Our current knowledge on ILCs in cancer is mostly based on studies in established tumors. However, early tumors and premalignant tissues are characterized by a different tissue environment than established tumors. This has a profound impact on ILC responses as these cells sense a large variety of tissue signals, which modulate their phenotype and function. Signals that could potentially activate ILC-responses at the pretumor stage are highlighted in this review and could serve as a starting point for future studies. In particular, studies in premalignant tissues of patients are needed to improve our understanding of the precancerous tissue microenvironment and the early immune responses against malignant transformation. It also remains to be determined if and to what extent signals found in the established tumor microenvironment, such as lactic acid and hypoxia (182, 183), shape ILC functions in precancerous lesions. A better understanding of ILC responses in early tumor development will also provide novel insights regarding the overall regulation of ILC responses in response to cellular stress.

Author contributions

Conceptualization, data curation, writing—original draft preparation, KW; Figure design, KW; writing – reviewing and editing, KW, MG, DC, NJ, and PO; Supervision, project administration, funding acquisition, PO. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

Publisher's note

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Underlying mechanisms of evasion from NK cells as rationale for improvement of NK cell-based immunotherapies

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Natural killer (NK) cells belong to the family of innate immune cells with the capacity to recognize and kill tumor cells. Different phenotypes and functional properties of NK cells have been described in tumor patients, which could be shaped by the tumor microenvironment. The discovery of HLA class I-specific inhibitory receptors controlling NK cell activity paved the way to the fundamental concept of modulating immune responses that are regulated by an array of inhibitory receptors, and emphasized the importance to explore the potential of NK cells in cancer therapy. Although a whole range of NK cell-based approaches are currently being developed, there are still major challenges that need to be overcome for improved efficacy of these therapies. These include escape of tumor cells from NK cell recognition due to their expression of inhibitory molecules, immune suppressive signals of NK cells, reduced NK cell infiltration of tumors, an immune suppressive micromilieu and limited *in vivo* persistence of NK cells. Therefore, this review provides an overview about the NK cell biology, alterations of NK cell activities, changes in tumor cells and the tumor microenvironment contributing to immune escape or immune surveillance by NK cells and their underlying molecular mechanisms as well as the current status and novel aspects of NK cell-based therapeutic strategies including their genetic engineering and their combination with conventional treatment options to overcome tumor-mediated evasion strategies and improve therapy efficacy.

KEYWORDS

NK cells, immune escape, immunotherapy, tumor, HLA

General features of NK cells

Natural killer (NK) cells are cytotoxic innate immune cells that were first described in 1973 by E. Klein and colleagues (1). They originate from multipotent hematopoietic stem cells (HSC) in the bone marrow (BM) and undergo different developmental stages gradually acquiring the expression of distinct surface markers defining the commitment to the lymphoid/NK cell lineage. Maturation of human NK cells is characterized by a loss of CD34 and c-KITC (CD117) expression followed by a sequential upregulation of CD94, CD16 and killer cell immunoglobulin-like receptors (KIRs) (2). NK cells comprise 5-10% of peripheral blood mononuclear cells (PBMCs), but they are also found with a variable frequency in various lymphoid and non-lymphoid tissues including BM, liver, lung, skin, kidney and spleen (3). NK cells have the capacity to form cytoplasmic lytic granules containing perforin and granzymes and produce a large number of cytokines, in particular interferon (IFN)-γ, but also proinflammatory and immune suppressive cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-10, chemokines and various growth factors like granulocyte-macrophage stimulatory factor (GM-CSF), granulocyte-stimulating factor (G-CSF) and IL-3. They exert their cytotoxic activity by distinct mechanisms, including the release of granzymes and perforin, secretion of IFN-γ and TNF-α, the expression of the FasL/Fas or TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptors and the antibody-dependent cell-mediated cytotoxicity (ADCC) *via* Fc receptors (CD16) recognizing antibodies bound to antigen-coated (tumor) cells (4–6). Based on their cytolytic function, NK cells play a key role in the first line of immune defense and are able to directly eliminate tumor or pathogen-infected cells. In this context it is noteworthy that NK cells have safety features, rarely elicit autoimmunity and promote immune homeostasis.

NK cells arise and progressively evolve from a limited diversity to highly differentiated and heterogeneous phenotypes, which are dictated by genetic factors and environmental stimuli, such as pathogen exposure, leading to distinct functions (7). In PBMCs, NK cells are generally subdivided into two major subsets based on their differential expression of CD56: (i) $CD56^{bright}$, $CD94^{+}$, $CD16^{-}$ NK cells, which are less abundant in PBMCs, are poorly cytotoxic, but produce high amounts of IL-1β, IFN-γ, IL-2, IL-12, IL-15, IL-18 and TNF-α upon stimulation, extensively proliferate in response to DC-derived cytokines and can extravasate from the circulation into tissues and (ii) $CD56^{dim}$, $CD16^{+}$ and KIR^{+} NK cells, which have a low proliferative capacity, but high cytotoxic activity accounting for most of the circulating NK cells (8, 9). Furthermore, terminally differentiated $CD57^{+}$ and adaptive $NKG2C^{+}CD57^{+}$ NK cells exist (10). Also, the discovery of memory-like NK cells being able to mount a robust secondary immune response upon activation has expanded the understanding of this innate immune cell population over the past decade (11, 12).

With the possibility of the in depth characterization of immune cell subpopulations by high-dimensional transcriptional and phenotypic profiling using (single cell) RNA-sequencing (RNA-seq) and mass cytometry an unexpected NK cell diversity was identified across different organs within individual donors regarding their function, maturation and interaction with stromal cells, which also provide a new framework for the analyses of NK cell responses under physiologic and pathophysiologic conditions (13–17). Interestingly, the diversity of NK cells was found both in the immune cell infiltrate of tissues and in peripheral blood (17, 18).

NK cell receptors and NK cell activity

NK cells are tightly regulated by a dynamic balance of transduced signals mediated by the physical interaction with adjacent cells. They express a number of germline-encoded activating and inhibitory receptors as well as cytokine and chemokine receptors on the cell surface, which influence the NK cell function, but knowledge of how these receptors convey signals and affect NK cell biology is still limited. There is evidence of a balance between activating and inhibitory receptors, which control the activity, cell diversity and function of NK cells (19, 20). These constitutively expressed NK cell receptors comprise non-HLA-specific receptors, HLA-specific receptors and homing receptors (20), and recognize their corresponding ligands expressed on the cell surface of target cells such as tumor cells or virus-infected cells (21), as summarized in Table 1.

Next to CD16 (FcγRIIIA), which interacts with Fc fragments of several IgG subclasses, triggering the ADCC (24), the natural cytotoxicity receptors (NCR) NKp30 (CD337), NKp44 (CD336), NKp46 (CD335), NKp80, DNAM-1 (CD226) and NKG2D (CD314) are the major activating receptors and are able to recognize induced self-ligands that are downregulated on healthy cells and highly expressed on tumor cells (25). There are a number of HLA class I-specific activating NK cell receptors (NKR) that recognize the non-classical HLA class I antigens HLA-E and HLA-F or epitopes shared by distinct HLA class I allotypes. For the activating receptor KIR2DS3, the ligand is still unknown. Other NK cell activating receptors include SLAMs, CD18, CD2 and the toll-like receptor (TLR) 3/9 (26, 27).

The primary inhibitory receptors on the cell surface of NK cells represent members of the killer cell immunoglobulin-like receptor (KIR) family, which consists of 14 polymorphic receptors. The different inhibitory KIRs can recognize classical HLA class I antigens, but for KIR2DL5 no ligand has yet been

TABLE 1 Major NK cell receptors and their ligands.

A: Activating NK cell receptors and their ligands

NK cell receptor type	Name	Ligands
non-HLA	NKp30 (CD337)	B7-H6, BHG6/BAT3, galectin
	NKp44 (CD336)	MLL5-Nidogen-1, PDGF-DD, PCNA
	NKp46 (CD335)	viral HA and HN, properdin
	NKG2D (CD314)	MICA, MICB, ULBPs
	FcyRIII (CD16)	IgG
	TLR3/9	microbial constituents, CpGs
	CD2	CD58
	α-integrin	vascular endothelial growth factor
	DNAM1 (CD226)	nectin2 (CD112), PVR (CD155)
	2B4	CD48
HLA I	KIR3DS2	HLA-C C1, HLA-A* 11:01
	CD94/NKG2C	HLA-E
	KIR2DS4	HLA-F, HLA-C, HLA-A* 11
	KIR2DS5	HLA-C C2?
	KIR3DS1	HLA-B* 51, HLA-F
	2B4	CD48
	KIR3DS1 (CD158b)	HLA-C2
	KIR2DL4 (CD158d)	HLA-G

B: Inhibitory NK cell receptors and their ligands

NK cell receptor type	Name	Ligands
HLA I	NKG2A (CD159a/CD94)	HLA-E
	KIR2DL1, DL2, DL3 (CD158a,b)	HLA-C, HLA-B
	KIR3DL1, DL2 (CD158e,k)	HLA-A, -B or -F
	ILT2/LIR-1 (CD85J)	HLA-G, different HLA class I allotypes
	LAG-3	MHC class II
non-HLA	TIM-3	galectin-9, HMGB1, CEACAM1
	PD-1 (CD279)	PD-L1, -L2, CD273
	TIGIT	PVR (CD155, CD274), nectin2 (CD112), nectin4, CD113
	Siglec 7 (CD328)	ganglioside DSGb5
	LAIR-1	collagen

The major NK cell receptors and their ligands are summarized as recently reviewed (21–23).

identified. Other inhibitory receptors include NKG2A, a member of the C-type lectin family, which heterodimerizes with CD94 and binds to the HLA-E antigen, the immunoglobulin-like receptor superfamily B member 1 (LILRB1, ILT2, CD85j), the T cell immunoglobulin and mucin domain containing molecule 3 (TIM-3), T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (TIGIT) (28–30), CD161, SIGLEC7, SIGLEC9, programed death receptor 1 (PD-1) and lymphocyte-activation gene (LAG-3) (31). These inhibitory receptors regulate the activation status and anti-tumoral immunity of NK cells by suppressing effector functions and augmenting Treg activity (32).

MHC class I molecules are ligands of the inhibitory receptors of NK cells thereby providing signals to self-tolerance resulting in NK cell inactivation and the discrimination between healthy, "self" and "non-self" cells including tumor or virus-infected cells. However, tumor or pathogen-infected cells often lack or downregulate MHC class I surface antigens, which results in an escape from recognition by $CD8^+$ cytotoxic T lymphocytes (CTL). In contrast, these MHC class I-negative cells could be recognized and eliminated by NK cells *via* the missing self-mechanism ("missing-self recognition"). However, the NK cell activation requires additional signals to induce self changes, e.g. by virus-encoded ligands or ligands upregulated by cellular stress, by DNA damage and alterations of suppressor genes (33) leading to the so-called "induced self-recognition" (34). Activated NK cells can eliminate target cells either directly *via* NK cell-mediated cytotoxicity or indirectly *via* proinflammatory cytokine-mediated killing by TNF-α and IFN-γ. In addition to

the interaction with tumor and pathogen-infected cells, NK cells could crosstalk with other immune cells, like macrophages, T lymphocytes and different dendritic cell (DC) subpopulations (35, 36). Over the last decade, the functional links between NK cells and myeloid cells have been broadly analyzed. This cooperative interaction triggers the innate and adaptive immune responses by stimulating the survival, maturation and tumor infiltration of DCs leading to "DC editing" (37–39). Vice versa, macrophages could shape NK cell differentiation and function (40).

NK cells as critical players for tumor immune surveillance

The primary role of NK cells is the recognition and elimination of tumor cells or virus-/pathogen-infected cells as the first line of defense against initiation of tumor formation and pathogen invasion without prior sensitization (41). Evidence for this hypothesis is an increased tumor incidence in human and experimental models with impaired NK cell function (42). NK cells are educated and licensed by inhibitory receptors that recognize classical MHC class I molecules, but could recognize MHC class I-deficient cells, which are then eliminated (43). Thus, NK cells are activated by tumor cells due to the decreased expression of MHC class I on tumor cells through the lack of inhibitory signals and by the induction of activating NK cell receptor ligands through their "missing-self" program (44) leading to productive cytotoxic responses. An additional major pathway involved in NK cell-mediated cytotoxicity is the FasL/Fas interaction, which provides a death signal to target cells leading to apoptosis. The activating receptor NKG2D on NK cells recognize the MHC class I-related surface proteins MICA and MICB as well as the UL-16-binding proteins (ULBPs; ULBP1-6), which are often upregulated in e.g. tumor cells countermanding any inhibitory signals and inducing NK cell-mediated cytotoxicity (45, 46).

Composition of the tumor microenvironment and NK cells

Detailed analysis of the tumor microenvironment (TME) in different cancer types demonstrated a complex network of immune effector cells, such as CTL and NK cells, but also immune suppressive cells, like regulatory T cells (Tregs), tumor-associated macrophages (TAMs), regulatory γδ T cells, myeloid-derived suppressor cells (MDSCs), soluble factors, extracellular matrix (ECM) components as well as suppressive molecules expressed on tumor cells. The interaction between the different immune cell subpopulations in the TME with tumor cells is diverse and orchestrated by the presence of specific chemokines and cytokines recruiting different immune suppressive cells into the TME and modulating immune effector cells, which is associated with tumor progression (47). This complex interplay is also shaped by changes in the metabolic activity of immune, stromal and tumor cells (48).

The distribution of NK cells is highly dynamic. Circulating NK cells can migrate into tissues *via* the expression of a broad number of receptors that control this recruitment (31). In different tissues, NK cells display specific phenotypic and functional features, which are altered by the physiologic and pathophysiologic micro-milieu. To reach the solid tumors, NK cells extravasate from the blood and traverse the ECM and the tumor stroma. In the tumor bed, NK cells are able to control tumor growth and metastasis (49). However, NK cell responsiveness is often reduced by the immune suppressive TME.

Although NK cells have been demonstrated to infiltrate into primary solid tumors, metastases and even into tumor-draining lymph nodes, the frequencies of NK cells in solid tumors were lower when compared to adjacent tissues and less abundant regarding the numbers of $CD4^+$ and $CD8^+$ T cells and B lymphocytes. The degree of NK cell infiltration in tumors is influenced by several factors (50), such as tumor localization, nature of cancer cells and expression of chemokine receptors/chemokines (51). In addition, NK cells recruited to the tumor core had a reduced cytotoxic potential compared to NK cells from normal tissues and are often associated with an unfavorable condition for survival (52, 53). The clinical relevance of tumor-infiltrating NK cells, e.g. their correlation with the patients' survival, depends on the expression of ligands for their receptors and is accompanied by a high variability of the different NK cell populations in distinct tumor entities (54). For example, NK cell frequencies are associated with an altered patients' survival in many tumor entities. NK cells highly infiltrating renal cell carcinoma (RCC) were dysfunctional in *ex vivo* cultures (14, 55, 56) (Table 2) and showed an increased expression of inhibitory receptors and a downregulation of activating receptors. Furthermore, low numbers of NK cells in head and neck squamous cell carcinoma (HNSCC) were associated with insufficient tumor elimination, while higher numbers of NK cells at the tumor site correlated with an increased patients' survival. Comparable results were also shown for colorectal carcinoma (CRC), gastric and esophageal cancer. Concerning non-small cell lung cancer (NSCLC), NK cells are less frequent in tumor tissues compared to normal lung epithelium, overexpress NK cell inhibitory receptors and show a $CD56^{bright}$ $perforin^{low}$ phenotype. The number of NK cells in NSCLC is of clinical relevance and linked to the tumor size, smoking history and a bad patients' prognosis (31, 68).

Bioinformatics of large RNA-seq datasets from The Cancer Genome Atlas (TCGA) revealed not only a link between NK cell numbers and patients' survival (31), but also identified a NK cell signature of 13 genes, which makes it possible to determine NK cell abundance across different tumor types and

TABLE 2 NK cell infiltration and its prognostic value.

Tumor	Method	NK cell identification	Tumor tissue	Infiltration tumor vs. metastasis/ normal cells	Prognostic factor/ survival	Reference
breast cancer	FC	$CD3^-$ $CD56^+$	primary	down	OS	(57, 58)
colorectal carcinoma	IHC/FC	$NKp46^+$	primary/ metastasis	down	OS, DFS	(59)
gastric and colorectal cancers	FC	$CD3^-$ $CD56^+$	metastasis	down	OS	(60)
endometrial cancer	FC/IHC	$CD3^-$ $CD56^+$	primary	down	DFS	(54, 61)
esophageal cancer	FC	$CD3^-$ $CD56^+$	primary	down		(62)
gastric cancer	FC	$CD3^-$ $CD56^{dim}$ $CD57^+$	primary	down	OS	(62)
melanoma	FC	$CD3^-$ $CD56^{dim}$	lymph node	up		(63)
non-small-cell lung carcinoma	FC	$CD3^-$ $CD56^+$	primary	down	tumor size, OS	(50, 64, 65)
renal cell carcinoma	FC/IHC	$CD3^-$ $CD56^+$ NKp46	primary	up	metastasis different, OS	(66)
several	FC	$CD3^-$ $CD56^+$	primary	diverse		(50)
lung adenocarcinoma	IHC	$CD57^+$	primary	down	OS	(67)

DFS, disease free survival; FC, flow cytometry; IHC, immunohistochemistry; OS, overall survival.

offers novel opportunities for NK cell-based treatment in specific cancer conditions (69, 70). Thus, strategies that increase the recruitment and activation of NK cells in tumors would be a suitable approach to enhance anti-tumor efficacy (71).

Impaired NK cell functions due to intrinsic mechanisms

Studies of different tumor entities demonstrated that the function of intra-tumoral NK cells is impaired, which might be due to aging, genetic defects and chronic infections (72–74), but also due to continuous exposure to tumor antigens (75–77). Tumor escape from NK cell-mediated immune surveillance could be due to impaired anti-tumor NK effector mechanisms, such as reduced production of proinflammatory cytokines, e.g. IFN-γ and TNF-α, proliferation and cytotoxicity due to a diminished expression of effector molecules, like perforin and granzymes. Various solid and hematopoietic cancers demonstrated a downregulation of the activating receptors NKp30, NKG2D, NKp46 and CD16 and an increase of soluble NKG2D ligands sMICA/B shed from the tumor cell surface, but high expression levels of the inhibitory receptor CD94/NKG2A, resulting in impaired NK cell cytotoxicity (78). This was associated with a poor prognosis of patients with breast cancer, chronic lymphocytic leukemia (CLL), ovarian cancer and acute myeloid leukemia (AML) (79). The presence of NK cells in the TME and higher expression levels of CD56, CD57, NKp30 or NKp46 at the tumor site were associated with a favorable patients' prognosis, while low NK cell numbers correlated with an increased risk of cancer recurrence after resection, and a reduced patients' survival (80). In NSCLC, overexpression of inhibitory NK cell receptors and a reduced number of NK cells was associated with a poor patients' outcome (64, 81) which was accompanied by a reduced cytotoxicity and promotion of tumor evasion. Next to the distinct expression pattern of NK cell receptors, the programmed death receptor PD1 has been well characterized as an exhaustion marker for T cells, but also for NK cells (82). The same applies to TIGIT, which is also associated with NK cell exhaustion (83). It is noteworthy that actin cytoskeleton remodeling and fragmented mitochondria in the cytoplasm of tumor-infiltrating NK cells can also lead to immune suppression (84, 85).

Impaired NK cell function due to extrinsic mechanisms

It is generally accepted that the TME shapes the innate as well as the adaptive immune responses, which are variable between distinct tumor types due to differences in the composition of infiltrating immune cells and soluble constituents. It is noteworthy that the critical function of NK cells to induce an effective anti-tumor immunity is a successful interaction between NK cells and DCs, and the production of chemokines. Both processes are negatively influenced by unique locoregional characteristics, in particular cellular and soluble components of the TME, which are associated with an immune escape due to a lack effector responses thereby promoting tumor cell metastasis (75–77). The chemokine milieu in the TME consists of reduced expression of CXCL2, CX_3CL1, CXCL1

and CXCL8 thereby attracting $CD56^{dim}$ NK cells and an increased CXCL9/10, CCL5 and CXCL19/21 expression driving the homing of $CD56^{bright}$ NK cells toward the stromal compartment (86).

Solid tumors often showed a high oxygen consumption, a low pH in the TME due to higher concentrations of lactate and a disorganized vascularization leading to hypoxia as well as an altered expression of genes involved in the regulation of metabolic processes. An acidic microenvironment (87) and a permanent or transient hypoxia leading to an upregulation of the transcription factor HIF-1α (88) due to the restricted access to nutrients and oxygen mediated by changes in the vascularization have been demonstrated to downregulate the expression of activating NCRs, reduce NK cell cytotoxicity and survival, which downregulates NK cell anti-tumor responses (89). This could be reverted by e.g. the treatment with an inhibitor of HIF-1α (89). In addition, NK cells may not penetrate into solid tumors including in low MHC class I-expressing tumors or once within the tumors become anergic or exhausted. Increased H_2O_2 levels lead to a decrease in the infiltration of $CD56^{dim}$ NK cells and impaired ADCC. Furthermore, NK cells in tumors can also acquire proangiogenic functions by secretion of vascular endothelial growth factor (VEGF), angiogenin and matrix metalloproteinases (MMPs) (90, 91). Although a proangiogenic NK cell phenotype has been identified, the potential of proangiogenic NK cell-driving tumor progression has not yet been analyzed in detail. However, tumor endothelium might improve NK cell recruitment to the tumor site as an indirect mechanism of targeting myeloid cells affecting NK cell recruitment and function (92).

Several immune suppressive cells, like MDSC, TAMs and Tregs negatively interfere with NK cell activation. This has been attributed to immune modulatory molecules present in the TME, such as indolamine 2, 3-deoxygenase (IDO) activity and transforming growth factor (TGF)-β, which can be secreted by MDSC, Tregs and anti-inflammatory macrophages. Additionally, IL-1β secreted by 6-sulfo LacNAc DCs induces cell apoptosis (93), while Tregs could also suppress NK cells by deprivation of IL-2 (94). Several other factors produced by tumor or tumor-associated cells, like prostaglandin E2, extracellular adenosine, IL-10 and IL-6, further directly or indirectly prevent NK cell activation (95). During infection and tumorigenesis, macrophages can modulate NK cell function by direct cell-to-cell contact or due to secretion of the cytokines IL-18, IL-12 and TGF-β (96). TGF-β modulates NK cell function *via* a decrease of NKG2D levels and CD16-mediated ADCC in tumors by impairing the cytotoxic potential as demonstrated in *in vivo* and *in vitro* co-culture experiments. In addition, TGF-β affects the expression of chemokine receptors thereby preventing NK cell recruitment as well as the NK cell metabolism by inducing a reduced glycolysis and oxidative phosphorylation that inhibits NK cell effector function. NK cell dysfunction has been associated with the inactivation of the glycogen synthase kinase-3 (GSK3). In contrast, IL-15 is chemotactic for NK cells and maintains NK cell activation by suppressing tumor escape mechanisms (97). However, sustained persistence of IL-15 in the TME could induce the expression of the cytokine-inducible SH2-containing protein, an IL-15 inducible IL-15 signaling inhibitor, leading to the degradation of IL-15R. This is associated with a diminished responsiveness of NK cells to IL-15 (98).

Strategies of tumor cells evading NK cell recognition

As described above, NK cells preferably recognize and kill malignant cells. But there exist many different strategies of tumors to directly evade NK cell recognition. On the one hand, these include the prevention of NK cell recruitment into tumors by physical barriers (laminin and collagen) of tumors or by preferential recruitment of immature NK cells *via* a chemokine gradient. On the other hand, tumors dampen the NK cell activation and effector function by a decreased expression of ligands for the activating NKRs or by generation of soluble activating receptor ligands, which block recognition. In contrast, inhibitory molecules, like the non-classical HLA class I molecules HLA-G and -E, Nectin-4 or PVR and inhibitory immune checkpoint (ICP) ligands, are often overexpressed in tumors thereby impairing not only T cell, but also NK cell responses (20, 21). High levels of HLA-E were found in many solid tumors and its overexpression correlated with a poor prognosis and NK cell exhaustion (99, 100), while the innate immunity is regulated by the engagement of HLA-G with the NK cell receptor KIR2DL4 or ILT2 (101, 102) leading to a reduced cytotoxicity. Many tumors express the MHC class I chain-regulated polypeptide A (MICA) and MICB, known as ligands for the activating receptor NKG2D on NK cells. However, tumors frequently shed MICA and -B thereby removing an activation signal and creating a soluble ligand, which can block the NK cell cognate receptors (103, 104). Thus, classical, non-classical as well as HLA class I-related molecules play a key role in NK cell functionality by either leading to immune escape or immune recognition. Characterization of these immune escape mechanisms represent the rational for the development of NK cell-based immunotherapies.

Different strategies to revert immune surveillance by NK cells-antibody-based approaches

Since NK cell anti-tumor function is frequently impaired in tumor patients, restoring their function is an obvious therapeutic option. Indeed, there exist different approaches to restore the

anti-tumor surveillance of NK cells (105). Agents that enhance NK cell function, like immune modulatory drugs, various stimulatory cytokines, STING agonists and TGF-β inhibitors have been recently summarized (106). In addition, a number of mAbs directed against key ICP ligands and their receptors have been designed, which prevent NK cell inactivation by e.g. decreasing inhibitory factors or increasing factors, which boost NK cell function. Recently, a humanized anti-NKGA mAb (monalizumab) has been developed, which exerts *in vitro* and *in vivo* anti-tumor efficacy as a single agent or with other therapeutics (107). The inhibition of NKG2A restores the cytotoxic activity against HLA-E-expressing target cells as well as the NK cell-dependent maturation of monocyte-derived DC and reduces the secretion of immune suppressive cytokines.

Major ICP-targeted therapies that affect NK cell-mediated anti-tumor immune responses are the immune checkpoint inhibitors (ICPis) PD1/PD-L1 and CTLA4. PD1 has been shown to be mainly expressed on T, B and myeloid cells, but also on about 25% of NK cells in healthy donors, but the molecular mechanisms leading to PD1 expression have not yet been identified (108). PD1 can also be expressed on tumor infiltrating NK cells of patients with different solid tumors (109). Blockade of PD1/PD-L1 interaction can enhance NK cell activity both *in vitro* as well as in animal models due to an enhanced ADCC-induced anti-tumor function leading to an increased tumor control. Moreover, NK cells play also a role in response to treatment with agonistic anti-CD137/4-1BB antibodies (Abs) (110). CD137 is expressed on primed NK cells, which upon ligation provides a powerful costimulatory signal (111). The addition of agonistic Abs increased NK cell proliferation and a synergistic effect was found between IL-15 and IL-21 upon CD137 engagement and the presence of APCs. Thus, CD137 triggering contributes to NK cell activation (112). These data suggest that restoring of the NK cell function by co-targeting immune modulatory pathways might be an important therapeutic strategy to prevent tumor immune escape.

Since intra-tumoral activated NK cells are often characterized by overexpression of TIGIT, which competes with the activating NK cell receptor DNAM1, TIGIT blockade might also be a promising approach (113) and has been described to increase patients' response (114). However, TIGIT and the activating receptor DNAM1 have CD155 as ligand suggesting a complex of CD155-mediated immune regulation *via* these receptors. Human tumor cells could express both membranous and soluble CD155. The latter binds preferentially and with a higher affinity to DNAM1 thereby inhibiting the DNAM1-mediated anti-tumor activity of NK cells (115). Recent studies also focused on increasing the infiltration and recruitment of NK cells by inhibiting soluble factors secreted by tumor cells, e.g. TGF-β (116). Furthermore, antibodies targeting the proteolytic site of MICA shedding can promote NK cell-driven tumor immunity (117).

In addition, Abs directed against inhibitory KIRs are potential therapeutic candidates, which might have fewer side effects compared to other therapeutic approaches. Recently, a humanized anti-NKG2A mAb monalizumab has been developed, which is explored in clinical trials (NCT02643550, NCT02921685). Other trials are addressing IPH4102 as an anti-KIR3DL2 (NCT02593045), lirilumab as an anti-KIR2DL1-3 (NCT01687387) antibody as well as different Abs directed against the PD1/PD-L1 axis.

Benefit and limitation in clinical NK cell-based immunotherapies – Adoptive cell transfer-based approaches

The translation of *in vitro* and *in situ* results of modulating NK cell activity and function into clinical concepts has been challenging and was investigated in a number of clinical trials. Over the past decades, considerable progress has been made in NK cell-based immunotherapies in haploidentical stem cell transplantation (haploSCT) or in the non-transplant setting, since allogeneic NK cells contribute to the graft versus leukemia/tumor effect (GvL/GvT) with generally no or only marginal graft versus host disease (GvHD) compared to allogeneic T cells (118–120). There are several sources for NK cells. They can be obtained from (i) healthy donors *via* leukapheresis followed by immunomagnetic purification (CD3-depleted, CD56-enriched), (ii) cord blood or (iii) induced pluripotent stem cell (iPSC) and administered unstimulated or cytokine-activated and expanded, respectively. After the first clinical trials in 2004 and 2005 using IL-2 activated donor NK cells, performed in parallel in Europe and the USA (121, 122), multiple clinical trials over the last 1.5 decades showed safety and feasibility of adoptive NK cell transfer for various hematological and oncological diseases, respectively (123). Despite the overall clinical benefit regarding GvL/GvT effect without GvHD, adoptive NK cell therapies are hampered by tumor immune escape mechanism, such as blocking of NKG2D by soluble MICA (124), exhaustion of NK cells in the immune-suppressive tumor microenvironment (125) and limited persistence of NK cells. In addition to the historical use of IL-2 for both *ex vivo* expansion of the NK cells during manufacturing and *in vivo* therapy, stimulation of NK cells with IL-12, IL-15, IL-18 and IL-21 enhanced cytotoxicity, successfully generated donor memory-like NK cells with enhanced persistence and improved anti-leukemia response. This could be demonstrated impressively in 4/8 pediatric patients with AML in a current clinical trial (126). Cytokine combinations are increasingly used for optimized manufacturing protocols (127). Nevertheless, the optimal cytokine cocktail after adoptive NK cell transfer to improve

cell expansion remains still unclear. Very recently, it has been shown in clinical trials that systemic IL-15 resulted in reduced clinical activity (128). The authors hypothesized that IL-15 promotes recipients $CD8^+$ T cell activation that finally leads to donor NK cell rejection.

Other trials are using NK cell subpopulations. Especially, cytomegalovirus (CMV) infection is one powerful stimulus promoting the functionality and phenotype of NK cells expressing the HLA-specific activating receptor CD94/NKG2C (129). Therefore, clinical protocols are currently developed based on the mechanisms underlying the generation of adoptive NK cells that involve NKG2C triggering to efficiently expand $NKG2C^+$ NK cells for therapy. Interestingly, adoptive NK cells appear to be resistant to MDSC and Treg suppression thereby providing them with a further advantage compared to CAR T cells for their use as therapeutics. Another benefit is the availability of NK cells for therapy from distinct sources. Multiple other approaches are ongoing to restore NK cell activity and reach long-lasting effects. These include the blockade of inhibitory receptors, blocking soluble activating receptors, combinational therapies with immune checkpoint inhibitors (130) and genetic engineering of the NK cells (131). In addition, cytokine-activated NK cells with upregulated NCRs and NKG2D are partly able to overcome tumor immune escape by restoring NKG2D-mediated NK cell cytotoxicity *via* scavenging of plasma MICA as demonstrated for neuroblastoma and head and neck cancer, respectively (124, 132).

Engineered NK cell-based immunotherapies

Genetic modification of immune effector cells has been demonstrated to be a promising strategy for the treatment of advanced cancers refractory to conventional therapies. In particular, chimeric antigen receptor (CAR) targeting cell surface antigens provide a suitable tool to increase the efficacy of effector cells. CARs are genetically engineered proteins composed of an extracellular domain specific for the respective/selected target antigen, a transmembrane domain and an intracellular signaling domain responsible for the transduction of the activating signal. During the last two decades, the CAR technology has been developed as next generation immunotherapeutic approach reaching impressive clinical results in two hematological disorders, the acute lymphoblastic leukemia and diffuse large B cell lymphoma. This led to more than 800 clinical trials worldwide (clinicaltrials.gov) (133) as well as to five approved CAR T cell products, the first four targeting CD19 and the last one directed against BCMA, respectively (134). In a similar way, engineered CAR NK cells redirected against several cancer epitopes including hematological and tumor targets resulted in improved NK cell cytotoxicity (135–138). Moreover, in addition to use the intracellular CAR T cell signaling, DAP10 and DAP12 give rise for more improvement for CAR NK cell cytotoxicity (139). There is a clear advantage of CAR NK cells over CAR T cells, since NK cells can be obtained from allogeneic donors, do not induce a cytokine storm, persist for more than one year and can be applied to the patients without development of GvHD and thus represent an "off the shelf" product for the treatment of patients (105, 137, 140). Another challenge is to overcome the high manufacturing costs of personalized autologous CAR T cell products by using one allogeneic CAR NK cell product for multiple applications in various patients. To date, more than 35 clinical trials using CAR NK cells are conducted (clinicaltrials.gov) against several cancer epitopes, such as CD19, CD19/22, CD33, CD7, HER2, MUC1, PDL1, NKG2D ligand, BCMA, ROBO1, PSMA, mesothelin and others using CAR NK cells from different sources, primary human NK cells, cord-blood derived and iPSC-derived NK cells as well as CARs from the cell line NK92 (141, 142).

While most of the trials are performed in China and USA, currently a phase I trial, CAR2BRAIN using lentiviral transduced CAR NK92 cells redirected against the human epidermal growth factor 2 (HER2, ErbB2) for treatment of recurrent patients with glioblastoma is conducted in Europe, in Frankfurt, Germany. The very well recognized study of Katy Rezvani, USA, employed cord-blood derived CAR NK cells redirected against CD19 for B cell malignancies. The promising results showed clinical responses in 8 out of 11 patients with no sign of cytokine release syndrome or neurotoxicity (143). Next to conventional CARs, additional genetic modifications are currently explored to enhance NK cell activity and homing into the tumor. Preclinical studies demonstrated an improvement of tumor cell infiltration through transgene expression of chemokine or adhesion receptors (144). Furthermore, the integration of the autocrine growth factor IL-15 as a down-stream cassette has been used, which led to an improved life span and persistence of those CAR NK cells in all patients (145). The overall cytokine and chemokine profile clearly differ between CAR T and CAR NK cells and supports the observation that allogeneic CAR NK cells do not contribute to any severe side effects, like cytokine release syndrome and toxicity. Nevertheless, NK cells are considered hard-to-engineer and hard-to expand compared to T cells. Recently, a novel viral envelope derived from the baboon endogenous virus (BaEV) showed superior efficacy as compared to other lentiviral envelope proteins to successfully genetically manipulate human NK cells (146, 147).

Finally, the question arises how to further improve both anti-tumoral activity, cytotoxicity and homing of CAR NK cells in the TME, which led to combinational therapies with ICPis. In the end of 2021, two clinical trials were started: (i) a phase II study using irradiated PD-L1 CAR-NK cells plus pembrolizumab for recurrent/metastatic gastric or head and neck cancer (NCT04847466) and (ii) FT576 (iPSC derived CAR NK cells) as monotherapy and in combination with

daratumumab in subjects with relapsed/refractory multiple myeloma (NCT05182073), respectively.

Conclusions

The enhancement of NK cell activity represents an important approach to control cancer growth. The increased understanding of the NK cell biology has led to the development of NK cell-based strategies to control tumors. New ways to enhance the NK cell targeting, their activation and cytolytic function are required, since the NK cells are becoming dysfunctional in the immune suppressive TME. Despite the potential of NK cell-based therapies it has become obvious that for the design of effective strategies using NK cells in the clinics, a detailed knowledge of NK cell receptors, NK cell subpopulations, tissue-specific NK cells and memory-like NK cells is required. Furthermore, the NK cell heterogeneity might influence the efficacy of NK cell-based therapies. Some preclinical and clinical studies suggest multifaceted opportunities of the implementation of NK cells for the treatment of cancer patients using combination therapies, which will lead to further clinical advances.

Author contributions

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

Conflict of interest

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

Publisher's note

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Glossary

Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
ALL	acute lymphoblastic leukemia
BM	bone marrow
CAR	chimeric antigen receptor
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
CR	complete revision
DC	dendritic cell
DFS	disease free survival
ECM	extracellular matrix
EGF-R	epidermal growth factor receptor
EV	extracellular vesicle
FC	flow cytometry
FDA	Food and Drug Administration
G-CSF	granulocyte-stimulating factor
GM-CSF	granulocyte-macrophage stimulating factor
GMP	good medical practice
Had	graft-versus-host disease
HLA	human leukocyte antigen
HSC	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
ICPi	immune checkpoint inhibitor
IDO	indolamine 2, 3-deoxygenase
IFN	interferon
IL	interleukin
iPSC	induced pluripotent stem cell
KIR	killer cell immunoglobulin-like receptors
LAG	lymphocyte-activation gene
mAb	monoclonal antibody
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MIC	MHC class I-related
MMP	matrix metalloproteinase
NCR	natural killer receptor
NK	natural killer
OS	overall survival
PBMNC	peripheral blood mononuclear cell
PD1	programmed death
PD-L1	programmed death ligand 1
RNA-seq	RNA-sequencing
TAM	tumor-associated macrophages
TCR	T cell receptor
TGF-β	transforming growth factor β
TIGIT	T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif
TMB	tumor mutational burden
TME	tumor microenvironment
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
Treg	regulatory T cell
ULBP	UL-16 binding protein
VEGF	vascular endothelial growth factor

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