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ORIGINAL ARTICLE

Shaping of NK Cell Responses by the Tumor Microenvironment

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Abstract Natural killer (NK) cells belong to the innate immune system and are potent cytolytic and cytokine-producing effector cells in response to tumor targets. NK cell based anti-tumor immunotherapy was so far mainly successful in patients with different types of leukemia. For instance, acute myeloid leukemia (AML) patients displayed a prolonged survival if transplanted with haploidentical stem cells giving rise to NK cells with a mismatch in inhibitory killer immunoglobulin receptors (KIRs) and recipients' HLA class I. Although promising results have been achieved with hematological tumors, solid tumors are in most cases poorly controlled by NK cells. Therapeutic protocols that aimed at improving NK cell responses in patients with solid malignancies succeeded in increasing NK cell numbers and functional responses of NK cells isolated from the patients' peripheral blood. However, in the majority of cases tumor progression and overall survival of patients were not significantly improved. There is increasing evidence that tumor-associated NK cells become gradually impaired during tumor progression compared to NK cells from peripheral blood and healthy tissues. Future protocols of NK cell based immunotherapy should integrate three important aspects to improve NK cell anti-tumor activity: facilitating NK cell migration to the tumor site, enhancing their infiltration into the tumor tissue and ensuring subsequent efficient activation in the tumor. This review summarizes the current knowledge of tumor-infiltrating NK cells and the influence of the tumor microenvironment on their phenotype and function.

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Introduction

Tumor initiation and progression is a complex process characterized by the interaction of malignant cells and infiltrating immune cells that shape each others' phenotype and responses. In many cases, infiltration of high numbers of certain immune cells, especially cytotoxic effectors such as NK or $CD8^+$ T cells, was shown to correlate with an improved prognosis for cancer patients [1, 2]. However, recent studies described phenotypic and functional features of immune cells in the tumor microenvironment that rather supported tumor growth [3]. Examples are tumor-associated macrophages (TAMs) that are often polarized towards an immunoregulatory phenotype, immature dendritic cells (iDCs) that promote tolerogenic responses and $CD8^+$ T cells that acquire an exhausted phenotype. In addition, tumors can promote the generation and recruitment of suppressive immune cells, such as regulatory T cells (Tregs) [4] and myeloid-derived suppressor cells (MDSCs) [5] that can suppress responses of anti-tumor effector cells. Thus, strategies of cancer immunotherapy should aim not only at increasing the numbers of effector cells in the tumors, but also at circumventing the immunosuppressive mechanisms that hamper effective anti-tumor immune responses at the tumor site.

NK cell based therapy relies on the ability of NK cells to efficiently distinguish tumor cells from healthy cells [6]. Transformed cells often express increased levels of ligands that engage activating NK cell receptors, while at the same time ligands of inhibitory receptors are downregulated. MHC class I molecules, that are expressed at high levels on normal cells and protect them from NK cell lysis, represent the main class of inhibitory ligands. It was shown that

the majority of acute myeloid leukemia (AML) cells expressed ligands for the activating NK cell receptors DNAM-1, NKp30 and NKp46 and thus, were susceptible to NK cell lysis [7]. Patients suffering from AML, who received grafts with a mismatch in NK cell expressed inhibitory killer immunoglobulin receptors (KIRs) and recipient expressed HLA class I ligands, survived significantly longer than recipients of grafts with an absence of mismatch [8]. Lessons from KIR-mismatched haploidentical transplantation indicated the importance to circumvent KIR-mediated inhibition in order to achieve full NK cell activation. Fully humanized antibodies against inhibitory KIRs have been developed and their safety was evaluated in phase I and II clinical trials with AML and multiple myeloma patients [9, 10]. Moreover, Venstrom et al. [11] recently demonstrated that the outcome of allogeneic hematopoetic stem cell transplantation in AML patients correlated with the expression of activating KIR genes of the donors. Donor KIR2DS1 positivity was associated with protection against relapse and donor KIR3DS1 with reduced mortality. Further examples of anti-cancer therapies that involve NK cells are the treatment of bladder cancer with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), the tyrosine kinase inhibitor Imatinib Mesylate (Gleevec) treatment of gastrointestinal tumors [12], DC-based immunotherapies [13] and antibody-based therapies [14]. Since in many cases NK cell based therapies of solid tumors remained unsuccessful, a better knowledge of the impact of the tumor microenvironment on NK cell activation is critical for the design of improved therapeutic protocols.

Tumor Cell Recognition by NK Cells

NK cells are often described as potent cytotoxic effectors that can eliminate tumor cells without prior sensitization [15, 16]. However, increasing evidence exists that effector functions of NK cells are more complex and regulated at multiple levels. During development, NK cells pass through a process of education, which results in the generation of mature effectors that attack malignant or stressed cells, but not healthy cells. It was shown that resting human NK cells can respond to certain stimuli [17], but their full activation is only achieved when multiple signals are properly integrated. Target cells initiate NK cell activation if they express sufficient amounts of ligands for activating NK cell receptors and low levels of ligands that engage inhibitory receptors [6]. In addition, NK cell priming with DCs [18], their interaction with $CD4^+$ T cells ([19] and our unpublished observations) or neutrophils [20, 21] or the presence of certain cytokines, such as IL-2, IL-12, IL-15, IL-18 or IL-21 [22], can further enhance their effector function.

The expression of numerous activating receptors (summarized in Fig. 1) enables recognition of an array of ligands widely expressed on transformed cells, while mainly absent in healthy tissues [6]. Activating receptors include NCRs (NKp30 and NKp44 in human, NKp46 in human and mouse), NKR (NK1.1 in mouse), NKG2D and DNAM-1 (in human and mouse). Other receptors, such as 2B4, CD48 or NTBA can also trigger and/or support NK cell activation. NKG2D, the best-characterized NK cell receptor in the context of tumor immunity, recognizes stress-induced ligands of the Rae1 protein family, H60 and MULT1 in mice, and MICA, MICB and members of ULBP family in humans. NKG2D ligands (NKG2D-Ls) are rarely expressed on healthy cells, but upregulated upon cellular transformation or viral infection [23, 24]. Furthermore, chemotherapeutic drugs or ionizing radiation that cause activation of the DNA damage pathway can further upregulate NKG2D-L expression on tumor cells [25]. In addition, activation of the DNA damage pathway also increases expression of ligands for the activating receptor DNAM-1 facilitating tumor cell recognition by NK cells [26]. DNAM-1- and NKG2D-mediated anti-tumor responses can be further enhanced by treatment with IL-2 or/and IL-12, respectively [27, 28]. Importantly, it was reported recently that the NKG2D receptor was essential for effective immunosurveillance of lymphoma and prostate carcinoma in mouse models of spontaneously arising malignancies [29].

The NCRs NKp46 and NKp30 are expressed on most NK cells, whereas NKp44 is induced after activation. Tumor-associated ligands for most NCRs remain unknown. Recently, BAT3 (the nuclear factor HLA-B-associated transcript 3, also called Bag6, BCL2-associated athanogene 6) and B7-H6, a B7-family member, were defined as ligands for NKp30. BAT3 is an intracellular protein that is released via exosomes from DCs and activates NK cells [30, 31]. BAT3 neutralization was shown to decrease the lysis of Raji cells by primary NK cells and to prevent tumor cell rejection by PBMCs in a multiple myeloma mouse model [30]. So far, the importance of BAT3 during anti-tumor immune responses in cancer patients remains unclear. B7-H6 is detected on the cell surface of several tumor cell lines and at low percentages of primary tumor cells of hematological origin derived from a small subset of patients [32]. B7-H6 was shown to activate cytotoxic NK cell responses by its interaction with NKp30. Three different isoforms of NKp30 were described. The final outcome of NKp30 activation depends on the NKp30 isoform expressed on the surface and can result in a quantitatively and qualitatively different response of NK cells [33].

Recently, Rosental et al. reported that proliferating cell nuclear antigen (PCNA) binds to NKp44 and inhibits NK cell function [34]. Expression of PCNA was associated with shorter overall survival of breast cancer patients [35] and might be involved in tumor immune evasion. The full effector potential of NK cells is mainly achieved upon

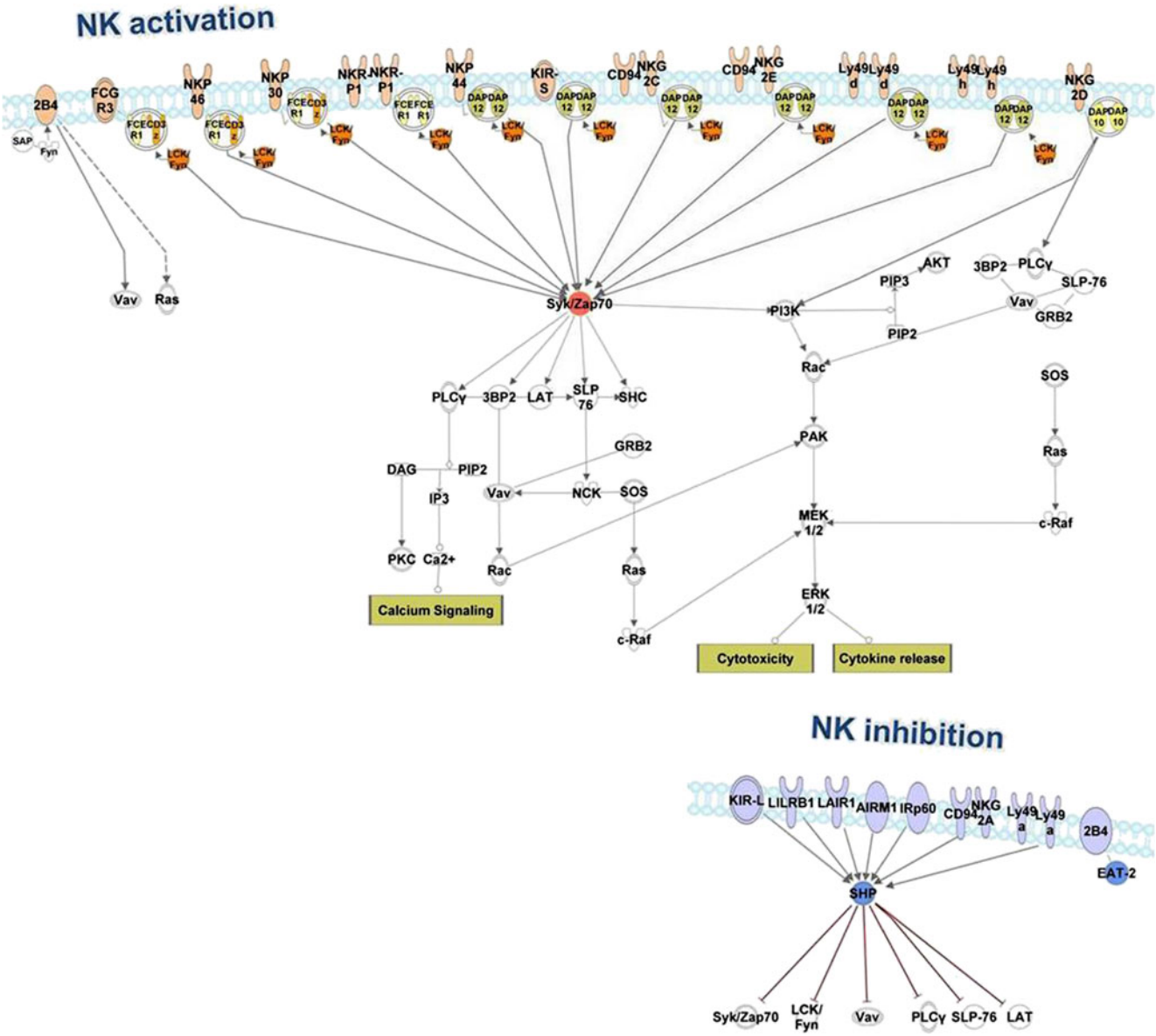

Fig. 1 NK cell activating and inhibitory receptors and their downstream signaling molecules. Signaling pathways downstream of activating NK cell receptors typically lead to NK cell cytotoxic responses and/or cytokine production. Central molecules involved in NK cell activation are often downregulated in the tumor microenvironment. These molecules include: *CD3ζ*, an adaptor molecule coupled to several activating receptors; contains an immunoreceptor tyrosine-based activation motif (ITAM) that becomes phosphorylated by Src family kinases upon engagement of activating receptor(s) by its ligands; *Lck kinase* that phosphorylates ITAMs of CD3ζ and FcεRIγ adaptor molecules; *Syk kinase* that is recruited to phosphorylated ITAMs and mediates downstream signaling; additional downstream molecules such as *Vav, PI3K or PLCγ*. Inhibitory NK cell receptors contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) and recruit phosphatases such as SHP-1 and SHIP upon ligand recognition. These events lead to the dephosphorylation of active components downstream of activating receptors counteracting NK cell activation. Tumor-associated NK cells often upregulate expression of inhibitory receptors that result in an enhanced threshold for NK cell activation. The network was generated using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com)

simultaneous engagement of several activating receptors [36]. As an example, the simultaneous blockade of DNAM-1 and NCRs leads to the complete abrogation of lysis of certain tumor cells by NK cells. Thus, the identification of additional ligands for activating receptors such as for the NCRs will open new possibilities for therapeutic targeting.

Tumor cells employ multiple mechanisms to escape from the direct recognition by NK cells. Tumor-infiltrating NK cells in cancer patients often display reduced surface expression of activating NK cell receptors compared to NK cells from peripheral blood [37]. The NKG2D-Ls can be shed from the surface of tumor cells, leading to increased levels

of soluble ligands in serum [38–40]. Patients with high levels of NKG2D-Ls in serum show decreased expression of the NKG2D receptor on both NK and $CD8^+$ T cells. Reduction of NKG2D levels on tumor-infiltrating and peripheral blood $CD8^+$ T cells from individuals with cancer was associated with circulating tumor-derived soluble MICA [39]. Paschen et al. showed that high serum levels of soluble ULBP2 in melanoma patients were associated with disease progression and poor prognosis [41]. The downregulation of NKG2D expression in response to the continuous exposure to its ligands can also lead to the impaired function of several other activating NK cell receptors including NKp46 and CD16 [42]. Moreover, NKG2D expression can be reduced by soluble factors such as TGF-β [43, 44] and L-kynurenine, a product of tryptophan degradation. L-kynurenine was also shown to reduce the expression of NKp46 on human NK cells [45]. Pietra et al. reported that the co-culture of NK cells with cell lines derived from melanoma skin lesions in the presence of IL-2 led to the inhibition of the IL-2-induced upregulation of NKp30, NKp44 and NKG2D, all of which are involved in melanoma cell killing [46]. The observed effect was mediated by tumor-derived indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) secreted by tumor cells. Expression of inhibitory KIRs and CD94/NKG2A receptors remained unchanged indicating that activating receptors were main targets of tumor-mediated suppression. We have evidence that expression of activating NK cell receptors in the tumor microenvironment is also regulated at the transcriptional level (our unpublished observation). In addition to the reduced expression of activating receptors, we also observed downregulation of the downstream signaling molecules indicating that the function of NK cells receptors in the tumor tissue can be modulated at multiple levels.

Efficient recognition of tumor cells by NK cells is instrumental for NK cell activation. Thus, up-regulation of activating ligands and/or down-regulation of inhibitory ligands on tumor cells would increase NK cell activation against tumors. For instance, we showed that activation of p53 with the small molecular compound, RITA, led to the upregulation of ULBP1 and ULBP2 in tumor cells resulting in enhanced NK cell-mediated target recognition [47]. Similarly, certain MIC and ULBP proteins can be upregulated by the oncogene Ras [48] or by application of histone-deacetylase inhibitors [49, 50]. The expression of NKG2D-Ls was shown to be controlled by certain miRNAs [51–53], some of which have broad tumor-suppressive functions. Several other compounds such as dacarbazine, a cytotoxic drug used for treatment of melanoma patients [54], hydralazine and valproate, tested in cervical cancer studies [55], 5-fluorouracil used for pancreatic cancer patients or IFN-α applied in melanoma patients [56] were shown to increase NKG2D-L expression on tumor cells. A detailed analysis of the molecular network that regulates expression of ligands for activating NK cell receptors might lead to the identification of novel targets for the modulation of NK cell/tumor cell recognition. Furthermore, a combination of agents known to upregulate activating ligands with other approaches of NK cell based therapies might improve the therapeutic benefit.

Anti-Tumor Effector Function of NK cells

NK cells lyse tumor cells upon engagement of activating receptors by the release of cytotoxic mediators, perforin and granzymes. Expression of death receptor ligands, FasL and TRAIL, enable NK cells to induce apoptosis in tumor cells expressing the corresponding receptors. Engagement of FcγRIII (CD16) by Ab-coated targets induces NK cell degranulation and is an important mode of action of Ab-based therapies. In addition, other mechanisms such as the release of exosomes that contain perforin and death receptor ligands [57] and the formation of nanotubes that enables contact with target cells over long distances [58] contributes to NK cell effector responses. Depending on the tumor origin and expression of cognate ligands, NK cells might use different mechanisms for tumor cell lysis. Besides tumor cells, immune cells infiltrating the tumor tissue can be direct targets for NK cells. Our previous study revealed that the mononuclear subset of myeloid derived suppressor cells (MDSCs) from mouse subcutaneous lymphoma expressed the NKG2D-L, Rae1, rendering those cells susceptible to NK cell lysis [59]. Similarly, NK cells were shown to kill immature DCs [60] that accumulate in the tumor tissue and thereby might prevent DC-mediated tolerance induction. In addition, NK cell cytolytic activity can also be directed towards activated T cells [61]. Although killing of activated T cells by NK cells can be beneficial for the control of immune responses and prevention of immunopathology, it might be detrimental for anti-tumor immune responses.

In addition to their cytolytic function, NK cells secrete IFN-γ, a cytokine with pleiotropic anti-tumor activity [62], which include direct suppression of tumor cell growth and metastases, induction of tumor cell apoptosis, anti-angiogenic activity, induction of T cell and macrophage differentiation and effector functions and upregulation of MHC class I and components of the antigen presenting machinery. NK cells are considered as early IFN-γ producers promoting the development of subsequent adaptive immune responses [63, 64]. Accordingly, protective immune responses in several transplantable tumor models in mice were abolished in the absence of IFN-γ [63, 65]. Recently, O'Sullivan et al. demonstrated that in the absence of adaptive immunity, NK cell derived IFN-γ contributed to tumor immunoediting mediated by innate immune system, through the induction of macrophage polarization [66].

Besides IFN-γ, NK cells secrete other pro-inflammatory cytokines including TNF-α and GM-CSF and chemokines such as CCL3 and CCL4 that recruit and activate other immune cells at the site of inflammation [67]. In the tumor tissue, NK cells can produce soluble factors that support tumor angiogenesis, such as VEGF and PDGF (our unpublished observation). In addition, human $CD56^{bright}$ NK cells can secrete IL-10 and IL-13 when stimulated with IL-2 and IL-15 [68, 69].

Many cytokines can have both immunoactivating and inhibitory function in the context of malignant disease. For example, IFN-γ was shown to induce the expression of the tryptophan-degrading enzyme, IDO, prostaglandin E2 (PGE2) and to enhance expression of HLA-G on tumor cells. High expression of IDO, cyclooxigenase-2, an enzyme that catalyzes key steps in PGE2 production, and HLA-G has been correlated with poor prognosis for cancer patients [70–72]. Activation of IDO leads to depletion of tryptophan from the microenvironment that is essential for immune cell functions. In addition, products of tryptophan degradation such as L-kynerunine and kynerunic acid can directly inhibit various immune cells, including T cells and NK cells. Both membrane-bound and soluble form of HLA-G that are increased in cancer patients bind to the inhibitory receptor ILT-2 expressed by NK cells [72–74]. Besides IFN-γ, other cytokines, such as GM-CSF, IL-10 and LIF contribute to the induction of HLA-G expression on malignant cells [75]. NK cell engagement of HLA-G can raise the threshold for NK cell activation by shifting the balance of inhibitory versus activating signals or can induce apoptosis of NK cells. In addition, HLA-G can be transferred by intercellular membrane exchange (trogocytosis) from APCs and tumor cells to NK cells creating $HLA\text{-}G^{+}$ NK cells with regulatory functions [76].

In conclusion, NK cell effector functions such as cytolysis and cytokine production can have both immunostimulatory and immunosuppressive effects in the tumor. The dynamics and nature of NK cell responses in the tumor tissue need to be further explored and factors shifting the balance towards immunosuppression need to be defined.

Shaping of NK Cell Function by the Tumor Microenvironment

There is increasing evidence that the phenotype and function of different immune cells is shaped by the tumor microenvironment. For instance, it was shown that tumor-specific T cells from the peripheral blood of cancer patients exert potent responses in vitro, but T cells isolated from the tumor tissue were rather unresponsive [77]. T cells exposed to chronic antigen stimulation, such as during viral infection and cancer progression, became functionally impaired and displayed an exhausted phenotype [78]. Importantly, exhausted T cells were detected in the tumor tissue, but not in the blood of melanoma patients [79, 80]. Transcriptional profiling revealed expression of certain markers including the inhibitory receptors PD-1, CTLA-4, LAG-3, CD160 and Tim-3 on exhausted T cells. Blockade of PD-1, CTLA-4, LAG-3 or Tim-3 as single treatments or in combinations restored T cell function both in vitro and in vivo. We observed that several T cell exhaustion markers such as PD-1, CTLA-4, LAG-3 and CD160 were upregulated by tumor-infiltrating NK cells compared to peripheral blood NK cells in a mouse model of subcutaneously transplanted lymphoma (our unpublished observation). Previously, Terme et al. [81] reported that tumor-derived IL-18 induced PD-1 expression on mouse NK cells that was associated with increased dissemination of metastases in mice. In multiple myeloma patients, tumor-associated NK cells were shown to express PD-1. Upon PD-1 blockade, increased killing of B7-H1-expressing tumor cells by NK cells was observed [82]. CTLA-4 inhibits the function of both T cells [83] and NK cells (our unpublished observation) underlining its importance as a therapeutic target. Of note, while LAG-3 inhibits T cell responses, evidence exists that it might have an activating role in NK cells [84]. Similarly, it was shown that in NK cells CD160 could trigger cytotoxicity and secretion of IFN-γ, TNF-α, IL-6, IL-8 and MIP1-β [85], while having mainly inhibitory function in T cells [86]. Different roles of Tim-3 in the regulation of NK cell responses has been reported. Ndhlovu et al. showed that cross-linking of Tim-3 diminished NK cell cytolytic responses [87], whereas engagement of Tim-3 by its natural ligand, galectin-9, induced IFN-γ release by human NK cells [88]. In conclusion, several molecules such as CTLA-4 and PD-1, were identified as inhibitory receptors in both NK cells and T cells, whereas others, such as LAG-3 and CD160, might exert different functions on NK cells or T cells. The blockade of inhibitory receptors that can simultaneously enhance functions of both tumor-infiltrating NK cells and T cells represents a promising approach to restore the activity of tumor-infiltrating lymphocytes against tumors.

In addition to the increased expression of inhibitory receptors, the impairment of T cell responses in the tumor tissue is characterized by changes in the signaling machinery of the TCR complex [77]. These include decreased expression of the adaptor molecule CD3ζ, tyrosine kinases Lck and Fyn and reduced expression of NF-κB family members that were correlated with impaired cytokine production by cytotoxic T lymphocytes. Similar signatures were observed in tumor-infiltrating NK cells. The signaling network downstream of activating and inhibitory NK cell receptors and central molecules targeted by tumor immunosuppressive mechanisms are depicted in Fig. 1. NK cells isolated from the ascites of ovarian carcinoma patients

expressed decreased levels of the adaptor proteins CD3ζ and FcεRIγ and Lck kinase compared to NK cells from peripheral blood [89]. These features correlated with their reduced ability to produce IFN-γ upon IL-2 stimulation. Similarly, in ovarian cancer patients, tumor-infiltrating NK cells displayed reduced levels of CD3ζ, produced less IFN-γ, IL-2 and IL-4, but more IL-10, compared to peripheral blood NK cells [90]. We also observed that in RMA-S lymphoma-infiltrating NK cells, mRNA levels of several signaling molecules downstream of activating NK cell receptors including Lck, PI3K, PLCγ and Vav, were decreased when compared to peripheral blood NK cells (our unpublished observations). Strategies that target signaling components are very challenging due to their promiscuous expression and function. Thus, it is important to identify the tumor-derived factors that cause impairments in the signaling machinery. Targeting these factors can potentially improve the function of multiple immune effector cells including NK and T cells.

In addition to the immunosuppressive mechanisms exerted by tumor cells, multiple immune cells with suppressive functions are detected within the tumor tissue (Fig. 2). These include immature dendritic cells (iDCs), tumor-associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs) and regulatory T cells (Treg). In the tumor tissue, NK cells can interact with different immune cells. The cross-talk of NK cells with iDCs leads to DC maturation and NK cell activation but, under certain conditions, influenced by the cell-to-cell ratio, previous activation status and environmental stimuli, results in the elimination of iDCs [91]. DCs in solid tumors often display an immature phenotype characterized by low expression of co-stimulatory molecules [92]. Rather than stimulating immune cells, iDCs in tumors often induce tolerance [93]. It is possible that activated NK cells in the tumor promote T cell activation by eliminating immature tolerogenic DCs and secreting IFN-γ that supports DC maturation. However, the outcome of the interaction between tumor-infiltrating DCs and tumor-infiltrating NK cells in vivo is currently unknown. Several factors produced within the tumor tissue such as VEGF, GM-CSF, M-CSF and IL-6 affect normal myelopoiesis and support the accumulation of immature myeloid cells in tumor-bearing mice and cancer patients [94]. A common feature of these cells is their ability to suppress T cell responses and these cells were called "myeloid-derived suppressor cells" (MDSCs). Depending on the tumor model and experimental set-up studied, MDSCs were reported to suppress [95] or promote NK cell responses in mice [59]. Similar to MDSCs, Tregs are enriched in the tumor tissue of cancer patients and tumor-bearing mice. Tregs were shown to control the homeostatic proliferation of NK cells and their anti-tumor immune responses [96]. Active recruitment and in situ proliferation contribute to the accumulation of Tregs in the tumor

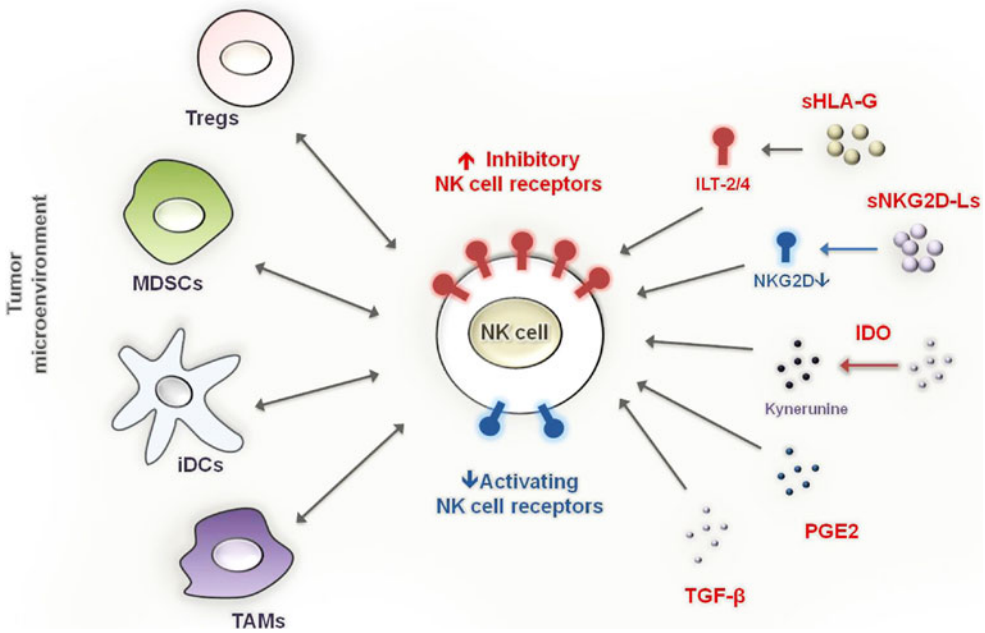

Fig. 2 Components of the tumor microenvironment that inhibit NK cell effector functions. Cells with suppressive function that are recruited and/or expanded in the tumor tissue such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), immature dendritic cells (iDCs) and tumor-associated macrophages (TAMs) interact with NK cells within the tumor tissue and can suppress NK cell effector function. The activation of NK cell cytotoxic responses and cytokine production can prevent the accumulation of immunosuppressive cells, e.g. by elimination of iDCs and monocytic MDSCs or by IFN-γ-mediated M1-polarization of TAMs. Soluble factors released by tumor cells as well as by infiltrating immune cells, such as soluble HLA-G (sHLA-G), soluble NKG2D ligands (sNKG2D-Ls), TGF-β, PGE2, the tryptophan-degrading enzyme, IDO, and its product kynurenine can inhibit NK cells by direct engagement of inhibitory receptors or by downregulation of activating NK cell receptors

tissue that is further supported by other suppressive cells in tumors, such as iDCs and MDSCs. It was demonstrated that Tregs downregulated NKG2D and suppressed IL-12-induced IFN-γ production by NK cells in a TGF-β dependent manner [97]. TGF-β is an important immunosuppressive factor produced by tumor and immune cells with broad effects on tumor-infiltrating immune cells [98]. It can also indirectly influence NK cell responses by affecting DC function, by inducing Tregs or by modulating the expression of adhesion molecules on endothelial cells in tumor vessels. Within the tumor microenvironment, TGF-β exerts its function mainly by its membrane-bound form presented by Tregs and MDSCs [95, 97].

In conclusion, inhibitory networks that impede NK cell activation in the tumor tissue include tumor cells, tumor cell derived factors and tumor-induced immune cells with suppressive functions (Fig. 2). The outcome is an impairment of NK cell function at multiple levels including expression of activating receptors, signaling molecules and effector molecules such as cytokines. Dissecting and counteracting this inhibitory network is critical for the design of novel strategies of anti-cancer immunotherapy.

NK Cell Recruitment to the Tumor Site

In peripheral blood of healthy individuals, two subsets of NK cells are defined: $CD56^{dim}$, comprising ~90 % and $CD56^{bright}$, comprising ~10 % of total NK cells. $CD56^{bright}$ NK cells are enriched in several tissues, such as in the lymph nodes and placenta. When stimulated, the $CD56^{bright}$ subset produces cytokines, whereas $CD56^{dim}$ NK cells exert potent cytolytic activity. However, depending on the stimuli, both cell subsets can display various effector functions. Several studies revealed that $CD56^{bright}$ NK cells were the dominant subset in the tumor tissue [99, 100]. It is currently unknown whether the enrichment of $CD56^{bright}$ NK cells is a consequence of differential recruitment to the tumor site or increased proliferation and survival. Moreover, it is possible that $CD56^{dim}$ NK cells acquire $CD56^{bright}$ features in response to factors produced within the tumor microenvironment. Accordingly, it was shown that the MUC16 glycoprotein that is present in ovarian tumors downregulated CD16 expression, which represents a hallmark of the $CD56^{dim}$ NK cell subset [101]. Furthermore, $CD56^{bright}$ NK cells display improved survival under conditions of oxidative stress, often present within tumor tissue [102]. We demonstrated that, similar to human malignancies, in mouse solid tumors, CD27-expressing NK cells were preferentially detected [103]. Mouse $CD27^{high}$ NK cells are considered to be the equivalent of the human $CD56^{bright}$ population.

In early studies addressing the NK cell infiltration of solid tumors and its association with prognosis, CD57 was used to identify NK cells. Of note, CD57 is expressed only by a subset of $CD56^{dim}$ NK cells and by a small subpopulation of T cells. On NK cells, CD57 expression correlates with high expression of inhibitory KIRs, low expression of the activating receptors NKp30, NKp46 and NKG2D and of the cytokine receptor chains IL-2Rβ and IL-12Rβ [104, 105]. Functionally, $CD57^{+}$ NK cells display reduced proliferative capacity, but are potent IFN-γ producers and show high lytic capacity when stimulated via CD16. Thus, high numbers of tumor-infiltrating $CD57^{+}$ cells were correlated with improved prognosis of cancer patients with several malignancies including lung, gastric and colorectal cancer [2, 106, 107]. These early observations suggested that $CD56^{dim}CD57^{+}$ NK cells might be effective during anti-tumor responses. However, the contribution of other NK cell subsets remained unclear. Several recent studies used NKp46 as marker to stain NK cells within the tumor. These data suggest that certain solid malignancies are infiltrated by a significant number of NK cells. Those include renal cell carcinoma [108] and gastrointestinal sarcoma (GIST) [33], where frequencies of NK cells among tumor-infiltrating leukocytes are found to be higher than in peripheral blood. Renal cell carcinoma-infiltrating NK cells displayed reduced cytotoxicity and increased surface levels of NKG2A/CD94 inhibitory receptor complex [108]. In GIST patients, NK cells were found to express predominantly the immunosuppressive isoform of the NKp30 receptor named NKp30c [33]. Compared to NKp30 isoforms a and b, which dominate in healthy individuals, triggering of NK30c with tumor cells or iDCs that express NKp30 ligands led to increased IL-10 production and diminished release of IFN-γ. Furthermore, the differential expression of NKp30 isoforms served as a predictive marker for the clinical outcome of GIST patients.

An important prerequisite for successful targeting of tumors is the efficient NK cell homing to the tumor site and infiltration of tumor tissue. In this context it was shown that certain cytokines such as IL-2, IL-12, IL-21, IFN-α as well as agents such as CpG or therapeutic mAbs increased numbers and/or activity of NK cells from peripheral blood [12]. However, low overall responses seen in treated patients with solid tumors might be the consequence of inefficient NK cell trafficking to the tumor site. In addition, NK cells are often not located in direct contact with tumor cells, but rather in the proximity of the blood vessels [109, 110]. Processes of cell migration and tissue infiltration are controlled by chemokines and chemokine receptors and factors that regulate cell–cell, cell–extracellular matrix (ECM) interactions and the modulation of ECM components [111]. Several of these molecules have been evaluated as therapeutic targets. CXCR3 and CX3CR1 are among the most important chemokine receptors implicated in NK cell recruitment to the tumor tissue. $CD27^{high}$ NK cells in mice

and CD56^{bright} NK cells in human express CXCR3 that might be responsible for their preferential accumulation in tumors [112]. We have shown that CD27^{high} NK cells accumulation in mouse subcutaneous lymphoma is CXCR3-dependent [103]. Lavergne and colleagues demonstrated increased infiltration of NK cells in CX3CL1-producing tumors correlating with reduced tumor growth [113]. A recent study by Pachynski et al. identified the protein chemerin as an important chemoattractant for NK cells [114]. Expression of chemerin correlated with good prognosis for melanoma patients and was downregulated during tumor progression in several types of solid tumors in humans. In mice, over-expression or exogenous application of chemerin led to higher NK cell infiltration of B16 melanoma tumors and to inhibition of tumor growth in a NK cell dependent manner.

Matrix metalloproteinase proteins (MMPs) have been implicated in processes such as destruction of extracellular matrix, basement membrane invasion and angiogenesis. Members of a disintegrin and metalloproteinase (ADAM) family proteins are membrane-bound proteases that cut and release ectodomains of transmembrane proteins including cytokines, growth factors and cell adhesion molecules [115–117]. Many ADAMs are overexpressed in tumors and affect different steps of tumor progression [117]. NK cell recognition of target cells is hampered by ADAM10 and ADAM17 activation, which were shown to be involved in the proteolytic release of soluble MICA and MICB from tumor cells [118, 119]. Grzywacz et al. suggested that NK cell encounter of target cells led to activation of MMPs that subsequently shed CD16 from the surface of NK cells [120]. Therefore, increased expression of MMP and ADAM enzymes in the tumor microenvironment might affect NK cell function and migration indirectly, by modifying the expression of cytokines, growth factors and cell adhesion molecules, or directly, by targeting NK cell receptors and their ligands.

Conclusions

Several studies support the view that NK cells are important effector cells against tumors. Individuals with a high NK cell activity have a reduced risk to develop cancer [121]. Moreover, increased numbers of NK cells in different types of tumors correlate with improved prognosis for cancer patients [2, 106, 107]. However, emerging evidence exists that NK cell function in the tumor microenvironment becomes impaired during tumor progression. Phenotypic and functional changes were observed at multiple levels including surface receptors, signaling and effector molecules. The suppressive network that affects NK cell function is complex and comprises tumor cells, tumor-derived factors and other immunosuppressive cells.

Successful strategies of NK cell based cancer therapy should integrate measures of efficient NK cell trafficking to the tumor site, infiltration of the tumor tissue, efficient recognition of target cells and activation of anti-tumor effector functions. To achieve these goals, a comprehensive knowledge about the suppressive networks impeding NK cell responses in different tumor entities during tumor progression is needed. In addition, markers for immunomonitoring that predict NK cell function within the tumor microenvironment need to be defined. The identification of checkpoints of both NK cell activation and loss of function in the tumors could identify novel targets expressed by NK cells. Their therapeutic manipulation could potentially unleash high effector function of NK cells against tumors within the tumor microenvironment.

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