Tricking the balance: NK cells in anti-cancer immunity

Jens Pahl *, Adelheid Cerwenka

Innate Immunity Group, D080, German Cancer Research Center, DKFZ Im Neuenheimer Feld 280, 69221 Heidelberg, Germany

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**Abstract**

Natural Killer (NK) cells are classically considered innate immune effector cells involved in the first line of defense against infected and malignant cells. More recently, NK cells have emerged to acquire properties of adaptive immunity in response to certain viral infections such as expansion of specific NK cell subsets and long-lasting virus-specific responses to secondary challenges. NK cells distinguish healthy cells from abnormal cells by measuring the net input of activating and inhibitory signals perceived from target cells through NK cell surface receptors. Acquisition of activating ligands in combination with reduced expression of MHC class I molecules on virus-infected and cancer cells activates NK cell cytotoxicity and release of immunostimulatory cytokines like IFN-γ. In the cancer microenvironment however, NK cells become functionally impaired by inhibitory factors produced by immunosuppressive immune cells and cancer cells. Here we review recent progress on the role of NK cells in cancer immunity. We describe regulatory factors of the tumor microenvironment on NK cell function which determine cancer cell destruction or escape from immune recognition. Finally, recent strategies that focus on exploiting NK cell anti-cancer responses for immunotherapeutic approaches are outlined.

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**Introduction**

Natural Killer (NK) cells have originally been described to belong to the innate arm of the immune system (Cerwenka and Lanier, 2001). More recently, conventional NK cells have been grouped among the emerging population of innate lymphocytes (ILC) as cytotoxic, interferon-γ (IFN-γ)-producing ILC (Artis and Spits, 2015). There is accumulating evidence that mature NK cells encompass a broad spectrum of phenotypic and functional diversity that may be shaped by epigenetic modifications by DNA methylation of NK cell genes and environmental influences (Bjorkstrom et al., 2010; Horowitz et al., 2013; Juelke et al., 2010; Lee et al., 2015; Luetteke-Eversloh et al., 2014; Schlums et al., 2015). This diversity of human NK cells extends the typical CD56brightCD16− (high cytokine producers) and CD56dimCD16+ (high cytotoxicity) NK cell subsets found in peripheral blood.

Like adaptive T and B lymphocytes, NK cells are thought to differentiate from the common lymphoid progenitor which arises
from hematopoietic stem cells in the bone marrow. However, this concept may be more complex since in a recent study in rhesus macaques it was suggested that the CD56dimCD16+ NK cell lineage develops from a different progenitor than CD56brightCD16- NK cells, T cells, B cells or myeloid cells (Wu et al., 2014). In the circulation, NK cells constitute 5–15% of peripheral blood lymphocytes in adult healthy individuals and can be detected at variable levels in peripheral tissues such as in the liver and the lung (Cerwenka and Lanier, 2001).

Typically, NK cells are involved in the first line of defense against infection and cancer. NK cells were discovered in the 1970s as large granular lymphocytes distinct to B and cytotoxic T lymphocytes with the ability to kill virus-induced murine leukemia cells without the need for prior sensitization to these target cells (Herberman and Ortaido, 1981; Kiessling et al., 1975). NK cells distinguish stressed, transformed and infected cells from healthy cells through an array of germline-encoded inhibitors, activating and adhesion receptors expressed on their cell surface (Vivier et al., 2011). In contrast to NK cells, adaptive T and B lymphocytes acquire a broad repertoire of antigen specificities by RAG recombinase-driven somatic recombination of their T and B cell receptor genes. Acquisition of NK cell receptor genes is independent of gene rearrangements. Yet, RAG proteins appear to play a critical role in NK cell functionality, since the lack of RAG1/2 activity during ontogeny affects genome stability and susceptibility to apoptosis of murine NK cells (Karo et al., 2014).

In the classical model of NK cell activation, NK cells are defined to respond to target cells with a reduced expression of MHC class I molecules, or an incomplete or incompatible repertoire of MHC class I molecules; a concept termed the ‘missing-self hypothesis’ (Karre et al., 1986; Vivier et al., 2008). Accordingly, NK cells recognize cells with a ‘non-self’ history such as from an allogeneic or haploidentical hematopoietic stem cell transplant. NK cells respond to virus-infected and malignant cells that frequently have reduced MHC class I expression. In addition, the responsiveness of NK cells is modulated by a complex spectrum of inhibiting and activating signals from target and accessory cells and their pro- and anti-inflammatory microenvironment (Fig. 1). Upon activation towards target cells, NK cells release cytotoxic proteins from pre-formed cytoplasmic granules by exocytosis into the immunological synapse at the NK–target cell interface (Krzeski and Strominger, 2008). After entry into the cytoplasm via the pore-forming protein perforin, members of the granzyme family of serine-proteases mediate target cell apoptosis through caspase-dependent and -independent pathways. In addition, target cell apoptosis can be mediated by Fas ligand or ‘necroptosis’-factor-related apoptosis-inducing ligand (TRAIL) expressed on the cell surface or released from the cytoplasmic granules of NK cells (Smyth et al., 2005a,b). In this context, PTEN was shown to be a negative regulator of NK cell cytotoxicity by limiting actin accumulation, polarization of the microtubule organizing center, and the convergence of cytoplasmic granules at the NK–target cell interface (Brierecheck et al., 2015).

NK cells are considered to bridge innate and adaptive immunity by the secretion of IFN-γ, which enhances MHC class I expression on tumor cells and MHC class II expression on antigen-presenting cells like monocytes/macrophages and dendritic cells (Vivier et al., 2008). Aside from their role in initial responses against infection and cancer, it has become evident, that NK cells also contribute to the induction of adaptive anti-cancer T cell as well as B cell responses (Diefenbach et al., 2001; Kelly et al., 2002; Krebs et al., 2009; Smyth et al., 2005a,b). In addition, NK cells can exert immunoregulatory functions under certain conditions. Several reports have shown that NK cells control the number of dendritic cells and activated CD4 and CD8 T cells and constrain the formation of memory T and B cell responses, as observed in murine lymphocytic choriomeningitis virus and cytomegalovirus infection models (Crouse et al., 2014; Ferlazzo et al., 2002; Narni-Mancinelli et al., 2012; Rydzynski et al., 2015; Schuster et al., 2014; Soderquest et al., 2011; Waggoner et al., 2012, 2014; Xu et al., 2014). Hence, to some extent NK cells are able to prevent excessive immune activation and autoimmune pathology. Their classification as solely innate immune cells is currently further challenged since there is now evidence that under certain conditions NK cells can acquire similarities to adaptive immunity such as expansion of specific subsets and antigen-specific responses, as will be further discussed below (Sun et al., 2014).

**Regulation of NK cell activity**

During development, NK cells that fail to express inhibitory receptors to at least one ‘self’ MHC class I type are rendered anergic to prevent reactivity against healthy ‘self’ cells; a concept referred to as ‘education’ or ‘licensing’ (Anfossi et al., 2006; Kim et al., 2005). NK cells that express inhibitory receptors in combination with activating receptors are able to react against abnormal ‘non-self’ cells. Transfer of NK cells from an MHC class I-sufficient mouse to an MHC class I-deficient mouse (and vice versa) can reset NK cell responsiveness (Elliott et al., 2010; Joncker et al., 2010). Hence, the fate of reactivity or hyporesponsiveness of mature NK cells appears to be continuously modulated by trafficking through environments with changing levels of inhibitory molecules. Consistent with this hypothesis, persistent failure of engaging inhibitory receptors in an MHC class I-deficient tumor microenvironment reduces NK cell responsiveness unless NK cell are re-stimulated with NK cell-activating cytokines like interleukin-2 (IL-2) or IL-12/18 (Ardolino et al., 2014).

Target cell recognition by NK cells is regulated by the net input of inhibitory and activating signals perceived through NK cell receptor and target cell ligand interactions (Gasser and Raulet, 2006; Moretta et al., 2006). Thus, lysis of cancer cells is triggered by low expression of ligands for NK cell inhibitory receptors, such as killer cell immunoglobulin-like receptors (KIR), TIGIT and CD96, in combination with induced/increased expression of ligands for NK cell activating receptors, such as NKG2D, DNAM-1 and ‘natural cytotoxicity receptors’ (NCR). HLA-A/B/C molecules bind to inhibitory KIR receptors (Thielen et al., 2012). The non-classical HLA-E molecule, presenting MHC class I-derived leader peptides, binds to the lectin-like inhibitory CD94–NKG2A receptor complex as well as to the activating CD94/NKG2C receptor complex (Braud et al., 1998; Kabat et al., 2002). The inhibitory KIR receptors and NKG2A contain cytoplasmic ‘immunoreceptor tyrosine-based inhibitory motif’ (ITIM). NK cells express numerous activating receptors that engage ‘stress’-induced ligands. These ligands are normally not expressed on healthy cells under non-inflammatory conditions but can be induced, for instance, in response to DNA damage as demonstrated for NKG2D ligands (Gasser et al., 2005). Human NKG2D binds to ‘MHC class I chain-related genes’ MICA and MICB as well as to the ‘ULBPI-binding proteins’ ULBP1–6 and signals through the adapter protein DAP10 (Bacon et al., 2004; Bauer et al., 1999; Chalupny et al., 2003; Cosman et al., 2001; Eagle et al., 2009). Murine NKG2D binds to its murine ligands retinoic acid early inducible 1 (Rae1), ULBP-like transcript (MULT-1) and minor histocompatibility antigen H60 and signals through DAP10 and DAP12 (Cerwenka et al., 2000; Diefenbach et al., 2000; Ullrich et al., 2013). The costimulatory adhesion receptor DNAM-1 binds to CD112 and CD155 and recruits the tyrosine kinase Fyn and the serine–threonine kinase PKC (Bottino et al., 2003; Martinet and Smyth, 2015). Activation through DNAM-1 is counteracted by the inhibitory receptors TIGIT (ITIM motif) and CD96 (ITIM-like motif), sharing the common ligands CD112/CD155 and CD155, respectively (Chan et al., 2014; Stanietzky et al., 2009). B7–H6 was the first identified
Fig. 1. Regulation of NK cell cytotoxicity during cancer immunosurveillance. NK cells interact with cancer cells through activating (in green) and inhibitory (in red) NK cell receptors with cognate activating (in green) and inhibitory (in red) ligands on target cells. A surplus of activating signals induces the release of perforin and granzymes. Target cell apoptosis is also induced via Fas ligand (Fas-L) and TRAIL expressed on NK cells. In addition, NK cells can kill antibody-coated tumor cells via FcγRIIIa/CD16, referred to as antibody-dependent cellular cytotoxicity (ADCC). During favorable pro-inflammatory conditions, activating cytokines (IL-15, IL-18, IFN-α; IL-2), produced by e.g. dendritic cells and CD4+ T cells, stimulate the cytotoxic function of NK cells. In the cancer microenvironment, NK cells are inhibited by a surplus of inhibitory ligands on tumor cells. Tumor-promoting and immunosuppressive conditions are generated by the presence of TGF-β, IL-10, prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF), nitric oxide (NOS) and reactive oxygen species (ROS), produced by immunosuppressive immune cells like regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC) and type 2 macrophages (MΦ). Soluble activating ligands shed from tumor cells impair NK cell activating receptors, favoring tumor cell escape from NK cell immunosurveillance. The following activating (in green) and inhibiting (in red) receptor-ligand interactions are depicted: NKG2D–MICA/B/ULBP1–6, DNAM-1–CD112/CD155, NKP30–87-H6, NKP44–NKP44-ligand(L); killer cell immunoglobulin-like receptor (KIR)–MHC class I molecules, NKG2A–HLA-E, CD96/TIGIT–CD155. Activating cytokines and inhibiting cytokines/tumor-promoting factors are depicted in green and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cellular ligand for an NCR expressed on the surface of cancer cells, binding to the activating receptors NKP30 (Brandt et al., 2009). Recently, a novel isofrom of the mixed-lineage leukemia-5 (MLL5) nuclear protein was proposed as a cancer cell-expressed ligand for NKP44 (Baychelier et al., 2013). However, how MLL5 is transported from the nucleus to the cell surface and how it is inserted into the cell membrane is unknown. NKP46 has been described to bind viral structural motifs (Arnon et al., 2004). The NCRs associate with ‘immunoreceptor tyrosine-based activating motifs’ (ITAM) and signal through CD3ζ and FcεRγ (Vivier et al., 2011). In addition to their ‘natural’ cytolytic activity, NK cells can kill antibody-coated cancer cells via the recognition of the Fc portion of antibodies by the NK cell-activating receptor FcγRIIIa/CD16 (de Landazuri et al., 1979; Lanier et al., 1988; Vivier et al., 1991).

NK cell activation is triggered by pro-inflammatory cytokines. IL-2, and IL-12, IL-15, IL-18, IFN-α produced during infection by CD4+ T cells and dendritic cells, respectively, promote the proliferation of NK cells and ‘prime’ their cytolytic activity and IFN-γ release. In addition to IL-12/IL-18, the CD160-HVEM axis has been suggested to be critical for the production of IFN-γ by murine NK cells and IFN-γ-dependent tumor rejection (Tu et al., 2015). IL-15, presented by dendritic cells in trans through surface IL15Rα, is crucial for NK cell development, survival and effector function (Huntington et al., 2007; Koka et al., 2003; Lucas et al., 2007). Regulatory T cells can control NK cell reactivity and proliferation by limiting the availability of IL-2 to NK cells due to their high expression of high affinity IL-2Rα (CD25), which is largely absent on resting mature human NK cells (Gasteiger et al., 2013).
Indications for adaptive immune properties of NK cells

Pre-activation of NK cells by the cytokine combination of IL-12/IL-15/IL-18 triggers CD25 up-regulation on NK cells and thus responsiveness to IL-2; furthermore, IL-12/IL-15/IL-18 improve and prolong the cytolytic and cytokine-producing potential of NK cells (Cooper et al., 2009; Leong et al., 2014; Ni et al., 2012; Romaine et al., 2012). This activation confers long-lasting enhanced effector functions and amplified responses to restimulation; characteristics previously only ascribed to adaptive T and B lymphocytes (Sun et al., 2014; Vivier et al., 2011).

In the human system, expansion and long-term persistence of a CD57+ NKG2C+ NK cell subset has been observed in individuals after infection and reactivation with human cytomegalovirus (HCMV) and is associated with epigenetic modifications in NK cell receptor genes (Foley et al., 2012; Lee et al., 2015; Lopez-Verdes et al., 2011; Luette-Eversloh et al., 2014; Schlums et al., 2015). This phenomenon may be based on a state of increased NK cell activation but is also proposed to reflect properties of adaptive lymphocytes. Expansion of the NKG2C subset after culture with HCMV-infected fibroblasts was shown to be mediated by monocytes, IL-12 and HLA-E expression on the infected cells (Rolle et al., 2014). In addition to NKG2C+ NK cell, expansion of NKG2C-negative NK cells with a distinctive activating KIR repertoire has been observed in HCMV-infected individuals (Beziat et al., 2013; Della Chiesa et al., 2014).

The seminal observation that at least murine NK cells are indeed able to acquire properties of adaptive immunity and memory was initially observed when hapten-specific contact hypersensitivity responses could be transferred from hapten-sensitized mice to naïve mice in a T and B cell-independent manner mediated by a subset of liver-resident NK cells (O’Leary et al., 2006). In a murine model of cytomegalovirus infection it was demonstrated that NK cells, activated after specific recognition of the m157 viral glycoprotein, exhibited the capability for expansion, contraction, long-time persistence and superior responses to secondary challenges in a virus-specific manner (Sun et al., 2009). This activation program requires Ly49H-DAP10 signaling (recognizing m157), DNAM-1 signaling, Bi-mediated apoptosis during the contraction phase, and pro-inflammatory signaling via IL-12, IL-18—MyD88 and the transcription factor Zbtb32 during the proliferation phase (Beaulieu et al., 2014; Min-Oo et al., 2014; Nabekura et al., 2014; Sun et al., 2012). Likewise, antigen-specific activation of NK cells was demonstrated in mice challenged with influenza virus-like particles or vesicular stomatitis virus (Paust et al., 2010). Hence, there is evidence that under certain conditions murine NK cells can acquire properties reminiscent of adaptive lymphocytes and memory. However, it remains to be determined whether human NK cells can possess ‘memory’ of infection. Whether NK cells remember exposure to cancer cells is unknown.

Evidence for NK cells in cancer immunosurveillance

A role for NK cells in the rejection of transplanted hematopoietic tumors or chemically-induced tumors was demonstrated in various mouse models, in which NK cells prevented tumor outgrowth and supported the formation of primary and secondary tumor-specific CD8 and CD4 T cell responses (Cerwenka et al., 2001; Dieffenbach et al., 2001; Kelly et al., 2002). Besides significant cytolytic activity of human NK cells against cancer cells in vitro, evidence for anticancer responses by NK cells in humans has been derived from a number of clinical studies. Importantly, individuals with low NK cell cytotoxic activity have a higher incidence of cancer, implying that NK cells interfere with cancer development (Imai et al., 2000). The biological relevance of the anti-cancer potential of NK cells was pointed out by the finding that, after haploidentical hematopoietic stem cell transplantation, alloreactive NK cells (generated from the donor graft in the recipient) support graft-versus-tumor effects and reduce leukemia recurrence in patients with acute myeloid leukemia (ALL) (Ruggeri et al., 2002). In this setting, NK cells are thought to become reactive to the tumor cells due to the incomplete ‘self’ HLA class I repertoire of the recipient’s tumor cells as proposed by the ‘missing-self hypothesis’.

Therapeutic antibodies like rituximab (anti-CD20), cetuximab (anti-epidermal growth factor receptor (EGFR)) and trastuzumab (anti-Her2/neu) improve the outcome of patients with B cell malignancies, colorectal and head-neck cancer, and breast cancer, respectively (Kirkwood et al., 2012). In addition to their direct anti-tumor effects, therapeutic antibodies can induce robust antibody-dependent cytotoxicity by NK cells from cancer patients against autologous tumor cells (Pahl et al., 2012). A functional role for CD16+ NK cells (and CD16+ macrophages) in the clinical effects of therapeutic antibodies has been suggested by the finding that patients with the 158V/158F gene polymorphism in FcγRIIa/CD16 exhibited the most favorable outcome; this gene polymorphism confers higher affinity to the antibody Fc portion and enhances NK cell cytolytic function (Bibeaux et al., 2009; Cartron et al., 2002; Dall’Ozzo et al., 2004; Koene et al., 1997; Musolino et al., 2008; Vance et al., 1993; Weng and Levy, 2003; Zhang et al., 2007). Moreover, the clinical response to antibody-based and tyrosine kinase inhibitor-based immunotherapy has been shown to positively correlate with NK cell activation, NK cytoxicity and tumor infiltration by NK cells in certain cancer entities (Arnould et al., 2006; Marechal et al., 2010; Rusakiewicz et al., 2013; Veeramani et al., 2011). Hence, multiple lines of evidence propose a direct role for NK cells in anti-cancer responses in humans and propose NK cells as attractive targets for immunotherapy.

Tumor escape from NK cells immunosurveillance

High numbers of tumor-infiltrating NK cells and cytotoxic T cells are often associated with a better prognosis for cancer patients (Stojanovic and Cerwenka, 2011). It is conceived that NK cells preferentially mount responses against hematological malignancies or cancer metastasis which are more easily accessible through the circulation. In contrast, solid cancers frequently lack adequate numbers of NK cells. During the development of large solid tumors, NK cells (and cytotoxic T cells) are frequently rendered functionally exhausted as a consequence of ‘cancer immunoeediting’ (Schreiber et al., 2011). In agreement with this model, defective cytotoxicity and reduced receptor expression was observed in tumor-associated and peripheral blood NK cells of cancer patients (prior to anti-cancer therapies) (Carlsten et al., 2009; Costello et al., 2002; Fauriat et al., 2007; Le Maux Chansac et al., 2005; Lee et al., 2004).

The inhibitory character of solid tumors is imposed by a complex composition of immunosuppressive molecules, such as transforming growth factor-β (TGF-β), IL-10, indoleamine 2,3-deoxygenase (IDO), prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF), nitric oxide (NOS) and reactive oxygen species (ROS), produced by regulatory immune cells, such as regulatory T cells, myeloid derived suppressor cells (MDSC) and type 2 macrophages and by tumor cells themselves (Vitale et al., 2014). These factors generate a chronic inflammatory and immunosuppressive milieu that supports tumor progression.

Tumor cells can interfere with the expression and function of several NK cell-activating receptors like NKG2D. This inhibition may in some cases be mediated by chronic tumor cell ligand–NK cell receptor engagement or tumor exosome-bound TGF-β (Clayton et al., 2008; Coudert et al., 2005, 2008; Oppenheim et al., 2005; Pahl et al., 2013). Melanoma cells and tumor-accessory fibroblasts were
shown to inhibit NK cell function by bivalent receptor–ligand interaction and soluble IDO and PGE2 (Balsamo et al., 2009; Carlsten et al., 2009; Pietra et al., 2012). Moreover, hypoxia reduces cancer cell susceptibility to NK-mediated lysis by a mechanism involving the activation of autophagy and the subsequent degradation of NK-derived granzyme B (Baginska et al., 2013; Messai et al., 2014).

Proteasome inhibitors, histone deacetylase inhibitors (HDACi) and certain chemotherapeutics have been shown to increase the expression of NKGD2 ligands on tumor cells and tumor cell sensitivity to NK cell recognition, whereas in fact HDACi decrease Nkp30 ligand expression (Fiegler et al., 2013; Ullrich et al., 2013).

Proteolytic shedding of NKGD2 and Nkp30 ligands (e.g. MICA, B7-H6, BAG6) from the tumor cell surface has been discussed to render tumor cells less sensitive to NK cell recognition; soluble ligands act as decoys, mask or down-regulate NK cell receptors and in some cases their amount in cancer patient sera correlates with poor outcome (Groh et al., 2002; Hilpert et al., 2012; Reiners et al., 2013; Salih et al., 2002; Schlecker et al., 2014). However, the concept of a solely inhibitory character of soluble ligands has been challenged in the context of murine MULT-1 (Deng et al., 2015): MULT-1 shed from tumor cells improved NK cell functionality and tumor rejection in mice by competing for NKG2D binding with Rae-1 expressed on myeloid cells in the tumor microenvironment. In this scenario, soluble MULT-1 appeared to be rather activating, presumably by relieving NK cells from immunosuppression (due to chronic Rae-1 engagement), leading to normal NKG2D expression.

The influence of the tumor microenvironment on immune responses is well established. As another influencing parameter, there is increasing evidence that the composition of the gut microbiota shapes anti-tumor responses. Whereas some bacteria predispose to tumor development, disruption of the microbiota by antibiotics was argued to compromise the response to chemotherapy and immunotherapy (Garrett, 2015; Viaud et al., 2014). It has recently been described that Fusobacteria nucleatum, associated with colon adenocarcinoma, inhibits NK cell cytotoxicity against tumor cells by engaging the NK cell inhibitory receptor TIGIT, indicating an additional layer of immunosuppression (Gur et al., 2015).

Altogether, NK cell responses against established and advanced solid tumors are often insufficient. In view of harnessing the full cytolytic potential of NK cells for cancer immunotherapy at least two prerequisites for effective NK cell anti-cancer responses are required: to improve targeting and infiltration of tumors by NK cells, and to restore and improve the quality of NK cell functionality.

**NK cell-based cancer immunotherapy**

**Checkpoint inhibition and therapeutic antibodies**

Cancer immunotherapy provides a multitude of potent and more specific treatments for cancer patients (Mellman et al., 2011). Antibody-mediated therapeutic inhibition of the T cell-inhibitory receptors CTLA-4 by ipilimumab and PD-1 by nivolumab, referred to as checkpoint blockage, disrupts T cell tolerance, boosts anti-tumor responses and improves patient prognosis (Hodi et al., 2010; Topalian et al., 2012). Combination of these antibodies probably further advances treatment outcome (Wolchok et al., 2013). Still, in many patients tumors evade from T cell recognition due to MHC class I down-regulation, engagement of other inhibitory T cell and (NK cell) receptors (e.g. TIGIT, CD96, Tim-3), lack of adequate (neo-)antigens, exclusion of anti-tumor immune cells and the presence of inhibitory accessory cells. Therefore, additional strategies are necessary to aid T cell-based immunotherapeutic approaches but also to control T cell resistant tumors.

Analogous to counteracting T cell anergy and exhaustion, antibody-mediated checkpoint blockage of inhibitory NK cell KIR receptors by an anti-KIR blocking antibody (lirilumab) is currently tested in clinical trials (Kohrt et al., 2014). This approach reduces the threshold for NK cell cytotoxicity against target cells, counteracts NK cell inactivation during cancer progression and exploits the concept of ‘missing-self’ recognition. It has been reported that inhibition of the TAM tyrosine kinase receptors (Tyro3, Axl and Mer) in NK cells can potentiate the anti-metastatic potential of NK cells in mice (Paulino et al., 2014). Blockage of CD96 improved NK cell function in mice (Chan et al., 2014). Moreover, rituximab and trastuzumab–mediated ADCC was augmented in preclinical studies by cross-linking of the activating receptor CD137, which is induced on NK cells upon ADCC in vivo (Kohrt et al., 2011, 2012).

Tumor-reactive therapeutic antibodies, binding to tumor surface antigens, enhance the susceptibility of tumor cells to NK cell cytotoxicity even if the tumor cells are weakly sensitive to antibody-independent NK cell cytotoxicity (Gennari et al., 2004; Gerdes et al., 2013; Veeramani et al., 2011). It has been suggested that therapeutic antibodies may mobilize the cytotoxic potential of those NK cells which are functionally sufficient for Fcγlla/CD16-dependent lysis but deficient for antibody-independent lysis, as a result of NK cell education or tumor encounter (Pahl et al., 2013; Parkhurst et al., 2011; Tarek et al., 2012). Importantly, NK cell activation by anti-EGFR-coated cancer cells stimulates IFN-γ release and triggers dendritic cell maturation and the priming of tumor antigen-restricted (EGFR and MAGE-3) CD8 T cell responses in vitro; in patients with head-neck cancer, anti-EGFR cetuximab therapy was associated with the accumulation of EGFR-specific CD8 T cells (Srivastava et al., 2013). Thus, in response to antibody therapy, NK cells may contribute to the generation of systemic anti-cancer T1 responses against in vivo-selected cancer epitopes. In particular, neoantigens are considered ideal targets for mobilizing anti-tumor T cell responses against tumor-specific antigens through cancer vaccination or engineered T cells (Carreno et al., 2015; Kreiter et al., 2015; Schumacher and Schreiber, 2015). As compared to T cells, NK cells have limited capacity for proliferation, survival, and eradication of large tumor masses. Yet, a critical contribution of NK cells in initiating T cell immunity for tumor control and for the efficacy of antibody therapy was emphasized in preclinical studies (Abes et al., 2010; Park et al., 2010; Yang et al., 2013). As a compromising byproduct, however, anti-EGFR cetuximab therapy was reported to additionally result in the accumulation of CTLA-4+ regulatory T cells associated with a less functional phenotype of NK cells in the tumor and a poor patient response to treatment (jie et al., 2015). In vitro, cetuximab–activated NK cells preferentially triggered the proliferation and expansion of regulatory T cells in the presence of TGF–β and dendritic cells. These findings underscore the need for combinations of synergistic immunotherapies, such as anti-CTLA-4 and tumor-targeted therapy, in order to counteract inhibitory effects and enhance anti-tumor responses.

**Adaptive transfer of NK cells**

To potentiate NK cell activity, the application of IL-2 in patients has remained challenging because high doses of IL-2 can result in serious adverse effects and expand regulatory T cells (Geller and Miller, 2011). As an alternative, NK cells can be (re-)activated ex vivo and used for adaptive cell transfer therapy (Ardolino et al., 2014; Parkhurst et al., 2011). In the case of T cells, adaptive transfer using autologous tumor-reactive T cells (e.g. anti-MART-1) and chimeric antigen receptor (CAR) T cells (e.g. anti-CD19–CD3ζ–CD28) achieved significant clinical responses in some patients with advanced melanoma or B cell malignancies (Maus et al., 2014; Rosenberg and Restifo, 2015). These T cells, however, fail to control epitope-negative variants and have the potential for long-time adverse effects on epitope-positive non-malignant cells. Similar to CAR T cells, genetically-engineered CAR NK cells are currently
explored to more specifically direct NK cell cytotoxicity toward cancer cells (Chu et al., 2014; Schonfeld et al., 2015). Analogous to therapeutic antibodies, this approach enables the killing of cancer cells which are otherwise poorly susceptible to NK cell recognition in addition to ‘natural’ cytotoxicity against epitope-negative cells.

Adaptive transfer of ex vivo cytokine-activated autologous or haploidentical NK cells resulted in favorable responses in a subset of pediatric and adult patients with hematological malignancies without causing graft-versus-host disease in the recipients (Curti et al., 2011; Geller and Miller, 2011; Rubnitz et al., 2010). Objective clinical responses in patients with solid tumors have so far not been documented, presumably due to the complex immunosuppressive character of solid tumors (Geller and Miller, 2011). In fact, in melanoma patients, IL-2-activated (autologous) NK cells lose cytotoxic activity after adaptive transfer unless re-activated ex vivo (Parkhurst et al., 2011). In mice NK cell functionality after adaptive transfer is augmented and prolonged by pre-activation with a cytokine cocktail of IL-12, IL-15 and IL-18; this superior activation required the presence of CD4 T cells and endogenous IL-2 production by the T cells (Cooper et al., 2009; Ni et al., 2012). A clinical trial applying human NK cells pretreated with these cytokines is currently ongoing in patients with acute myeloid leukemia (clinical trial no. NCT01520558).

Strategies to counteract immunosuppression

Disruption of immunosuppression may be suitable to sustain NK cell activation in the cancer microenvironment. Depletion of M2-type macrophages or regulatory T cells has been suggested to counteract immunosuppression and tumor progression (Germano et al., 2013; Morse et al., 2008; Qian et al., 2011). An acute infection or infection-mimicking immunostimulators may facilitate the conversion to a more pro-inflammatory milieu that supports recruitment and anti-cancer activity of immune cells (Matzinger, 2002). Oncolytic viruses are able to selectively kill cancer cells, trigger immune cell recruitment and induce anti-cancer (and anti-viral) responses by NK cells and T cell (Alvarez-Breckenridge et al., 2012; Breitbach et al., 2011; Heo et al., 2013; Prestwich et al., 2009). Furthermore, oncolytic viruses have been found to synergize with immunotherapies in pre-clinical models, resulting in potent systemic anti-tumor responses by NK cells and T cells (Parrish et al., 2015; Zamarin et al., 2014).

For the success of NK cell-based immunotherapy, it will be necessary to improve the recruitment and infiltration of NK cells, which is poor in solid cancers but in many studies positively associated with patient outcome (Rusakiewicz et al., 2013). Oncolytic virotherapy can improve the infiltration of murine NK cells and T cells even at tumor sites devoid of local virus replication (Zamarin et al., 2014). Induction of chemokines like chemerin and CXCR3 ligands has been emphasized to improve NK cell infiltration and NK cell-dependent tumor ablation in mice (Pachynski et al., 2012; Wendel et al., 2008). Moreover, NK cells deficient in PTEN showed improved survival, proliferation and trafficking of murine NK cells to tumor sites (Leong et al., 2015). Antibody-based immunotherapy and tyrosine kinase inhibitor-based immunotherapy enhance tumor infiltration of NK cells in mice (Gennari et al., 2004; Rusakiewicz et al., 2013; Veeramani et al., 2011).

Conclusion

In conclusion, multiple approaches have been under investigation to uncover and exploit mechanisms to sustain and improve anti-tumor functionality of NK cells. In the future, elucidation of (inhibitory) checkpoints in NK cell activation will advance strategies to counteract immunosuppressive pathways in the cancer microenvironment. Tumor penetration has remained one major hurdle, warranting novel strategies to enhance the recruitment, targeting and infiltration of NK cells into solid cancers. In view of introducing highly tumor-reactive NK cells in cancer patients, it will be necessary to establish the appropriate timing for adoptive cell transfer. This could minimize suppressive effects on NK cell functionality by conventional anti-cancer therapies, which may be exploited to achieve smaller tumor masses as found in conditions of minimal residual disease. Collectively, enhancing and prolonging NK cell activation and tumor penetration remain major challenges for advanced NK cell-based immunotherapies for cancer patients that could possibly be solved by combination therapies.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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