Title

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Permalink

https://escholarship.org/uc/item/3k45b7m5

Journal

Annual Review of Cancer Biology, 3(1)

ISSN

2472-3428

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Publication Date

2019-03-04

DOI

10.1146/annurev-cancerbio-030518-055653

Peer reviewed

ANNUAL REVIEWS

Annual Review of Cancer Biology

Natural Killer Cells in Cancer Immunotherapy

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Keywords

natural killer cell, immunotherapy, inhibitory receptor, IL-15, IL-2

Abstract

Natural killer (NK) cells have evolved to complement T and B cells in host defense against pathogens and cancer. They recognize infected cells and tumors using a sophisticated array of activating, costimulatory, and inhibitory receptors that are expressed on NK cell subsets to create extensive functional diversity. NK cells can be targeted to kill with exquisite antigen specificity by antibody-dependent cellular cytotoxicity. NK and T cells share many of the costimulatory and inhibitory receptors that are currently under evaluation in the clinic for cancer immunotherapy. As with T cells, genetic engineering is being employed to modify NK cells to specifically target them to tumors and to enhance their effector functions. As the selective pressures exerted by immunotherapies to augment CD8^{+} T cell responses may result in loss of MHC class I, NK cells may provide an important fail-safe to eliminate these tumors by their capacity to eliminate tumors that are "missing self."

INTRODUCTION

The ability of lymphocytes to mediate natural killing of certain tumors and virus-infected cells was first discovered by several laboratories in the 1970s (Herberman et al. 1975, Kiessling et al. 1975, Ortaldo et al. 1977). This activity is predominantly mediated by natural killer (NK) cells, which are the founding members of the innate lymphoid cell (ILC) family (Spits et al. 2016), which includes NK cells, ILC1, ILC2, and ILC3. NK cells and all ILCs are distinct from T cells in that they do not productively rearrange or express T cell antigen receptor genes, and they develop in mice lacking the *Rag1* and *Rag2* genes required for the development of T cells and B cells. While NK cells are most similar in function to CD8$^+$ cytotoxic T lymphocytes in their ability to directly recognize and kill tumors and pathogen-infected cells by mechanisms using granzymes, perforin, and TNF family death effector molecules (Sun & Lanier 2011), ILC1, ILC2, and ILC3 are considered functional counterparts of the Th1, Th2, and Th17 subsets of CD4$^+$ T cells based on their secretion of characteristic cytokines and the requirement of certain transcription factors for development (Klose & Artis 2016). NK cells and ILCs derive from a common progenitor cell, with NK cell development being dependent on interleukin (IL)-15-mediated signaling, whereas ILCs require IL-7-mediated signaling (Seillet et al. 2016). Although most of the innate immune responses to tumors have previously been attributed to NK cells, ILC1 have also been implicated in antitumor immunity in some cases (Dadi et al. 2016). As ILC1 and NK cells share many phenotypic properties and are often difficult to distinguish based on cell surface markers (Spits et al. 2016), both NK cells and ILCs should be investigated in future studies of cancer immunity. Indeed, tumors may avoid NK cell–mediated attack by secreting transforming growth factor beta (TGFβ), which might convert NK cells into noncytolytic ILC1-like cells that promote tumor survival (Cortez et al. 2017, Gao et al. 2017). Although NK cells likely evolved to provide host defense against viruses and other microbial pathogens, they can use the same receptors and effector molecules to also discriminate between healthy and transformed cells, thus providing a barrier to cancer [reviewed by Lopez-Soto et al. (2017), Malmberg et al. (2017), and Morvan & Lanier (2016)].

The best-characterized subsets of NK cells in humans are identified as immature CD3$^-$CD56^{bright} CD16$^-$ cells and mature CD3$^-$CD56^{dim}CD16$^+$ cells within the lymphoid population. The immature NK cells are most responsive to activation by IL-2, IL-15, and IL-12 to induce the production of interferon gamma (IFNγ) and other factors, whereas mature NK cells are less responsive to cytokine stimulation alone but have preformed stores of granzymes and perforin to quickly mediate potent cytolytic function. However, mature NK cells can produce abundant IFNγ and other cytokines after triggering by activating NK receptors. Recent studies have revealed that NK cells are remarkably diverse. Using mass cytometry analysis and combining antibodies against 37 markers expressed by NK cells, Blish and colleagues estimated up to 30,000 phenotypic subsets of NK cells in the peripheral blood of healthy humans (Horowitz et al. 2013; Strauss-Albee et al. 2014, 2015). Much of this heterogeneity may reflect different developmental states and activation statuses of the circulating NK cells, rather than stable, functionally distinct subsets. In mice, NK cells are identified as CD3$^-$NKp46$^+$ lymphocytes or, more commonly in C57BL/6 mice, as CD3$^-$NK1.1$^+$ lymphocytes. Many of the receptors expressed by NK cells are also present on ILC1, although high levels of CD200R1 expression on ILC1 may distinguish them from NK cells, which express lower amounts of CD200R1 (Weizman et al. 2017). Studies in mice and humans have identified subsets of NK cells that possess immunological memory and have been termed "memory" or "adaptive" NK cells (O'Sullivan et al. 2015). In humans, this subset of adaptive NK cells that have been expanded by cytomegalovirus (CMV) infection has been shown to lack expression of the FcεRIγ signaling protein (Zhang et al. 2013) and to have undergone epigenetic modification, resulting in NK cells with enhanced antibody-dependent cellular
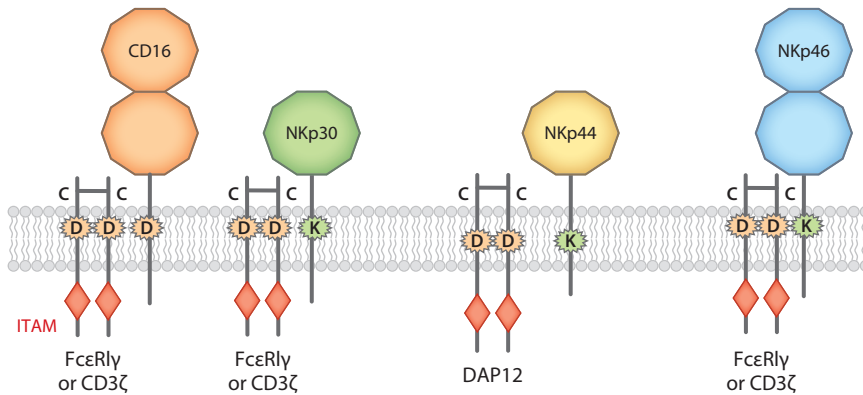
Figure 1

Immunoreceptor tyrosine-based activation motif (ITAM)-based activating natural killer (NK) receptors.
NK cells express several receptors that transmit intracellular activating signals by their association with
ITAM-bearing adapter proteins, including FcεRIγ, CD3ζ, and DAP12. Upon receptor engagement, these
adapters recruit the Syk or ZAP70 tyrosine kinases and initiate downstream activation, similar to the T cell
antigen receptor or surface immunoglobulin in T and B cells.

cytotoxicity (ADCC) function (Lee et al. 2015, Schlums et al. 2015). Here, we review how NK
cells recognize tumors, as well as the current immunotherapeutic strategies to engage them in
cancer treatment.

NK CELL RECOGNITION

Immunoreceptor Tyrosine-Based Activation Motif–Based Activating NK Receptors

Early studies of NK cells revealed that they express the CD16-activating Fc receptors that allow
them to kill immunoglobulin G (IgG)-coated tumors by the process of ADCC. CD16 signals by
association with the immunoreceptor tyrosine-based activation motif (ITAM)-containing FcεRIγ
signaling adapter in humans and mice, as well as with CD3ζ in humans (**Figure 1**) (Bournazos
et al. 2017, Kurosaki & Ravetch 1989, Lanier et al. 1989). Upon engagement of CD16 by IgG
bound to target cells, FcεRIγ and CD3ζ are phosphorylated and recruit Syk and ZAP70, resulting
in downstream signaling using the same pathway as T cell receptors on T cells and surface Ig on
B cells. As discussed below, many of the existing cancer therapeutic antibodies (e.g., rituximab,
daratumumab) may rely on NK cells for their efficacy, and other therapeutic agents target-
ing tumors are under development to engage CD16 with bispecific antibodies or other agents
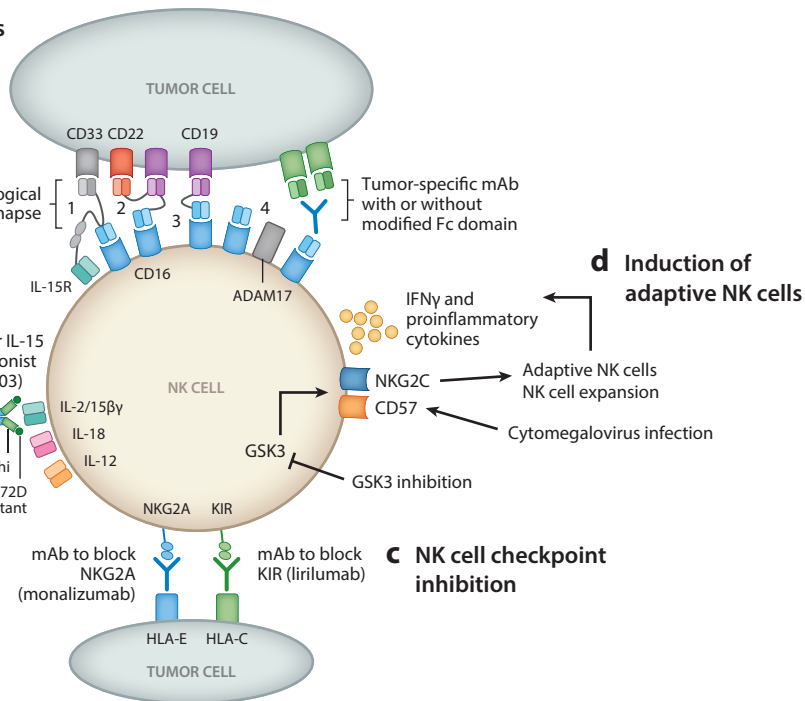(**Figure 2**).

Other activating receptors that use ITAM-induced signaling pathways to activate NK cells
include the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 (**Figure 1**). Like
CD16, NKp30 (Pende et al. 1999) and NKp46 (Pessino et al. 1998) associate with and signal
through FcεRIγ and CD3ζ, whereas NKp44 (Vitale et al. 1998) associates with and signals through
another ITAM-bearing adapter, DAP12 (Lanier 2009). NKp46 and NKp30 are constitutively
expressed on NK cells, whereas NKp44 is only expressed after activation, for example by IL-2
(Vitale et al. 1998). Using in vitro assays, antibodies against NKp30, NKp44, and NKp46 have been
shown to partially inhibit human NK cells killing certain tumor cell lines, implying the existence of
NCR ligands on tumors. However, the precise nature of these NCR ligands on tumors has proven

a Design of CD16 target agents to increase cytotoxicity

1. TriKEs that incorporate an IL-15 linker
2. TriKEs that bind two tumor antigens
3. BiKEs
4. ADAM17 inhibitors

b Use of cytokines to expand NK cells and increase their activation

c NK cell checkpoint inhibition

**d Induction of adaptive NK cells**

Figure 2

There are many strategies available to enhance natural killer (NK) cell antitumor activity in adoptive immunotherapy: (*a*) engaging activating receptors through tumor-specific monoclonal antibodies (mAbs), bispecific killer engagers (BiKEs) or trispecific killer engagers (TriKEs) that link CD16 on NK cells to tumor antigens, and a disintegrin and metalloprotease 17 (ADAM17) inhibitor that prevents CD16 shedding in order to increase cytotoxicity of NK cells; (*b*) using cytokines, particularly interleukin 15 (IL-15), to expand NK cells ex vivo and using ALT-803, which binds to IL-15, to increase NK cell activation; (*c*) blocking killer cell immunoglobulin-like receptors (KIRs) to release inhibitory signals and activate NK cells; and (*d*) expanding NK cells through inhibition of glycogen synthase 3, which promotes NKG2C^{++} and CD57$^+$ NK cells and increases NK cell activation.

elusive, with the exception of the B7-H6 ligand of NKp30. B7-H6 is expressed on certain tumors (Brandt et al. 2009) but is not restricted to tumors, as TLR activation also induces B7-H6 on monocytes and neutrophils (Matta et al. 2013). Genes encoding NKp30 and NKp44 are present in humans, but not mice, limiting researchers' ability to perform preclinical studies of NKp30 and NKp44 using autologous mouse tumor models to explore these receptors' therapeutic potential. As with CD16, bispecific antibodies and other agents are under investigation to target NK cells bearing ITAM-based NCRs to tumors for therapeutic application.

Activating NK Receptors Not Requiring Immunoreceptor Tyrosine-Based Activation Motif–Based Adapters

While T cells and B cells express dominant ITAM-based antigen receptors that regulate their clonal expansion and responses, NK cell responses are often dictated by cooperative signaling through multiple activating receptors, which alone are insufficient to initiate cytolytic function, proliferation, or cytokine secretion, particularly when opposed by signals from their inhibitory receptors. In an elegant study, Bryceson et al. (2006b) demonstrated that when ligated alone by agonist monoclonal antibodies (mAbs) against activating NK receptors, only CD16 was capable
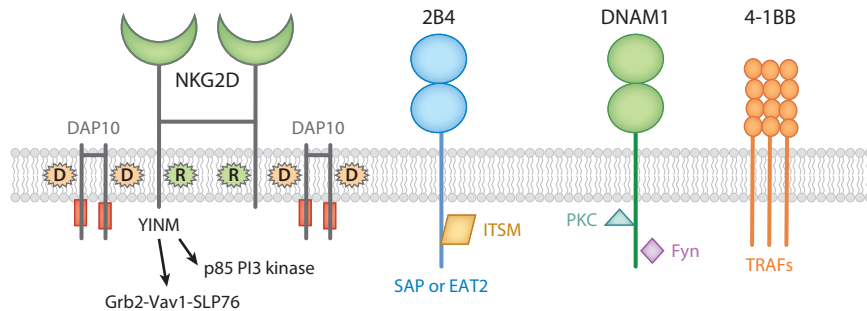
Figure 3

Activating natural killer (NK) receptors not requiring immunoreceptor tyrosine-based activation motif (ITAM)-based adapters. NK cells express several receptors that can provide activating signals that do not depend on ITAM-containing adapters, including NKG2D, 2B4, DNAM1, and 4-1BB, which use other signaling pathways. The YINM motif in the DAP10 adapter associated with NKG2D recruits a p85 PI3 kinase and the Grb2-Vav1-SLP76 signaling complex. The immunoreceptor tyrosine-based switch motif (ITSM) in the cytoplasmic domain of 2B4 recruits SAP and EAT2 signaling proteins. Motifs in the cytoplasmic domain of DNAM1 recruit PKC and Fyn. Motifs in the cytoplasmic domain of 4-1BB recruit TNF-associated factors (TRAFs) to initiate signaling.

of inducing degranulation and cytokine production by freshly isolated peripheral blood NK cells. Responses could be elicited by other activating receptors but required the simultaneous ligation of multiple receptors in distinct combinations, including NKp46, NKG2D, 2B4, CD2, and DNAM1. These results predict that tumors will only trigger a productive response by resting NK cells if they possess an appropriate array of ligands for these receptors at a critical threshold on their cell surface. After IL-2 priming, however, cross-linking these individual receptors with agonist antibodies induced degranulation and cytokine production, suggesting that cytokine-priming of NK cells in vivo may be necessary to achieve a therapeutic benefit mediated by NK cells upon treatment with particular agonist antibodies. Several costimulatory receptors on human and mouse NK cells have been identified, and their ligands may be constitutively expressed, induced, or upregulated by cellular stress (**Figure 3**).

NKG2D (CD314)

NKG2D (CD314) and its ligands are the most extensively studied of the activating receptors on NK cells. In healthy individuals, NKG2D is also expressed on γδ T cells, NKT cells, and CD8^{+} T cells. In response to certain stimuli, NKG2D can be induced on some CD4^{+} T cells and some myeloid cells (reviewed by Lanier 2015). In humans, NKG2D signals by association with the DAP10 adapter protein, which recruits PI3 kinase and a Vav1 signaling complex (Upshaw et al. 2006, Wu et al. 1999). In mice, an alternatively spliced transcript of NKG2D expressed in activated mouse NK cells can pair with either DAP10 or the ITAM-bearing DAP12 adapter (Diefenbach et al. 2002, Gilfillan et al. 2002); however, this does not occur in humans (Rosen et al. 2004). Humans and mice possess several genes that encode NKG2D ligands (e.g., the Rae-1, Mult1, and H60 ligands in mice and the MICA, MICB, and ULBP1–6 ligands in humans), and the regulation of expression of these proteins is quite complex, involving both transcriptional and posttranscriptional mechanisms and differing by cell type (Raulet et al. 2013). The NKG2D ligands are typically not expressed, or are expressed at only low levels in healthy adult tissues, but are frequently upregulated during infection and are often expressed by tumors (Raulet et al.

2013). Cancer cells and virus-infected cells have devised numerous strategies to disrupt NKG2D-mediated immunity [reviewed by Lanier (2015) and Raulet et al. (2013)]. Therapeutic strategies are currently being developed to enhance the expression of NKG2D ligands on the surface of tumors, to augment NKG2D function in NK cells and T cells, and to engineer NKG2D chimeric receptors for adoptive cell therapies. To avoid detection by NK cells, tumors frequently shed their NKG2D ligands; for example, membrane proteases on tumor cells can cleave the MICA and MICB proteins from the tumor surface, rendering them resistant to NKG2D-dependent NK cell killing. To preserve the expression of these NKG2D ligands on tumors, monoclonal antibodies have been developed against the proteolytic cleavage site on the MICA and MICB proteins, preventing their shedding and rendering them susceptible to NK cell attack (Ferrari de Andrade et al. 2018).

DNAM1 (CD226)

DNAM1 is a costimulatory receptor expressed by NK cells, T cells, platelets, and myeloid cells (Shibuya et al. 1996). Proteins of the nectin family, CD112 and CD155, serve as its ligands and are broadly distributed in healthy tissues (Bottino et al. 2003, Tahara-Hanaoka et al. 2004). Interactions between DNAM1 and its ligands serve to enhance adhesion and provide costimulation to T cells and NK cells. A role for DNAM1 in tumor immunity and immunosurveillance was documented by studies in DNAM1-deficient mice (Gilfillan et al. 2008, Guillerey et al. 2015, Iguchi-Manaka et al. 2008, Tahara-Hanaoka et al. 2005). The function of DNAM1 is countered by the inhibitory receptor TIGIT, which also binds to CD112 and CD155 and disrupts interactions with DNAM1 (Johnston et al. 2014, Stengel et al. 2012, Yu et al. 2009). Within this family, another receptor expressed by NK cells and T cells, CD96, also binds to CD155 (Fuchs et al. 2004). While CD96 has an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain, initial reports have suggested that it is an activating receptor in human NK cells (Fuchs et al. 2004). Recent studies have shown that CMV-induced adaptive NK cells express lower levels of TIGIT compared to canonical NK cells, making them inherently resistant to CD155-expressing myeloid-derived suppressor cells, highlighting the unique functional attributes of this subset of NK cells (Sarhan et al. 2016). Based on preclinical studies in mouse tumor models demonstrating that blocking TIGIT can enhance tumor immunity (reviewed by Dougall et al. 2017), antibodies against TIGIT are presently in clinical trials as a checkpoint blockade for cancer treatment.

2B4 (CD244)

2B4 (CD244) is a member of the SLAM family of receptors that includes CD150, CD229, CD84, NTB-A, and CD319 (Wu & Veillette 2016). 2B4 is expressed in NK cells, T cells, and myeloid cells and is a receptor for CD48, which is present on most hematopoietic cells. Signal transduction by 2B4 is complex and can be either activating or inhibitory, depending on the expression of the EAT2, SAP, or ERT (a pseudogene in humans) signaling proteins in the cytoplasm. Mice lacking the *Eat2*, *Sap*, and *Ert* genes are unable to efficiently mediate the so-called "missing self" rejection of MHC class I–negative cells (Karre et al. 1986) and fail to reject hematopoietic tumors (Dong et al. 2009). In the absence of these adapters, 2B4 is an inhibitory receptor that can recruit SHP-1 to suppress stimulation through other activating NK receptors (Dong et al. 2009). In mature human NK cells, 2B4 predominantly functions as a costimulatory receptor, working in conjunction with other costimulatory or activating receptors on NK cells, such as CD16, to lyse target cells (Bryceson et al. 2006a).

4-1BB (CD137)

4-1BB (CD137) is a member of the TNF family that is expressed on activated T cells and NK cells, as well as on myeloid cells in humans and mice, and can deliver costimulatory signals to T cells. Agonist mAbs to 4-1BB induced impressive CD8^{+} T cell tumor rejection in mouse models (Melero et al. 1997), leading to the development of agonist antihuman CD137 mAbs (urelumab and utomilumab) for therapeutic use in cancer patients (Chester et al. 2018). With regard to NK cells, in a preclinical model, Kohrt et al. (2011) demonstrated NK cell–dependent elimination of B cell lymphomas in mice by combined treatment with anti-CD20 and the agonist anti-CD137. Similar NK cell–dependent results were observed in xenograft models by using cetuximab and anti-CD137 to treat epithelial growth factor receptor (EGFR)-expressing human tumors (Kohrt et al. 2014) and by using trastuzumab and anti-CD137 to treat HER2-expressing breast tumors (Kohrt et al. 2012). While the initial trials with urelumab were halted due to severe liver toxicity, trials with utomilumab (a less potent agonist) in combination with rituximab are underway in B cell lymphoma patients. It should also be noted that Campana and colleagues (Imai et al. 2005) engineered K562 to express membrane-tethered IL-15 and 4-1BB ligand (4-1BBL) to use as feeder cells in order to expand human NK cells for adoptive immunotherapy. Signals through 4-1BB on activated NK cells greatly enhanced NK cell proliferation and survival. In our own studies, we have observed that 4-1BB can significantly increase human NK cell effector functions other than ADCC. Thus, costimulation of human NK cells provides an attractive strategy to enhance their antitumor activity.

In summary, the activating receptors expressed on NK cells and T cells provide attractive targets for the development of agonists to enhance tumor immunity, provided this can be done without unmanageable toxicity.

Inhibitory NK Receptors and Checkpoint Blockade

It is as important to restrain an immune response as it is to initiate an immune-mediated attack in order to avoid collateral damage and pathology. Thus, all immune cells have evolved a diverse array of inhibitory receptors to counter the activity of the activating receptors. Many of these inhibitory receptors are characterized by an ITIM in their cytoplasmic domain, which upon receptor engagement recruits phosphatases to dampen cell activation (Ravetch & Lanier 2000) (**Figure 4**).

KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS

Karre et al. (1986) first described the ability of NK cells to preferentially kill tumors that lacked MHC class I, which they termed recognition of "missing self." These seminal observations predicted the existence of inhibitory receptors for MHC class I that dampened or prevented NK cell activation but allowed for a more robust response against virus-infected cells or tumors that had lost or blocked expression of MHC class I in response to selective pressures caused by CD8^{+} T cells. Subsequently, the Ly49 receptors in mice (Karlhofer et al. 1992) and killer cell Ig-like receptors (KIRs) in humans (Colonna & Samaridis 1995, D'Andrea et al. 1995, Wagtmann et al. 1995) were identified as the inhibitory receptors recognizing polymorphic MHC class I ligands. Ly49 (*Klra*) and KIR are multigene families with extensive allelic polymorphisms, and the receptors are expressed on overlapping subsets of NK cells (and some T cells) (Lanier 2005, Orr & Lanier 2010). In humans, the KIR2D subfamily of receptors recognizes polymorphic HLA-C ligands, whereas the KIR3D subfamily of receptors binds to polymorphic HLA-A and HLA-B ligands. Blocking these ITIM-bearing inhibitory receptors with mAbs can disrupt interactions with MHC class I on tumors, allowing the tumors to be killed and cytokines to be secreted,
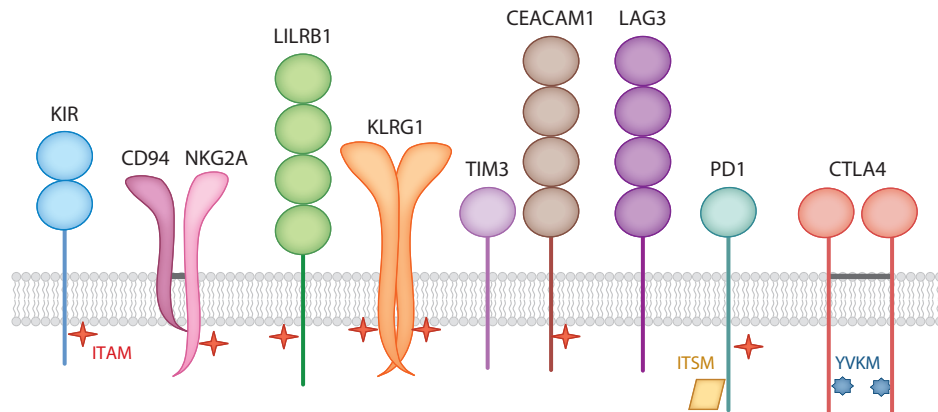
Figure 4

Inhibitory natural killer cell receptors. NK cells possess numerous inhibitory receptors, some of which are characterized by ITIMs in their cytoplasmic domains. Upon ligand engagement, the ITIMs recruit tyrosine phosphatases, such as SHP-1, to counter signals transmitted by activating NK receptors. Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; KIR, killer cell immunoglobulin-like receptor; NK, natural killer.

provided that the tumor displays sufficient activating ligands to trigger the activating receptors on the NK cell. This has been used as the basis for the development of a blocking antibody against KIR2D (lirilumab) for therapeutic use in patients with acute myeloid leukemia (AML) and multiple myeloma (**Figure 2**) (Benson et al. 2011, 2012, 2015; Romagne et al. 2009; Vey et al. 2012). Initial results using lirilumab as a monotherapy in AML and multiple myeloma have not indicated significant efficacy, and in a phase II trial, NK cells from treated patients appeared to be hyporesponsive; in vitro experiments suggested the possibility of KIR stripping from the cell surface by a mechanism of Fc-mediated trogocytosis (Carlsten et al. 2016). While the combination in recent studies of lirilumab with anti-PD1 (Opdivo®) gave encouraging hints of clinical benefit in head and neck cancer patients, subsequent phase II trials failed to confirm efficiency beyond anti-PD1 monotherapy. Further studies are underway to evaluate KIR blockade in other cancers and with other agents. These negative results might reflect that the tumors in these patients had already been immune edited by NK cells and thus lack expression of the relevant ligands for the stimulatory receptors on NK cells to initiate a response, or that the NK cells may be suppressed by other redundant evasion mechanisms to prevent NK cell attack.

CD94-NKG2A

Another inhibitory receptor is the CD94-NKG2A receptor, which recognizes the human HLA-E and mouse Qa-1 MHC molecules (Braud et al. 1998, Lee et al. 1998, Vance et al. 1998). The NKG2A subunit of the receptor contains an ITIM, which is responsible for its inhibitory function. Unlike KIR, CD94-NKG2A and its MHC ligands are not polymorphic, simplifying the generation of reagents to block this receptor. A blocking antibody (monalizumab) has been produced against human NKG2A and is in phase II clinical trials (McWilliams et al. 2016, Ruggeri et al. 2016). CD94-NKG2A, like KIR, is only expressed on a subset of NK cells and T cells; however, prior studies reported that NKG2A can be induced on NK cells by IL-12 (Saez-Borderias et al. 2009) and on activated antigen-specific T cells by IL-15 (Mingari et al. 1998) or by TGFβ

(Bertone et al. 1999). These findings suggest that the inhibitory CD94-NKG2A might be upregulated by cytokines in the tumor microenvironment to limit NK cell or T cell responses against tumors; therefore, blocking this receptor may have therapeutic utility. Currently, monalizumab is in combination trials with PD-L1-blocking antibodies and with ADCC-inducing antibodies to EGFR (cetuximab) in head and neck carcinomas; preclinical data suggest that monalizumab may augment NK cell–mediated ADCC by cetuximab (**Figure 2**).

LILRB1

LIRLB1 (also designated as CD85j, LIR1, or ILT2) is an ITIM-bearing inhibitory receptor recognizing MHC class I that is present on a subset of NK cells and T cells, as well as broadly expressed on B cells and myeloid cells (Colonna et al. 1997, Cosman et al. 1997). LILRB1 binds to all human HLA class I proteins, but with the highest affinity for HLA-G (Shiroishi et al. 2006). LILRB1 is expressed at much higher cell surface density and on many more B cells and myeloid cells compared to T cells or NK cells, making it less specific to cell type for potential application in checkpoint blockade applications in cancer.

KLRG1

Initially identified as an ITIM-based inhibitory receptor on mast cells that can suppress IgE Fc receptor signaling (Soto & Pecht 1988), KLRG1 is expressed on mouse and human NK cells, as well as T cells (Corral et al. 2000, Voehringer et al. 2002). In mice, Klrg1 marks the activation and differentiation status of NK cells (Huntington et al. 2007) and has been shown to be stably expressed at high levels on CMV-induced memory NK cells (Sun et al. 2009). KLRG1 binds to cadherins and in vitro has been shown to suppress NK cell lysis of cadherin-bearing tumors (Grundemann et al. 2006, Ito et al. 2006, Tessmer et al. 2007). As cadherins may be abundantly expressed in tumor microenvironments, blockade of KLRG1 may have therapeutic application in some solid tumors.

TIM3

NK cells express several other inhibitory receptors whose functions are less well understood or have less well-defined ligands. TIM3 is constitutively expressed by all mature human NK cells, and its expression is upregulated by type I IFN and cytokines, including IL-2, IL-12, IL-15, and IL-18 (Ndhlovu et al. 2012). Cross-linking TIM3 with mAbs can suppress NK cell activation triggered by CD16, suggesting that it is an inhibitory receptor; however, TIM3 has no characteristic inhibitory signaling motifs in its cytoplasmic domain (Ndhlovu et al. 2012). TIM3 has been shown to form heterodimers with CEACAM1, which contains conical ITIMs in its cytoplasmic domain, possibly explaining the inhibitory function of TIM3 (Huang et al. 2015b). When expressed on activated human and mouse NK cells, CEACAM1 itself can serve as an inhibitory receptor when CEACAM1 on NK cells binds to CEACAM1 on tumors in a *trans*-homophilic manner (Hosomi et al. 2013). TIM3 is also expressed on myeloid cells and subsets of T cells, and numerous ligands have been proposed for TIM3, including galectin-9, phosphatidylserine, HMGB1, and CEACAM1 (Das et al. 2017). There is evidence that in some circumstances, TIM3 may serve as an activating receptor on T cells, but the basis for these contrasting functional behaviors has not been resolved (Das et al. 2017). Based on preclinical studies, mAbs against TIM3 are presently in clinical trials in cancer patients.

LAG3

LAG3 was initially cloned from a human NK cell clone (Triebel et al. 1990) and was shown to bind MHC class II ligands (Baixeras et al. 1992). It is also expressed on activated T cells, B cells, and plasmacytoid dendritic cells. Despite binding to MHC class II, there is no evidence that NK cells are affected by the expression of MHC class II on tumors, in contrast to MHC class I. NK cells in mice deficient in LAG3 were reported to have impaired killing of certain tumor cell lines, which would imply an activating role for LAG3 in NK cells (Miyazaki et al. 1996). The molecular basis for this has not been established, as it does not appear to involve MHC class II. In T cells, it has been suggested that LAG3 has an inhibitory function, and based on preclinical mouse tumor studies blocking mAbs and LAG3-Fc, fusion proteins are being evaluated in the clinic as a checkpoint blockade therapy for cancer, despite not having a mechanism to account for the inhibition (Miyazaki et al. 1996).

PD1

The human gene encoding PD1 was originally cloned from the human YT NK-like leukemia cell line (Shinohara et al. 1994). While the expression and function of PD1 have been extensively studied in T cells (Sharpe & Pauken 2018), less is known about the role of PD1 in regulating NK cell responses. Unlike T cells, PD1 is not frequently expressed at significant levels on activated NK cells, although PD1 has been detected on human and mouse NK cells in some circumstances, including on tumor-infiltrating NK cells (Beldi-Ferchiou et al. 2016, Benson et al. 2010, Huang et al. 2015a, Iraolagoitia et al. 2016, Pesce et al. 2017, Wiesmayr et al. 2011). The first report by Benson et al. 2010 of PD1 expression on NK cells in patients with multiple myeloma using the CT-011 clone (pidilizumab) is confounded by the finding that this antibody in fact does not bind PD1, but rather a Delta-like ligand. Thus, NK cells may contribute to the efficacy of PD1 checkpoint blockade in some cancer patients, but more research is needed.

CTLA4

The first checkpoint blockade therapy for cancer approved by the US Food and Drug Administration (FDA) involved disruption of interactions between the inhibitory CTLA4 receptor and its B7 ligands (CD80 and CD86). The inhibitory function of CTLA4 is considered to be mainly due to its ability to bind to its B7 ligands and prevent the binding of the lower-affinity activating CD28 receptor, as well as its ability to rapidly internalize these ligands, removing them from the surface of the APC by trans-endocytosis (Qureshi et al. 2011). Unlike T cells, human and mouse NK cells do not typically express significant amounts of either the activating CD28 or inhibitory CTLA4 receptors, although in some circumstances, IL-2-activated mouse NK cells have been reported to express CTLA4, and CTLA4 was detected on tumor-infiltrating NK cells in a mouse tumor model (Stojanovic et al. 2014). There are no intrinsic NK cell effects reported in CTLA4-deficient mice, thus it is likely that CTLA4 predominantly regulates T cell activation. Nonetheless, studies examining CTLA4 in tumor-infiltrating NK cells in human cancer patients may provide new insights into this possibility.

Collectively, most of the checkpoint receptors currently in clinical development are expressed both by NK cells and T cells. Studies to determine the mechanisms involved in the success of these drugs should explore the potential contribution of NK cells to these immune therapeutics.

Cytokine Activation of Autologous and Haploidentical NK Cells for Cancer Therapy

NK cells respond to cytokines to induce differentiation, homeostatic control, and activation. Thus, it is logical to assume that cytokines should support NK cell persistence and expansion to improve cancer therapy. Early direct therapeutic strategies involved the use of IL-2. Based on preclinical studies, Rosenberg and colleagues (Grimm et al. 1982, Lotze et al. 1981, Yron et al. 1980) found that incubation of mouse splenocytes or human peripheral blood mononuclear cells with IL-2 led to the generation of lymphokine-activated killer (LAK) cells that lysed syngeneic or allogeneic tumors. Repeated injections of IL-2 into mice with established tumors induced LAK activity and led to significant tumor regression (Rosenberg et al. 1985). While the antitumor effects of LAK cells and IL-2 in mice were impressive, significant barriers impeded attempts to translate this into human therapy. While limited clinical response was observed in cancer patients treated with IL-2 alone, combination therapy with LAK cells and IL-2 resulted in objective responses in 22% of patients with a variety of advanced metastatic cancers. However, toxicity associated with higher doses of IL-2 (Rosenberg et al. 1987), specifically life-threatening vascular leak syndrome or liver toxicity, limited efficacy (Ettinghausen et al. 1988, Hoffman et al. 1989). As an alternative to direct application of cytokines, cytokines can be manipulated indirectly in vivo. Based on pioneering studies at the National Cancer Institute (NCI), Restifo and colleagues (Dudley et al. 2002) discovered that sublethal radiation in mice induced lymphodepletion to make space, depleted suppressor cells, and, importantly, released a cytokine sink. This finding was translated into human clinical trials in melanoma patients treated with tumor-infiltrating lymphocytes. In these trials, use of high-dose cyclophosphamide with fludarabine resulted in higher response rates.

Because LAK cells represent a mixture of T cells and NK cells, their proportional contributions to antitumor responses remain unclear. Studies in mice suggested that NK cells were the major contributors to antitumor activity. Several groups began manipulating autologous NK cells in the unique setting of recovery after autologous hematopoietic stem cell transplantation. In this circumstance, NK cells are the dominant lymphocytes reconstituting in the first month. Although use of low-dose IL-2 to expand NK cells after autologous transplantation resulted in robust in vivo expansion of NK cells (Miller et al. 1997, Soiffer et al. 1994), analysis of these phase II autologous NK cell studies showed limited efficacy. Although ex vivo activation with high doses of IL-2 improved in vivo function, definitive efficacy was lacking (Burns et al. 2003). This could be a result of inhibitory signals delivered by self-MHC class I and of the fact that even low-dose IL-2 dramatically stimulates a population of suppressive regulatory T cells (Tregs). This finding, consistent with pioneering work by Velardi and colleagues (Ruggeri et al. 2002) showing that NK cell alloreactivity after haploidentical transplantation can control myeloid leukemias, sparked the hypothesis that use of allogenic NK cell therapies from healthy donors might increase the persistence of a population of NK cells in vivo that would not be suppressed by self-MHC of the residual tumor.

We were the first to infuse related HLA-haploidentical NK cells in a nontransplantation setting (Miller et al. 2005). Apheresis products were CD3 depleted (subsequently to include CD19 depletion to avoid passenger lymphocyte syndrome) and activated with 1,000 IU/mL IL-2 ex vivo overnight. Patients received either low-dose fludarabine for five days (25 mg/m^2/day) or high-dose cyclophosphamide for two days (60 mg/kg/day), followed by the same dose of fludarabine (Hi-Cy/Flu) prior to NK cell infusion. This treatment protocol was based on studies from the NCI discussed above. IL-2 was administered daily (1.75 million units/m^2) for 14 days [subsequently modified to 6 higher doses (10 million units without m^2 correction) over 2 weeks]. Only patients receiving Hi-Cy/Flu had in vivo detectable donor-derived NK cells (defined as 100 donor-derived

NK cells per microliter of blood 14 days after adoptive transfer). In some patients, NK cells persisted for up to one month. This outcome was associated with a surge in endogenous IL-15, which was highest in patients receiving Hi-Cy/Flu at the time of NK cell infusion and which diminished as lymphocytes recovered. Fludarabine alone was insufficient to induce this effect. These results are proof-of-concept that human NK cells in some patients could survive for up to one month when infused after lymphodepleting chemotherapy and the administration of IL-2. Infusion of allogeneic NK cells was safe without the risk of graft-versus-host disease (GVHD). In this initial study, 5 out of 19 patients with poor prognosis AML went into complete remission. Clinical efficacy was associated with in vivo expansion of donor NK cells. Thus, the persistence and in vivo expansion of NK cells became a surrogate marker for testing other approaches aimed at increasing the survival of NK cells.

With the application of haploidentical NK cell therapy to treat ovarian cancer, breast cancer, and refractory lymphoma, we found that host Tregs persist after conditioning and expand rapidly when IL-2 is administered after adoptive NK cell transfer (Bachanova et al. 2010, Geller et al. 2011). This is a result of high-affinity IL-2 receptor (IL-2R) alpha chains (CD25) on Tregs driving their proliferation. Tregs can inhibit NK cell function indirectly by limiting the bioavailability of IL-2 (Gasteiger et al. 2013, Sitrin et al. 2013) or directly through their secretion of TGFβ (Ghiringhelli et al. 2005). To overcome the inhibitory effects of Tregs identified in preclinical studies in the mouse (Zhou et al. 2009), we conducted a trial using recombinant IL-2 diphtheria toxin (DT) fusion protein (IL2DT; trade name Ontak®). Our objective was to target DT to Tregs through IL-2 binding to CD25. Because IL2DT had a short half-life compared to other methods of Treg depletion using antibodies, it was used in combination with lymphodepletion. Our rationale was that inhibitory effects would be eliminated by the time NK cells, also binding IL-2, were infused. Fifty-seven refractory AML patients were treated with high-dose chemotherapy followed by IL-2 administration. Donor NK cell expansion was observed in 10% (4 of 42) of patients receiving haploidentical NK cell infusions and IL-2 alone, whereas NK cell expansion occurred in 27% (4 of 15) of patients also given IL2DT. Importantly, the addition of IL2DT was associated with higher complete remission rates and improved disease-free survival at 6 months postadoptive NK cell transfer (Bachanova et al. 2014). These results demonstrate that Treg expansion as a result of IL-2 administration limits NK cell expansion in vivo and may limit their therapeutic effects. However, this outcome may be dependent on the immune status of the patient and may vary by disease type.

It may be possible to surmount the obstacle of Treg expansion following administration of IL-2 by using IL-15. IL-15 was originally identified as a T cell growth factor that binds the common beta and gamma chains of IL-2R (**Figure 2**) (Grabstein et al. 1994). Thus, IL-2 and IL-15 are distinguished by their binding to unique alpha chains, IL-2Rα and IL-15Rα, respectively. IL-15 is physiologically *trans*-presented by membrane-bound IL-15Rα on dendritic cells or other myeloid cells (Mortier et al. 2008, Prlic et al. 2002). Preclinical studies demonstrated that IL-15 can control homeostasis and stimulate NK cell and antigen-specific CD8+ T cell activity without causing activation-induced cell death or promoting Treg cell function (Waldmann et al. 1998). Recognition of these properties led the 2007 NCI Immunotherapy Workshop to designate IL-15 as the immunotherapeutic with highest potential for clinical development. However, dose escalation was limited by unexpected toxicities encountered in the first-in-human clinical trial of recombinant human (rh) monomeric IL-15 given as an intravenous (IV) bolus infusion (Conlon et al. 2015). Nonhuman primate experiments suggested that subcutaneous (SC) administration should lower peak concentrations and improve clinical tolerance. A recently published trial sought to establish a safe outpatient regimen of IL-15 for cancer patients (Miller et al. 2018). Nineteen patients were treated subcutaneously with rhIL-15, Monday through Friday for two weeks at dose levels of 0.25, 0.5, 1, 2, and 3 μg/kg/day, where 2 μg/kg was determined to be the maximal

tolerated dose (compared to 0.3 µg/kg when given by IV bolus infusion). The treatment induced a profound expansion of circulating NK cells and a proportional, but less dramatic, increase of circulating CD8+ T cells.

Many IL-15 products are now reaching the clinic. As an example, Altor BioScience Corporation (a NantWorks company in Miramar, Florida) has developed an IL-15N72D/IL-15Rα-Fc superagonist complex (ALT-803). A major advantage shown in preclinical studies is its substantially increased in vivo half-life compared to rhIL-15 due to the human IgG1 Fc fragment fused to the IL-15Rα in the complex. In addition, studies have shown enhanced NK cell function in vitro and in mouse models after treatment with ALT-803. ALT-803 rescued the functional activity of NK cells derived from ovarian cancer patient ascites (Felices et al. 2017). ALT-803 also enhanced in vivo CD16-triggered NK cell clearance of B cell lymphomas when treated with an anti-CD20 mAb (Liu et al. 2016). In conjunction with the postulated role of gamma chain cytokine–mediated bypass of some checkpoint inhibition pathways (Cheng et al. 2013), the ability of IL-15 to potentiate CD16-mediated functions on NK cells makes this cytokine an attractive immunotherapeutic target. We have recently completed first-in-human testing of ALT-803 in hematologic malignancy patients who relapsed more than 60 days after allogeneic transplantation (Romee et al. 2018). The hypothesis was that stimulating donor immunity would induce a graft-versus-tumor response to treat cancer relapse. ALT-803 was given to 33 patients via IV or SC administration once weekly at four doses (dose levels of 1, 3, 6, and 10 µg/kg). ALT-803 was well tolerated. In this clinical setting, no dose-limiting toxicities or treatment-emergent GVHD requiring systemic therapy was observed. Adverse events following IV administration included constitutional symptoms temporally related to increased amounts of serum IL-6 and IFNγ. The SC route was tested to mitigate these effects. SC delivery resulted in self-limited injection site rashes infiltrated with γδ T cells without acute constitutional symptoms. Pharmacokinetic analysis revealed prolonged (>96 h) serum concentrations following SC delivery, but not IV injection. In this setting, SC delivery mimicked a continued infusion lasting nearly a week. ALT-803 stimulated activation, proliferation, and expansion of NK cells and CD8+ T cells without increasing Tregs. Responses were observed in 19% of evaluable patients, including one complete remission lasting 7 months. This immunostimulatory IL-15 superagonist to augment antitumor immunity, both alone and combined with other immunotherapies, warrants further investigation. Another IL-15 product developed by George Pavlakis containing the heterodimer of IL-15/IL-15Rα (Bergamaschi et al. 2018) was recently acquired by Novartis. Clinical development is underway. An alternative to IL-15 is the use of PEG (polyethylene glycol)-ylated IL-2 [developed by Nektar as NKTR-214 (Charych et al. 2016)] or mutant IL-2 proteins that preferentially bind to the IL-2R beta and gamma chains, thereby avoiding stimulation of Tregs. These agents are also currently undergoing evaluation in the clinic.

Priming NK cells with a short ex vivo treatment with a combination of IL-15, IL-12, and IL-18 prior to adoptive transfer is another cytokine-based approach that has shown promise in mouse cancer models. IL-12 synergizes with IL-18 to epigenetically prime NK cells for enhanced IFNγ production (Chan et al. 1991, Luetke-Eversloh et al. 2014, Okamura et al. 1995). Cerwenka and colleagues (Ni et al. 2012) tested the in vivo antitumor activity of cytokine preconditioning by incubating syngeneic mouse NK cells with either IL-15 alone or IL-12, IL-15, and IL-18 for 16 hours ex vivo before adoptive transfer into MHC class I–deficient RMA-S tumor-bearing mice. Sustained tumor clearance and NK cell persistence was observed in 22% of the mice that received IL-12/15/18-preactivated NK cells. No beneficial effect was observed in mice that received IL-15- or IL-2-preactivated NK cells. These cells are often designated as cytokine induced memory-like (CIML) NK cells. This approach has been translated into human clinical trials based upon favorable preclinical data (Cooper et al. 2009). Fehniger and colleagues (Romee et al. 2012)

reported that CIML NK cells exhibit enhanced IFNγ production and cytotoxicity against primary AML blasts regardless of KIR/KIR ligand interactions. In a first-in-human trial, NK cells expanded in vivo, and some patients with refractory AML experienced remissions (Romee et al. 2016).

NK Cell Expansion Strategies Allow the Development of Diverse NK Cell Products

Several groups have promoted the use of cell lines derived from malignant NK cell leukemia or lymphoma (i.e., NK-92, NKL, KYHG-1, YT, and NKG). These transformed NK cell lines maintain cytolytic function and lack the expression of inhibitory KIR. Based on unlimited growth of the NK cell lines, genetic modification introducing CD16 or chimeric antigen receptors (CARs) is accomplished easily. Among the lines, NK-92 cells have been tested in clinical trials that included patients with renal cell carcinoma and malignant melanoma (Arai et al. 2008). In a phase I dose escalation study treating 12 patients, investigators reported only transient toxicities and stable disease in 33% of patients. However, the in vivo activity of the NK-92 cells was difficult to establish (Cheng et al. 2013), and the requirement that the NK-92 cells be irradiated prior to infusion limits their in vivo persistence.

Multiple strategies are under investigation to make off-the-shelf NK cells a reality. These efforts depend upon robust expansion of NK cells. Imai et al. (2005) engineered the MHC class I–negative K562 cell line to express 4-1BBL and membrane-bound IL-15 capable of 1,000-fold expansion of blood NK cells after three weeks in culture. K562 cells expressing membrane-bound IL-21 and 4-1BBL (K562-mb21-4-1BBL) have also been highly successful for ex vivo expansion of NK cells (Denman et al. 2012). These K562-expanded primary NK cells have been tested clinically and found to be safe. They do not induce GVHD. Results have been encouraging in remission maintenance along with allogeneic hematopoietic stem cell transplantation (Ciurea et al. 2017). Another approach involves using particles derived from plasma membranes of the K562-mb21-4-1BBL cells (PM21 particles). This technique offers the advantage of off-the-shelf feeder cell support without the requirement for live feeder cells (Oyer et al. 2016).

A potential problem with highly expanded NK cells is whether they generate terminally differentiated cells or cells that may be more functionally immature. More attention should be devoted to developing products with defined functional characteristics. Focusing on the functional attributes of adaptive NK cells, Cichocki et al. (2017) discovered that adding a small-molecule inhibitor of glycogen synthase kinase 3 to peripheral blood NK cells from a CMV-seropositive donor expanded ex vivo with IL-15 for one week resulted in an enriched population of adaptive $CD57^+NKG2C^{++}$ NK cells. This outcome was linked to the expression of several transcription factors associated with late-stage NK cell maturation, including T-BET, ZEB2, and BLIMP-1, without affecting cell viability or proliferation. These cells contained high levels of granzyme and were highly functional in mediating potent ADCC, especially in long-term (up to 40 h) killing assays (Cichocki et al. 2017). This discovery has been translated into a phase I clinical trial to investigate systemic administration of these adaptive NK cells for AML and solid tumors, as well as intraperitoneal delivery in ovarian cancer.

Continued improvements in NK cell expansion technology may make off-the-shelf NK cells a reality. Starting material can be from adult peripheral blood or from cord blood progenitors, where hematopoietic progenitors may have more expansion potential and are more amenable to gene transfer (Ciurea et al. 2017, Dolstra et al. 2017, Kang et al. 2013). Recently, Rezvani and colleagues (Liu et al. 2018) initiated a clinical trial using NK-CAR against CD19 with a construct also providing IL-15. However, even progenitor-derived NK cells are heterogeneous in function and not clonal. Potentially, this obstacle may be overcome by deriving NK cells from

self-renewing human embryonic stem cells or, alternatively, induced pluripotent stem cells (iPSCs) (Knorr & Kaufman 2010, Woll et al. 2009). These cells have unlimited scale and are easy to genetically modify, and NK cell clones can be selected based on functional attributes. An example of a gene modification is based on the biology that activated NK cells lose CD16 expression and the homing receptor CD62L through proteolytic cleavage by membrane-bound disintegrin and metalloprotease 17 (ADAM17) (Romee et al. 2013). This CD16 receptor shedding is mediated by the ADAM17 cleavage between Ala[195] and Val[196] at a membrane-proximal site of the CD16 receptor (Lajoie et al. 2013). Pharmacologic inhibition of ADAM17 enhanced NK cell–mediated ADCC function by preserving CD16 on the surface. An alternative approach is to engineer iPSCs with CRISPR technology to express a cleavage-resistant high-affinity CD16. Repurposing CD16 on NK cells with greater specificity by using anticancer antibodies or immune engagers will result in an NK cell product with incredible diversity.

In summary, the use of cytokines, adoptive cell transfer, and a form of antigen-specific off-the-shelf NK cells will allow repeated dosing of NK cells as a living drug. The main barrier remaining is how to maintain the persistence and expansion needed to provide a meaningful effector-to-target ratio in vivo and to overcome the rejection of allogeneic NK cells when transferred into immuno-competent patients. While lymphodepleting conditioning has provided a proof-of-concept on the potential of NK cells to expand in vivo and survive for up to one month, the use of Hi-Cy/Flu is too toxic for solid tumor patients who are treated on an outpatient basis, and even in these patients, the immune rejection is still strong. What is needed is more potent transient T cell blunting for one to two weeks that is safe and nontoxic. Some have proposed that conditioning patients with anti-CD3 mAb OKT3 might be appropriate for this purpose, but OKT3 is no longer readily available and can cause a toxic cytokine release syndrome. Optimal immune blunting would need to be specific to T cells without affecting NK cells. Given the similar biology between these cell types, this remains a challenge and an aspiration for future research.

Bispecific and Trispecific Killer Cell Engagers

NK cells can broadly recognize stressed malignant targets through a collection of receptors but may be limited therapeutically by a lack of specificity. This assumption is supported by the recent success and FDA approval of genetically engineered CAR-expressing T cells, which are specific for CD19^{+} B cell malignancies (Glienke et al. 2015, Hermanson & Kaufman 2015). However, these T cell–personalized approaches are expensive (around $500,000) because they require formidable infrastructure to produce individualized cell products. The FDA has approved a bispecific T cell engager named binatumomab, which engages CD3 on T cells and CD19 on B cell malignancies, as a proof-of-concept for an alternative T cell approach. Engaging the CD3 complex on T cells results in both an activation and a proliferation signal, resulting in clinical efficacy in patients with acute lymphocytic leukemia (Topp et al. 2015), and this dual function may be key to efficacy. These outcomes have motivated several groups to develop strategies to engage activating receptors on endogenous and adoptively transferred NK cells (**Figure 2**).

We have been particularly interested in bi- and trispecific killer cell engagers (BiKEs and TriKEs) (**Figure 2**). These molecules are designed to form an antigen-specific immunological synapse between NK cells and tumor cells to trigger antigen-specific NK cell–mediated killing of tumor targets. Unlike full-length bispecific antibodies (300–450 kDa), these constructs (50–75 kDa) are composed of a single-chain variable fragment (scFv) made up of a variable heavy and variable light chain of an antibody connected by an inert short peptide linker (Holliger & Hudson 2005). This configuration facilitates combination with the scFvs of one (BiKE) or two (TriKE) antibodies of different specificities. As an alternative approach, one of the scFvs can be replaced

by a functional cytokine or even added to a TriKE with multiple specificities to create a TetraKE with four function domains.

The mAb-driven low-affinity Fc engagement of CD16 to trigger ADCC has changed the standards of cancer care (**Figure 2**). Accordingly, the biology of alternative approaches to repurpose the CD16 receptor must be explored. BiKEs and TriKEs were designed with agonistic anti-CD16 scFv components, leading to the hypothesis that an anti-CD16 scFv could bind and trigger CD16 with a stronger interaction than the natural low-affinity binding of CD16 to the Fc of IgG1 antibodies. This concept was demonstrated when comparing the binding affinity of CD16 for the Fc-portion of an anti-HER2 antibody to the binding affinity of the anti-HER2 × anti-CD16 bispecific antibody to CD16, which demonstrated a 3.4-fold increase in binding of the bispecific antibody to CD16 (Moore et al. 2011). Additionally, BiKEs and TriKEs have several potential advantages. These include increased biodistribution compared to mAbs due to their significantly smaller size, their possibly smaller immunogenicity, and their ability to be engineered with great flexibility.

Our group has focused on BiKEs and TriKEs that engage CD16 along with CD19 and CD22 on B cell non-Hodgkin's lymphoma (Gleason et al. 2012); CD33 on AML (Gleason et al. 2014) and myelodysplastic syndrome (Wiernik et al. 2013); EpCAM on prostate, breast, colon, and head and neck carcinoma (Vallera et al. 2013); and CD133 on cancer stem cells, as well as a combination of EpCAM and CD133 for a broad-spectrum molecule (Schmohl et al. 2016, 2017). Because CD16 engagement alone does not deliver a proliferative signal, newer-generation TriKEs and TetraKEs all incorporate an IL-15 moiety that substantially induces NK cell proliferation and survival, much like the intracellular CD28 or 4-1BBL domains essential to CAR T constructs that sustain and amplify an immune response. This investigational decision was motivated by clinical studies testing IL-15 against a variety of cancers that found predominant NK cell activation and proliferation without stimulating Treg expansion. The TriKE and TetraKE molecules containing IL-15 demonstrate clear advantages in CD16 engagement over BiKE predecessors by adding a costimulus to cytotoxicity and inducing secondary inflammatory cytokines that may be important to orchestrate a broader immune response. When compared to its BiKE counterpart, one of these molecules, an anti-CD16 × IL-15 × anti-CD33 TriKE, has demonstrated better tumor control and NK cell maintenance and expansion in a preclinical AML xenogeneic mouse model containing human NK cells. It is tempting to speculate that the IL-15 moiety may also enhance a bypass of inhibitory checkpoint signaling. In support of this finding, a recently published study has shown that IL-15/IL-15Rα-Fc superagonist complexes (ALT-803) can augment PD1 blockade therapy in patients with advanced lung cancer (Wrangle et al. 2018). By the end of 2018, the anti-CD16 × IL-15 × anti-CD33 TriKE is scheduled to reach the clinic in a phase I trial at the University of Minnesota for patients with refractory AML and high-risk myelodysplastic syndrome. Other camelid constructs with different agonistic binding to CD16 are currently being tested to generate second-generation TriKEs with higher specific activity. In addition to our own platform, Affimed has clinically tested NK cell engagers with a different tetravalent structure, binding CD16A on NK cells rather than the broader CD16A/CD16B binding using our TriKE approach (Rothe et al. 2015). Constructs binding CD16A/B, as opposed to only CD16A, may also bind to neutrophils in addition to NK cells, leading to a drug sink with unknown clinical consequences. Comparative studies using these NK cell engagers are needed to reconcile this question.

Current TriKEs and TetraKEs trigger NK cell responses via CD16 ligation and cytokine signaling. Additional components can be added to manipulate the immune synapse between NK cells and their targets. Some have suggested that engaging NKG2D through fusion proteins (Smits et al. 2016, von Strandmann et al. 2006) may be more advantageous. In addition, scFvs that block NK cell–specific or broad checkpoints like KIRs, TIGIT, NKG2A, or PD1 may be optimal.

Alternatively, an scFv-neutralizing TGFβ could be included to reduce negative signaling in the tumor microenvironment. Another interesting concept is the incorporation of an scFv functionally blocking ADAM17—a matrix metalloproteinase involved in CD16 shedding to maximize CD16-mediated killing. Besides IL-15, several other cytokines such as IL-12 that differentially modulate NK cell biology could be included (Vallera et al. 2016), as it has been shown in mouse model systems that IL-12 is essential to generate memory NK cells specific for CMV (Sun et al. 2012). The choice of options for optimally manipulating the immune synapse will be based on careful studies in NK cell biology.

While the BiKE and TriKE strategies discussed above use an anti-CD16 scFv that binds both CD16A and CD16B, it remains unclear whether CD16B engagement on neutrophils will be clinically important. While BiKEs and TriKEs bind CD16B, which may be a drug sink, thus rendering them biologically unavailable, they do not appear to activate neutrophils or inhibit the ability of BiKE- or TriKE-mediated NK cell–mediated killing; however, alternatives to overcome this have been proposed. Constructs that are specific for CD16A engagement are already in the clinic, paired with anti-CD30 for treatment of Hodgkin's lymphoma (Arndt et al. 1999; da Costa et al. 2000; Hartmann et al. 1997, 1998; Hombach et al. 1993; Reiners et al. 2013; Renner & Pfreundschuh 1995; Renner et al. 1997, 2000; Sahin et al. 1996). The future for off-the-shelf immune engagers is exciting. Other types of multispecific antibody constructs have been generated to engage CD16 in conjunction with CD20 and CD19 on B cell non-Hodgkin's lymphoma (Bruenke et al. 2005; Glorius et al. 2013; Johnson et al. 2010; Kellner et al. 2008, 2011; Kipriyanov et al. 2002; Portner et al. 2012; Schlenzka et al. 2004; Schubert et al. 2012), CD19 and CD33 on mixed lineage leukemia (Schubert et al. 2011), CD33 or CD33 and CD123 on AML (Kugler et al. 2010, Silla et al. 1995, Singer et al. 2010), HLA class II on lymphomas (Bruenke et al. 2004), EGFR on EGFR+ tumors (Elsasser et al. 1999, Ferrini et al. 1993), HER2 (neu) on metastatic breast cancer and other HER2-expressing tumors (Moore et al. 2011; Weiner et al. 1995a,b), and MOV19 on ovarian cancer (Ferrini et al. 1991).

PERSPECTIVES AND CONCLUSIONS

NK cells are not known to cause autoimmune disease and are well regulated to behave and only become maximally activated during pathogenic infections. Unlike viral infections where there is abundant secretion of IFNs and cytokines such as IL-2, IL-12, IL-15, and IL-18 that can awaken naïve or resting NK cells, these inflammatory cytokines are rarely present in the tumor microenvironment. Activated NK cells are also unable to secrete autocrine growth factors that allow their expansion, instead relying on other hematopoietic and nonhematopoietic cells to provide factors needed for their proliferation and survival. Thus, to fully engage NK cells in cancer immunotherapy, it may be necessary not only to target NK cells to tumors but also to provide sources of these factors to enhance their effector functions and proliferation. Numerous strategies are in preclinical and clinical testing to develop new therapeutics to engage NK cells in the attack against cancer. Based on exponential growth in translational research in immuno-oncology and NK cell biology, it is likely that drugs enhancing endogenous NK cell functions or NK cell products themselves will be commercialized in the next five years.

DISCLOSURE STATEMENT

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Contents

Errata

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