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Review

# Positive and negative regulation of Natural Killer cells: Therapeutic implications

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**Abstract**

Natural Killer (NK) cells can mediate numerous anti-tumor and anti-viral effector functions as well as play important immunoregulatory roles in various disease states. Promoting the ability of NK cells to respond in an immunotherapeutic setting has often been sought by the addition of NK cell-stimulating factors. However, such therapies are often found to be insufficient, which may in part be due to the presence of inhibitory influences on the NK cell. NK cells can respond to a plethora of cytokines which are generated by numerous cell types and these interactions can markedly affect NK cell survival and activity. NK cells also possess multiple activating and inhibiting receptors which can alter their function. Whether the NK cell will become activated or not can depend on a complex balance of activating and inhibitory signals received by the cell and modulation of these signals may shift the balance on NK activation. This review discusses the various activating and inhibitory stimuli which can act on NK cells, and suggests that future NK cell-based therapies consider not only activating stimuli but also removal of possible inhibitory elements which could prevent optimal NK cell function and/or survival.
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*Keywords:* Natural killer cells; Cytokines; Immunoregulation

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## 1. Introduction

Natural Killer (NK) cells are large, granular lymphocytes classified as members of the innate immune system. These cells play an important role in the response to viruses, parasites, microbial pathogens and neoplastic cells by the release of cytotoxic granules as well as secretion of a wide range of cytokines [1,2]. NK cells were originally identified functionally when lethally irradiated and unsensitized F1 hybrid mice were shown to reject parental or allogeneic bone marrow allografts [3,4]. This observation was found to be in disagreement with classical transplantation laws which stated that F1 offspring are obligated to accept co-dominantly inherited parental major histocompatibility complex (MHC) determinants. This phenomenon became known as 'hybrid resistance,' in that the F1 mice (H-$2^{bxd}$) were found to reject engraftment of either parental (H-$2^b$ or H-$2^d$) bone marrow cells (BMC) [1]. Subsequent studies found that the cells mediating this bone marrow rejection were radioresistant and identical to the lymphoid cells that were found to kill tumors *in vitro* in an MHC-unrestricted manner [2,5,6]. These cells became known as 'Natural Killer' cells for their ability to spontaneously lyse target cells without need of prior sensitization [7]. Later studies demonstrated that mice with severe combined immune deficiency (SCID), which lack functional B and T cells due to a mutation preventing productive V(D)J recombination, but have fully functional NK cells, were able to reject bone marrow but not skin allografts indicating that NK cells appear to recognize determinants on hematopoietically derived cells [8]. NK cells have been shown to be pivotal mediators of innate immunity and play an important role in mediating resistance to viruses, tumors, and other infectious disease pathogens. Early in a viral infection, NK cells are activated and suppress viral spread [9]. The role of NK cells and cancer has also been extensively examined and NK cells have been shown to help control tumors and reduce the spread of metastases [10]. In mice depleted of NK cells, growth of some tumors as well as tumor metastases were increased [10,11]. The versatility of NK cells makes them attractive cells to exploit clinically in order to treat a multitude of disease states, particularly in cancer. However, the reported ability of NK cells to target virally infected, neoplastic, or bone marrow cells appears to vary greatly from one model system to the next. This is most likely in part due to the numerous activating and inhibitory stimuli which act on the NK cell (Fig. 1), both directly and through the surrounding environment which affects overall NK cell function. The species differences between mouse and man may also contribute to the differences seen with *in vitro* and *in vivo* responses in preclinical models and clinical trials. This review will examine NK cell biology in terms of differences between human and murine cells, development from progenitors, factors affecting their activation and inhibition, and possibilities for clinical application.

## 2. Human and murine NK cells—similarities and differences

The mouse is the most common preclinical model used in NK cell studies and has distinct advantages and disadvantages with regard to extrapolation to human NK cell biology. One of the primary advantages of murine studies-*versus*-higher vertebrates is that they are relatively easy to maintain and significantly lower in cost and provide rapid readouts for many disease states, including cancer. The ability to work with the highly characterized mouse genome and genetically identical inbred mice under controlled conditions also make murine studies highly reproducible. Murine studies also allow for *in vivo* assessment which is critical when examining pathogenic situations. However, there are also significant differences that need to be considered when attempting to extrapolate murine NK cell data to the human system. Initial differences between mouse and human NK cells can be seen with their phenotypic differences. Murine NK cells can be defined as NK1.$1^+$C$D3^-$ or DX$5^+$C$D3^-$ cells. Most NK cells in mice also express asialoGM1, but other cells, including macrophages and activated T cells, are also asialoGM$1^+$ making this marker not NK cell-exclusive [12,13]. Many laboratories, however, still use asialoGM1 antibodies for the *in vivo* depletion of NK cells if NK1.1 is not present in the strain, as in Balb/c mice. In contrast, human NK cells are also defined as being CD3 negative but express the neuronal cell adhesion molecule CD56 (CD$56^+$C$D3^-$). However, the extent of expression of CD56 varies on different populations of NK cells, and there is a strong correlation between CD56 expression and NK cell cytolytic function. CD$56^{dim}$ NK cells are present in peripheral blood and contain large amounts of perforin and granzymes. These cells are highly cytotoxic but produce low levels of cytokines, while, on the other hand, CD$56^{bright}$ NK cells contain much less perforin and granzymes and are poorly cytotoxic but produce high levels of cytokines. These CD$56^{bright}$ cells are primarily present in the lymph nodes and represent a significant percentage of the total amount of NK cells in the body [14]. Interestingly, resting splenic murine NK cells also do not appear to express significant amounts of perforin or granzymes in contrast to human CD$56^{dim}$ NK cells. There are no known equivalents of the CD$56^{dim}$ and CD$56^{bright}$ NK cells in mice, and there is a paucity of NK cells in mouse lymph nodes.

Several studies have suggested NK cell and dendritic cell (DC) interactions to be critical for human NK cell-mediated responses, primarily through the production of IL-12 by the DCs which has been shown to stimulate both NK cell cytotoxicity and IFN-γ production [15,16]. NK cell stimulation by DCs in mice is generally thought to occur through type I interferons (IFNs) for cytotoxicity and IL-2 [17] or IL-15 and IL-12 for IFN-γ production [18]. However, a recent study demonstrated that GITR–GITR–ligand interactions may also contribute to DC-mediated stimulation of NK cells [19]. Cytokine production may be an important role for the CD$56^{bright}$ NK cell population rather than direct lysis of target cells.

Another important difference between human and murine NK cells is their method of self-recognition of major histocompatibility complex (MHC) proteins. There are three families of receptors which are specific for self MHC: (1) killer cell immunoglobulin-like receptors (KIRs), (2) Ly49 receptors, and (3) NKG2/CD94 receptors. In mice, there are no known functional KIR receptors, although they do express both Ly49 and NKG2/CD94 receptor families. Conversely, in man KIRs and

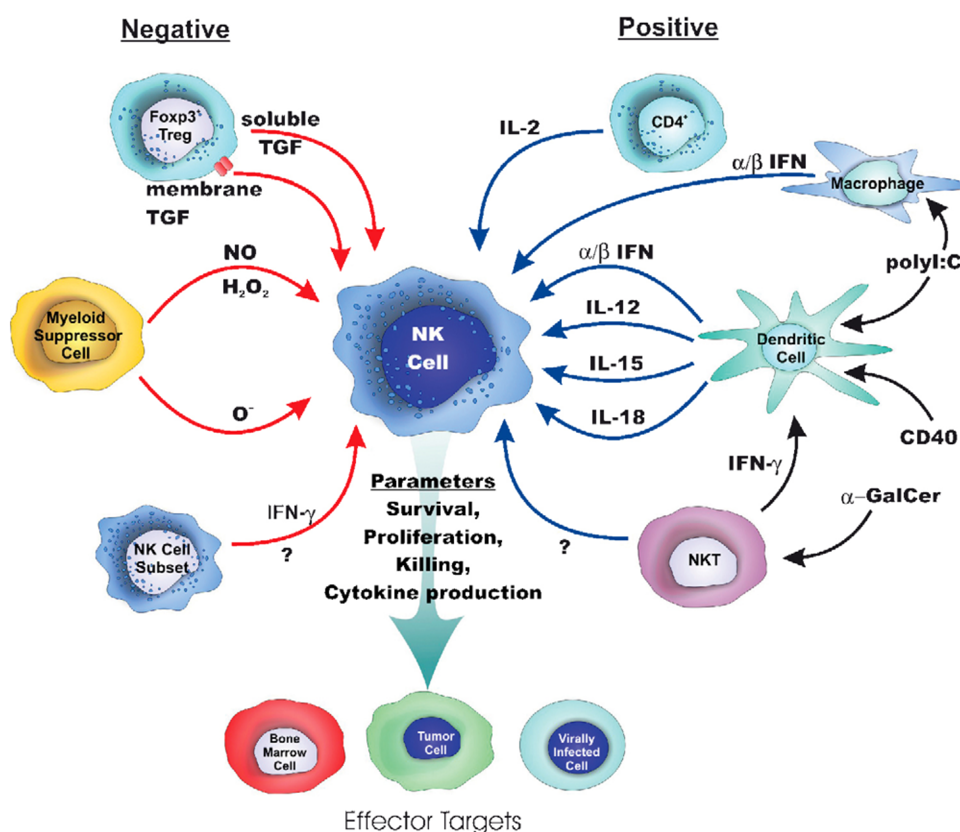


Fig. 1. Activating and inhibitory stimuli on NK cells. NK cells effector mechanisms against tumors, virally infected cells, or BMC are affected by numerous activating and inhibitory stimuli. NK cells are activated, either directly or indirectly, through $CD4^+$ T cells, monocytes/macrophages, dendritic cells, or NKT cells. Similarly, NK cells can be inhibited by regulatory T cells, myeloid suppressor cells, or other NK cell subsets. The combination of both signals determines overall NK cell function.

NKG2/CD94 are expressed and not Ly49 molecules. However, a single, non-functional Ly49 pseudogene does exist in humans [20]. Interestingly, although Ly49 receptors and KIR receptors are evolutionarily quite different with respect to sequence and structure, both receptors have evolved to perform a similar function, suggesting the critical importance of self-MHC recognition in NK cell biology.

Aside from phenotype and self-MHC recognition, human and mouse NK cells also have other differences. Studies examining pregnant mice have found that there are perforin-expressing cells with an NK cell phenotype in the uterus as well as the lungs and salivary glands, although these cells are not present in non-pregnant, resting mice [21]. Therefore, while *ex vivo* NK cells isolated from human peripheral blood exhibit strong cytolytic capability with abundant perforin, resting murine NK cells obtained from the spleen are very poor killers with low perforin [21,22]. When activated, however, murine splenic NK cells will express perforin and showed increased lytic capability. The high levels of perforin found in human NK cells could be due to the numerous genetic as well as environmental differences between the species. Laboratory mice are generally inbred, housed in specific pathogen-free conditions, and are used at a relatively young age (8–12 weeks) as opposed to human studies. These controlled situations may be too far removed from the human environment to make appropriate conclusions about the role or phenotype of NK cells based solely on data from murine models.

Another important difference in the NK cells between mouse and man can be found in the ability of human NK cells to produce clones that grow for an extended time *in vitro*. Human NK cells can survive in extended cultures of IL-2 while maintaining KIR receptor expression [23]. Normal murine NK cells do not form clones and cannot survive in long-term cultures, usually dying after two weeks of culture even under optimal growth conditions. However, NK cells generated from p53 knockout mice have been shown to be stable in long-term cultures for more than 2 years [24].

Therefore, these important differences between the NK cells in mouse and man provide critical caveats for researchers to consider when looking to extrapolate data from pre-clinical murine models to the clinic.

## 3. NK cell development

In order to understand NK cell function it is important to know the origins and their development of NK cells. NK cells develop from hematopoietic stem cells (HSC) located within the bone marrow (BM). NK cells appear to need the

BM microenvironment for successful development. Ablation of bone marrow by administration of estradiol or the bone-seeking radio-isotope ($^{89}Sr$) inhibits NK cell development, while not markedly affecting other hematopoietic lineages including B and T cells [25,26]. This has been thought to be due to NK cell dependence on the bone marrow stromal cells for development, while cells from other lineages can develop in extramedullary sites, such as the spleen or liver. NK cells develop properly in athymic nude mice or in mice that have undergone a splenectomy [27,28]. In the fetus, however, NK cells have been shown to develop in the thymus and the liver [29,30] although overall development and function of fetal NK cells is unclear. Functionally, fetal thymic NK cells lack many Ly49 receptors, with the exception of Ly49E, which is expressed at 10- to 30-fold higher levels [31]. Fetal NK cells exhibit poor cytolytic function and can only lyse $MHC^{-/-}$ tumors by prior cross-linking their activating receptors. The role of fetal NK cells is still poorly understood although it is possible that they may help protect the developing fetus from infection before the maturation of the adaptive immune system. Neonatal NK cells are also poor killers, although some studies have shown that both fetal and neonatal NK cells use Tumor Necrosis Factor (TNF)-related apoptosis inducing ligand (TRAIL) as a key effector molecule in some tumor models [32]. Lysis of the NK cell-sensitive cell line L929 by fetal and neonatal NK cells was found to be inhibited by anti-TRAIL neutralizing antibodies, although not by anti-FasL neutralizing antibodies, or by the granule inhibitor concanamycin A [32]. This is in contrast to NK cells from adults where perforin/granule-mediated killing predominates.

Early developing $CD34^+$ HSCs have been shown to require stromal cell contact for NK cell development, while more differentiated $CD7^+CD34^+$ cells can develop into NK cells by the addition of interleukin-2 (IL-2) alone [33]. These results, as well as other studies, demonstrated that once human or mouse NK cell precursors (NKP) were committed they could develop into NK cells following stimulation with IL-2 or IL-15 [34,35]. Later studies showed that the stromal cell requirements could be bypassed by the addition of stem cell factor, (SCF, also known as c-kit ligand), fetal liver kinase 2 ligand (FLK2, also known as FMS-like tyrosine kinase 3 ligand or FLT3L) and IL-7 [35]. These receptor tyrosine kinases for these cytokines, c-kit and Flt3, respectively, are present on developing hematopoietic stem cells (HSCs) as well as NK cell precursors (NKPs), but not mature NK cells (Flt3) or only on a small subset (c-kit) [36]. These cytokines have been shown to aid in the development of NK cells and their loss results in a reduction in murine NK cell numbers [37,38]. However, the loss of either c-kit or Flt3 does not completely eliminate NK cells, suggesting that neither is essential for NK cell development.

IL-15 is a particularly critical cytokine for NK cell development and survival. IL-2 alone has been shown to be capable of generating mature NK cells from progenitor cells *in vitro* and activated NK cell function *in vivo*, and thus was thought to be critical for NK cell development. However, IL-2 deficient mice have been shown to retain a functional NK cell population, though activity was reduced [39]. In contrast, mice that are deficient for IL-15 completely lack NK cells and transfer of NK cells into IL-15-deficient mice results in their loss [40]. Being that stromal cells and dendritic cells can either make or present IL-15 to the NK cells, this may be a critical and limiting pathway in NK cell development and function, and analogous to IL-7 with T cells, regulate NK cell numbers in the periphery.

## 4. NK cell activation

NK cell activation leads to a dramatic increase in cytolytic activity [41]. Besides cytolytic activity, activated NK cells can also produce a multitude of cytokines with the ability to modulate immune responses to pathogens and directly affect the target cell as well. Pivotal among these cytokines is interferon-γ (IFN-γ). IFN-γ is a pleiotropic cytokine capable of influencing both the innate and adaptive immune systems. It has also been shown to favor the development of a proinflammatory, cell-mediated immune response by the stimulation of antigen presenting cells (APCs) as well as driving the differentiation of $CD4^+$ T-helper 1 (Th1) lymphocytes. Both viral and fungal resistance have been shown to be mediated by NK cells in part due to their production of IFN-γ. NK cells have been demonstrated to be an early host defense mechanism to fungal diseases such as *Aspergillus* [42]. The role IFN-γ produced by NK cells was also shown using the intracellular bacterium *L. monocytogenes* in SCID mice, where IFN-γ production by NK cells led to the antimicrobial function of infected macrophages [43]. Early after infection, IFN-γ is primarily detected in NK cells, and this rapid production of IFN-γ by NK cells contributes to host defense prior to the emergence of an effective adaptive immune response [44,45]. The rapid NK cell activation is can be linked to the activation of professional antigen presenting cells (APCs), which upon activation, produce IL-12. IL-12 stimulation of NK cells leads to increased cytotoxicity of NK cell-sensitive and insensitive tumor targets, as well as increased production of IFN-γ [46].

Although NK cells can respond directly to some pathogens, responses to microbial pathogens generally require the assistance of APCs. APCs and other phagocytic cells, express pattern recognition molecules known as Toll-like receptors (TLRs) which can bind products from a wide range of pathogens. In response to these pathogens, TLRs signal the APC to produce multitude cytokines including interleukin-1β (IL-1β), IL-12, IL-15, IL-18, and tumor necrosis factor α (TNFα). The combination of IL-12 and IL-18 has been shown to synergize resulting in large amounts of IFN-γ production by NK cells [47–49]. These cytokines also result in significant anti-tumor responses as well [50].

In addition to IFN-γ, activated NK cells also produce granulocyte–monocyte colony stimulating factor (GM-CSF). GM-CSF is a hematopoietic growth factor that stimulates the development of neutrophils, monocytes, and DCs and promotes the proliferation and development of early erythroid megakaryocytic and eosinophilic progenitor cells [51].

Human NK cells constitutively transcribe GM-CSF at low levels and transcription of GM-CSF increases dramatically following IL-2 or CD2 stimulation [52]. Human NK cell lines stimulated with IL-2 and IL-12 also show synergized production of IFN-γ and GM-CSF [53]. The activating stimuli to NK cells

can affect survival and proliferation of the NK cell itself, as well as function as reflected by cytotoxic capability and cytokine production. GM-CSF production by NK cells may also be a means by which NK cells contribute to DC and adaptive immune responses.

### *4.1. NK cell activating receptors*

It has been demonstrated that NK cells can markedly lyse cells lacking expression of MHC class I. It was believed that NK cells were selectively 'turned off' or inhibited by MHC class I expression, and thus NK cells are perpetually in an active state and needed to be "turned off" to avoid target killing. This was followed with the characterization of inhibitory receptors on NK cells. In recent years, a number of activating receptors have been identified on NK cells that complement these inhibitory receptors. Signaling through activating receptors is balanced with signals received through inhibitory receptors, and the final killing event by an NK cell could be determined by weighing input between these opposing stimuli.

In order for NK cells to receive an activation signal from a potential target, ligation of the activating receptor must occur. However, there is debate as to whether activating signals of some type are absolutely necessary for NK cell function. One receptor of particular interest is the activating receptor NKG2D. NKG2D is a homodimeric type II transmembrane receptor which is found on human and rodent cells [54,55]. In addition to NK cells, NKG2D expression can be found on $CD8^+$ T cells, γδ T cells, NKT cells, and activated macrophages [55]. Unlike other NKG2 family members (NKG2A, NKG2B, NKG2C, and NKG2E), NKG2D does not form heterodimers with CD94, and is specific for different ligands[56]. The ligands responsible for triggering NKG2D have been shown to be expressed as 'stress' ligands [57]. These molecules are believed to have evolved to aid the body by signaling the immune system when cells become stressed, due to either exogenous (*e.g.* viruses) or endogenous (*e.g.* neoplastic transformation) factors. In humans, NKG2D has been found to interact with distinct MHC class I family members, specifically MICA and MICB. Recently, NKG2D has been shown to play a role in murine bone marrow graft rejection [58]. The administration of anti-NKG2D monoclonal antibodies allowed the engraftment of parental BALB/c mice by F1 hybrid mice, demonstrating that NKG2D is involved with NK cell-mediated bone marrow rejection [58]. It was also found that transgenic expression of NKG2D ligands in C57BL/6 mice prevented engraftment into syngeneic C57BL/6 mice, though engraftment could occur if blocking antibodies for NKG2D were used [58]. This suggests a novel role for NKG2D in bone marrow transplantation but may be dependent on strains examined and NKG2D may work in concert with other receptors.

2B4 (CD244) is an interesting member of the CD2 subfamily that has been shown to be capable of transducing both activating and inhibitory signals to the NK cell [59]. The cytoplasmic domain of 2B4 binds to a small protein named SAP, which is crucial for 2B4-mediated activation. If 2B4 engages its murine target ligand CD48 (CD58 in man) in the absence of the SAP protein, 2B4 will transduce an inhibitory signal [60]. The recruitment of the SAP protein to 2B4 has been found to prevent the binding of SHP-1 phosphatase to the cytoplasmic domain of 2B4. In the absence of 2B4, SHP-1 binding leads to dephosphorylation of downstream elements involved in NK cell activation [61]. People with X-linked lymphoproliferative disease (XLP), which has been correlated with a mutation in the SAP-encoding gene, have normal immune function until they are exposed to Epstein-Barr Virus (EBV) [62], at which time XLP patients often get lethal infectious mononucleosis as a result of NK cell unresponsiveness to EBV-infected cells. It has been observed that NK cells from patients with XLP lack the ability to kill $EBV^+$ B cell lines, and that the addition of monoclonal antibodies to 2B4 restores NK cell-mediated killing of $EBV^+$ B cell lines [62].

The Fc receptor, CD16, is also an activating receptor present on both murine and human NK cells. One of the early observations with NK cells was that nearly all NK cells express CD16 (FcγRIII), a receptor with a low affinity for the Fc portion of antibodies known to associate with the signaling molecule FcεRIγ. Upon binding to the Fc portion of an antibody, NK cells are triggered to mediate antibody-dependent cellular cytotoxicity (ADCC) [63]. Antibody triggering also leads to signaling through FcεRIγ, an adaptor protein which leads to the production of IFN-γ and GM-CSF, as well as degranulation of the NK cell [64].

Although Ly49 receptors are primarily known for their inhibitory effect on murine NK cells upon engagement of MHC class I, there are also two known activating Ly49 receptors. Ly49D is specific for several MHC class I molecules, including $H\text{-}2D^d$ [65], and $Ly49D^+$ NK cells from C57BL/6 mice are activated by $H\text{-}2D^d$ expressing cells. Ly49H is also a NK cell activating receptor. Upon infection by murine cytomegalovirus (MCMV), cells often down-regulate MHC class I expression to prevent cytolysis by T cells. However, this now exposes the virally infected cells to NK cell-mediated killing. The virus, in response, evolved the ability to produce decoy "MHC-like" molecules. One of these decoy molecules, m157, is structurally similar to MHC class I molecules [66]. Presenting this decoy receptor allows MCMV to evade killing by NK cells. This evasion by MCMV was countered by Ly49H, which is specific for m157 and upon engagement triggers a strong cytolytic response [67]. The evolution of this virus and subsequent evolution of the NK cell is a fascinating example on the importance of these molecules in the immune system.

Other cell surface molecules can affect NK cell function *in vivo*, including those activating via indirect pathways (by triggering other cells that produce factors which activate NK cell functions). Similar to viral activation, NK cells can also be activated by synthetic oligodeoxynucleotides such as polyinosinic–polycytidylic acid (poly I:C), which is a synthetic polymer of inosine that resembles the RNA of infectious viruses and is used to stimulate the production of interferon by the immune system [68] or CpG dinucleotides which mimic bacterial DNA. Poly I:C has been shown to induce the production of IFN-α by dendritic cells and macrophages, which leads to the subsequent activation of NK cells [69]. This correlation was demonstrated in studies in which mice were administered poly I:C with or without silica (which has been shown to reduce

macrophage numbers in mice) [70]. Treatment of mice with silica prior to poly I:C administration prevented NK cell activation and bone marrow rejection. Engagement of poly I:C by DCs or macrophages is mediated through TLR3 [71]. Poly I:C therapies have been used clinically to induce interferons which subsequently induce NK cell function [72,73], and it remains an attractive therapeutic due to its potential for NK cell activation through IFN production by APCs. However, potential problems with toxicities may limit its use in the clinic [74]. Poly I:C has long been shown to augment NK cell activity *in vivo* due to effects on macrophages. CpG dinucleotides act on Toll-like receptors and behave in a similar manner. The end result of many of these agents is augmentation of NK cell function *in vivo*.

Stimulation of CD40, a molecule expressed by numerous cell types including B cells, monocytes and DCs, has been shown to activate NK cells indirectly *in vivo*. Murine studies with an agonist monoclonal antibody to CD40 have shown anti-tumor and anti-metastatic responses to be dependent on NK cells in some tumor models [75]. NK cells are negative for CD40 and it is unlikely that anti-CD40 antibody was acting directly on the NK cell. However, anti-CD40 antibodies are known to activate APCs, resulting in the production of many potent NK cell activating cytokines by monocytes or DC [76].

### 4.2. *Cytokine activation of NK cells*

Activation of NK cells can be accomplished by direct stimulation with numerous cytokines (Table 1). First among these cytokines is IL-15, a pleiotropic cytokine generated primarily by stromal cells and dendritic cells. IL-15 was shown to stimulate proliferation of an IL-2-dependent cell line [77] and it was shortly thereafter shown to stimulate human NK cells by augmenting proliferation and cytotoxicity. [78]. Exposure to IL-15 causes the upregulation of the anti-apoptotic protein bcl-2 which gives IL-15-stimulated NK cells a survival advantage [40]. In addition to its critical role in NK cell development, IL-15 is also important for homeostatic proliferation and survival of NK cells. This effect was dramatically demonstrated in a study in which NK cells were transferred to IL-15$^{-/-}$ mice (which lack NK cells) or IL-15$^{+/-}$ littermate controls (which have functional NK cells) [40]. While a substantial population of the adoptively transferred NK cells survived for up to 5 days in IL-15$^{+/-}$ mice, NK cells in IL-15$^{-/-}$ mice could not be detected 5 days after adoptive transfer. These studies identified the critical role IL-15 plays in NK cell survival and homeostatic proliferation. One noteworthy finding is that NK cells do not require expression of the high affinity IL-15Rα chain. IL-15R α-chain is expressed on monocytes/macrophages, and interactions between these cells and NK cells expressing IL-2/IL-15Rβγ (CD122 + CD132) is capable of generating a proliferative signal to the NK cells [79].

IL-2 is another cytokine that has been demonstrated to augment NK cell function. As with IL-15, IL-2 also up-regulates bcl-2 expression in NK cells [80]. IL-2 has been shown to induce IFN-γ production by human [81] and murine NK cells [82]. In addition, many studies have shown the beneficial effects

Table 1
Activation and inhibition of Natural Killer cells

| Cytokine | Species | Cytotoxicity | Cytokine production | Proliferation | Survival |
|---|---|---|---|---|---|
| IL-2 | Human | + [172] | + [84] | + [173] | + [83] |
| | Mouse | + [174] | + [53] | + [85] | + [175] |
| IL-12 | Human | + [176] | + [92] | + [92] | − [80] |
| | Mouse | + [177] | + [53] | + [97] | |
| IL-15 | Human | + [78] | + [78] | + [178] | + [179] |
| | Mouse | + [79] | | + [170] | + [40] |
| IL-18 | Human | + [99] | + [99] | + [99] | |
| | Mouse | + [97] | + [47] | + [97] | |
| IL-21 | Human | + [100] | | + [100] | |
| | Mouse | + [101] | + [101] | + [102] | − [102] |
| IFN-α/β | Human | + [72] | | + [180] | |
| | Mouse | + [180] | + [181] | + [182] | |
| IFN-γ | Human | + [183] | | | |
| | Mouse | + [184] | + [112] | | |
| TNF-α | Human | + [185] | | | − [121] |
| | Mouse | | + [186] | | |
| Poly I:C | Human | + [187] | + [187] | | |
| | Mouse | + [68] | + [68] | + [182] | |
| CD40 Stimulation | Human | + [188] | + [189] | | + [190] |
| | Mouse | + [75] | | | |
| TGF-β | Human | − [142] | − [142] | | |
| | Mouse | − [140] | − [191] | − [192] | |

List of factors suggesting activation (+) or inhibition (−) of NK cells by various stimuli with references in square brackets.

of IL-2 on NK cell proliferation and cytotoxicity [83–86]. Following BMT, most studies have shown NK cells can prevent GVHD while promoting GVT. Pre-clinical studies in mice have shown that adoptive transfer of IL-2-activated NK cells and administration of recombinant human IL-2 prevented GVHD while maintaining beneficial graft-*versus*-tumor (GVT) effects [87]. However, there are murine studies in which the prevention of GVHD by high dose IL-2 regimens was independent of NK cells [88]. These differences may be due to multiple cellular pathways affected by high dose IL-2 which may compensate for each other. Case in point, IL-2 has been demonstrated to induce suppressive T regulatory cells [89].

Interleukin (IL)-12, a heterodimeric cytokine of 70 kDa comprised of covalently linked p40 and p35 subunits, was originally discovered in 1989 [46]. IL-12 is an important regulator of Th1 responses and is predominately produced by dendritic cells (DCs), monocytes, and macrophages [90]. IL-12 is able to stimulate NK cell production of IFN-γ as well as augment NK cell proliferation and cytotoxicity [91,92]. The production of IFN-γ by NK cells following IL-12 treatment is considered important for anti-tumor responses in murine models [93]. In man, IL-12 can enhance the NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) of antibody-coated Her2-expressing tumor cell lines [94]. NK cells produce abundant IFN-γ, TNF-α, MIP-1α, and GM-CSF in response to IL-12 and antibody-coated tumor targets [94]. Importantly, depletion of NK cells within hosts given exogenous IL-12 attenuates the anti-tumor effect, suggesting a vital role for NK cells in the anti-tumor activity of IL-12 [95].

The proinflammatory cytokine IL-18 is also capable of directly activating NK cells. IL-18 produced by macrophages and dendritic cells plays an important role in the Th1 response, primarily based on its ability to induce IFN-γ production by T cells and NK cells [49,96,97]. In mice bearing a melanoma cell line, IL-18 treatment significantly reduced tumor burden. In this study, co-treatment with the NK cell-depleting antibody asialoGM-1 abrogated the observed anti-tumor effects of IL-18 [98]. Other studies have shown a heightened proliferative and cytotoxic response of NK cells to IL-18 [97,99].

Another cytokine capable of activating NK cells is IL-21. IL-21 is a product of activated T cells and can play a role in proliferation and maturation of NK bone marrow progenitors, as well as activation of peripheral NK cells in human assay systems [100]. IL-21 is not necessary for development of NK cells since IL-21R knockout mice possess normal NK cell numbers and function in response to IL-15 *in vitro* or poly I:C *in vivo* [101]. IL-21 has been shown to enhance the cytotoxicity and IFN-γ production of activated murine NK cells [101]. However, this increase in activity did not correlate with an increase in NK cell survival. *In vitro* cultures of IL-15-activated NK cells cultured with IL-21 showed a decrease in cell number as well as an increase in apoptosis, while IL-15 stimulated NK cells remained viable [101,102]. Thus, it is possible that one needs to disassociate effects on survival-*versus*-function with regards to NK cells (Table 1).

### 4.3. Dendritic cells

Over the last several years there has been much interest in the crosstalk that occurs between dendritic cells and NK cells. In a study with mice bearing MHC I-negative tumors, adoptively transferred bone marrow-derived DCs or *in vivo* Flt3L-expanded DCs increased NK cell-dependent anti-tumor activities both *in vivo* and *in vitro* [103].

It has been found that many of the cytokines responsible for NK cell activation, including IL-2, IL-12, IL-15, IL-18, and type I IFNs, are produced by dendritic cells [104,105]. Dendritic cells are a major source of IL-15, a cytokine essential for NK cell development, proliferation, and survival [106,107]. At sites of inflammation, NK cells and immature DCs can interact with one another. The cellular contact between the two cell types appears to be important for promoting a series of events, including DC-induced NK proliferation [108]. During this interaction, DCs go through a period of maturation leading to the release of cytokines that influence the functional behavior of nearby NK cells. For example, IL-15 bound to IL-15Rα chains on the surface of activated dendritic cells has been shown to induce proliferation of NK cells [18]. Also, the release of IL-12 by DCs has been shown to be critical for the induction of IFN-γ release by NK cells and, in conjunction with IFN-α/β, for the enhancement of NK cell-mediated cytotoxicity [109]. The *in vivo* importance of DC-NK cell crosstalk is still being determined, but targeting DCs may be another mechanism to modify NK cell effects for immunotherapies. A recent study has demonstrated that GITR–GITRL interactions by NK cells and immature DCs leads to activation of NK cell function [19].

Studies by Dalod et al. have shown that plasmacytoid DC (PDC), which produce abundant IFN-α, TNF-β, and IL-12 following MCMV infection, induce rapid NK cell activation in response to MCMV [110]. Co-culture of NK cells with PDCs isolated from spleens of infected mice led to increased cytotoxicity against Yac-1 targets and increased IFN-γ production compared to co-culture with PDCs from uninfected mice [110]. NK cells are capable of responding to IFN-α/β by upregulating TRAIL on their surface [111]. TRAIL promoter analysis has shown the presence of an IFN-stimulated response element (ISRE) which binds to interferon stimulated gene factor-3 (ISGF3), a transcription factor stimulated by IFN-β. Additionally, virally infected cells, but not control cells, were susceptible to NK cell-mediated lysis *in vitro* [111]. The interactions between DCs and NK cells suggest that the stage of activation or type of DC can have a dramatic impact on whether NK cells are activated during interactions.

### 4.4. Natural Killer T cells

Natural Killer T (NKT) cells make up less than 1% of mouse T lymphocytes, yet they have been shown to be involved in a variety of immune responses [112–114]. The properties of NKT cells have been imprecisely characterized since some investigators have identified these cells as $TCR^+$ and $NK1.1^+$. However, conventional CD8 T cells can express NK1.1 after activation.

The unique property of NKT cells is a CD1d-restricted TCR. NKT cells express the Vα14-Jα18 rearrangement and react to the glycosphingolipid α-galactosylceramide (αGalCer) when it is presented by the class I-like molecule CD1d [115]. One of the main features of NKT cells that separate them from conventional T cells is their ability to secrete a variety of cytokines within a few hours after activation [116]. In response to TCR stimulation, NKT cells are most well known for producing copious amounts of IFN-γ and IL-4, though they have also been shown to produce IL-2, TNF-α, IL-5, IL-13, and GM-CSF [112]. The NKT cell's ability to produce IFN-γ and IL-4 rapidly is partially attributed to their possession of mRNA for both cytokines even before activation [117].

The activation of NKT cells *in vivo* leads to the subsequent activation of NK cells. It has been shown that within hours of αGalCer stimulation in mice the bulk of the resulting IFN-γ in the sera is due to the activity of NK cells [118]. In addition to stimulating NK cells to produce IFN-γ, αGalCer administration causes NKT cells to aid in NK proliferation and to increase their cytotoxic potential [118–120]. However, there are various subpopulations of NKT cells, some of which are suppressive and these may inhibit NK cells.

## 5. Inhibition of NK cells

Use of NK cells in protecting against viral, bacterial, and neoplastic insults can be mediated through numerous activating stimuli, but there exists a second checkpoint which needs to be overcome: the inhibitory signal. In some instances, inhibitory stimuli may be the over-riding factor affecting NK cell function. This may be an important regulatory pathway to circumvent autoreactive responses. NK cells receive many activating and inhibitory stimuli simultaneously, and the shift in the balance towards activation may result in demonstrable NK cell activity. The majority of means to increase NK cell function have relied solely to augment activation. However, removal of persistent inhibitory stimuli may greatly complement activation, such that effective therapies could be achieved using significantly lower doses of activating methodologies, resulting in lower toxicities. Importantly, there may be other issues in NK cell homeostasis and survival, such as also occur in T cell responses which need to be taken into consideration. It is possible that cytokines such as TNF-α and IFN-γ may also lead to contraction of NK cell responses and NK cells may undergo an activation-induced cell death (AICD) similar to T cells [121] or that loss of IL-15 leads to NK cell contraction. Thus far there is very little known with regard to regulation of NK cells *in vivo* and how they are controlled during infection.

### 5.1. Inhibitory NK cell receptors

Early studies with NK cell receptors primarily found receptors which generated an inhibitory response upon engagement of MHC class I molecules. The "Missing-Self" hypothesis by Ljunggren and Karre [122] was based on the observation that tumor cells lacking MHC class I molecules are more susceptible to killing by NK cells, unlike the MHC-dependent killing by CD8+ cytotoxic cells. The investigators observed that variants of a murine leukemia cell line lacking expression of the murine MHC molecules, H2, were more susceptible to NK cell-mediated lysis than H2-bearing leukemia cells [123]. This suggested a negative signal could result to the NK cell upon binding MHC class I.

The first identified inhibitory receptors identified on murine NK cells were the Ly49 receptors. These receptors interact with MHC class I molecules and transmit a potent inhibitory signal to the NK cell [124]. Ly49A was the first Ly49 characterized. This study found that IL-2-activated Ly49A$^+$ NK cells lysed a tumor cell line which normally express the MHC class I of the b-allele (H2$^b$), but not the same cell line transfected to express H2D$^d$, suggesting the binding of H2D$^d$ inhibited NK cell function [124]. The addition of anti-Ly49A antibody F(ab$'$)$_2$ fragments restored lysis of the H2D$^d$-transfected tumor cells, thus demonstrating that the Ly49 receptor is capable of inhibiting NK cell cytotoxicity. This study was quickly followed by additional discoveries of inhibitory Ly49 receptors including Ly49C, Ly49I, and Ly49G2 among others [125]. BMT rejection studies demonstrated similar patterns of MHC inactivation *in vivo* with these subsets.

Inhibitory receptors are generally expressed on overlapping subsets of NK cells, such that there are not clear definitions of 'activated' NK cells and 'inhibited' NK cells, but rather a variegated population of NK cells with a range of potential targets [126]. One characteristic of inhibitory receptors on NK cells is the connection with the immunoreceptor tyrosine-based inhibitory motif, or ITIM [127]. Engagement of an ITIM-associated receptor leads to the recruitment and activation of SHP-1, an SH2 domain containing tyrosine phosphatase which inhibits activation of hematopoietic cells [128].

Similar to the inhibitory Ly49 molecules on murine NK cells, there are inhibitory killer cell immunoglobulin-like receptors (KIRs) on human NK cells. As with murine NK cells, human NK cells are also inhibited by MHC class I molecules, and this inhibition can be removed by the addition of antibodies to specific KIRs on the NK cell [129]. In the transplant situation, some donor NK cells can express inhibitory KIRs for which the host does not have the appropriate ligand [130]. This is expected to result in an increase in NK cell function due to the reduction in inhibitory signals. This presents a powerful tool for the clinician who wants to utilize NK cell-based therapies. In addition to giving an activating stimulus to the NK cells, such as IL-2, reduction of inhibitory signals generated by the KIRs should shift the balance of the positive/negative regulation to favor a more positive outcome. In contrast to murine BMT studies demonstrating roles of Ly49 subsets in BMC rejection, clinical roles of KIR subsets in BMT rejection have been lacking. This may in part be due to extensive immunosuppressive conditioning regimens used in clinical transplants.

### 5.2. Regulatory T cells

Earlier studies have demonstrated that SCID and athymic nude mice, which lack T cells yet still possess NK cell activity,

had a heightened ability to reject BMC allografts compared to normal conterparts [131]. The conundrum of why NK cells from these mice were able to reject BMC to a greater extent than their normal counterparts may have been resolved by the characterization of regulatory T cells (Tregs). Tregs are a small subset of $CD4^+$ cells that constitute approximately 10% of the peripheral $CD4^+$ T cell population. Treg cells have typically been classified as $CD4^+$, $CD25^+$, $PD1^+$, and uniquely express the transcription factor, Foxp3 [132]. Treg cells are potently immunosuppressive and are postulated to maintain self tolerance [132], regulate homeostasis of the T cell pool [133], contribute to tolerance induction after solid organ transplantation [134], and provide protection from GVHD lethality in bone marrow transplantation models [135]. T cell inhibition by Treg cells can occur in an antigen-restricted [136] or antigen-unrestricted manner [137]. Researchers have recently shown that large numbers of Treg cells can be found in the tumor milieu of mice [138]. This is in part due to the migration of Tregs into the tumor environment, but may also be due to conversion of $CD4^+CD25^-$ T cells into Tregs by factors produced by the tumor itself. Further studies of the tumor environment indicated that high levels of TGF-β are capable of activating the quiescent Foxp3 transcription factor within the $CD4^+$ T cells, thus initiating a pathway leading to conversion of a $CD4^+$ T cell into a Treg cell [139]. Treg cells have been shown to inhibit anti-tumor responses within tumors, such that intra-tumoral depletion of Tregs results in reduced tumor burden [138].

Recently, Tregs have been shown to directly inhibit NK cell function directly in both tumor and BMT models, demonstrating a link between adaptive and innate immune cells [140–142]. The mechanism of Treg inhibition of NK cells in mice has not been completely elucidated, with some researchers reporting that IL-10 and soluble TGF-β mediate inhibition [143], while other researchers report surface-bound TGF-β as being the primary mediator of inhibition [140,143,144]. This study demonstrated that murine NK cells have reduced cytolytic activity in response to surface-bound TGF-β. This reduction was at least partially due to NKG2D down-regulation, although this was not observed in other studies [140]. Other studies have shown that soluble TGF-β also mediates reduction of NK cell cytotoxicity and perforin gene transcription [145]. Cytokine production by IL-12 activated NK cells has also been shown to be downregulated by co-culture with Tregs *in vitro* [142]. It is possible that both surface-bound and soluble TGF-β play a role in NK cell suppression, with surface bound TGF-β regulating NKG2D expression on murine NK cells, while soluble TGF-β down regulates the natural cytotoxicity receptor p30 on human NK cells, a protein which has been found to be critical for NK cell-mediated lysis of immature dendritic cells [142,146]. As NK cells themselves also produce TGF-β, it is curious if this is another means by which they can regulate themselves. Treg-mediated suppression of NK cells is not limited to functional inhibition but also affected NK cell homeostatic proliferation. Removal of Treg cells with a depleting anti-CD25 antibody results in an expansion and increase in cytotoxicity of NK cells [142]. The suppression generated by Tregs on NK cells was not complete, however. Although cytokine production by IL-12-activated NK cells was shown to be inhibited by Tregs, NK cells could be rescued by activation through IL-2Rγ chain-dependent cytokines, including IL-2, IL-4, IL-7, and IL-15 [142]. NK cells isolated from patients were cultured with purified Treg cells for 6 h, at which time cytokine was added, supernatants were harvested 18 h later and analyzed for IFN-γ by ELISA. While NK cells cultured with IL-12 alone produced IFN-γ, co-culture with Treg cells or soluble TGF-β inhibited IFN-γ production. Co-culture of NK cells with Tregs and IL-2Rγ chain-dependent cytokines successfully produced IFN-γ [142]. This suggests that the inhibition of NK cells is pathway specific, and thus Treg inhibition of NK cells may only occur in certain situations. It also leads one to speculate that as Treg cells inhibit IL-12-mediated activation of NK cells preferentially over IL-2Rγ chain-dependent cytokines, synergistic NK cell-mediated effects may be seen if Treg depletion is coupled with IL-12 therapy [140].

We have recently shown that Treg cells are capable of suppressing NK cell-mediated bone marrow rejection *in vivo* [141]. Treg inhibition of NK cells was found to occur in full MHC-mismatched allogeneic transplant models, as well as in a hybrid resistance model, where only NK cells are responsible for BM rejection. In these studies, reduction of Treg cells by treatment with anti-CD25 or anti-CD4 antibody augmented NK cell rejection of marrow allografts. This was demonstrated by engraftment by colony-forming unit-granulocyte/monocyte (CFU-GM) assays and long-term donor chimerism. Similar results were observed when mice were given neutralizing antibody to TGF-β, in that there was enhanced NK cell-mediated rejection of allogeneic or parental bone marrow. Furthermore, the addition of purified Tregs to the bone marrow graft inhibited activity of NK cells to reject parental bone marrow [141]. While it was shown that the inhibition of NK cells is partly mediated by TGF-β, it is likely that Treg-mediated inhibition of NK cells is due to multiple events *in vivo*. These studies do suggest that Tregs may represent a key inhibitory pathway in NK cell function and in cancer and in normal NK cell homeostasis.

### 5.3. *Myeloid suppressor cells*

Myeloid suppressor cells (MySCs) may also inhibit responses by NK cells. MySCs have a myeloid morphology and have been shown express the markers Gr-1 (Ly6-G) and CD11b (Mac-1). These cells accumulate in secondary lymphoid organs during immune responses, where they can inhibit T cell and B cell responses [147,148]. In addition, MySCs are known to produce several soluble mediators, including inhibitory cytokines, reactive oxygen intermediates, hydrogen peroxide, and nitric oxide, all of which can inhibit immune responses [149]. These products of MySCs, including reactive oxygen intermediates [150] and hydrogen peroxide [151], have been shown to inhibit both human and murine NK cells. To date there are not many studies suggesting interactions between MySCs and NK cells [152], but the role of MySCs in tumor-bearing animals suggests that elimination of these negative regulators may increase the effects of NK cell adoptive immunotherapies.

### 5.4. Natural Killer cells

Some intriguing studies have also suggested a role that NK cell subsets can inhibit other NK cells. Certain Ly49-bearing subsets of NK cells affect the ability of other subsets of NK cells with regard to BMC rejection [153]. Studies with $H2^d$ mice demonstrated that prior removal of the Ly49C/I subset, which is inhibited by $H2K^b$ and not involved in the rejection of $H2^b$ BMCs, significantly augmented rejection of the $H2^b$ BMCs by the remaining subsets [153]. A similar and converse situation was seen when $H2^b$ strain mice which were depleted of the Ly49G2 (specific for $H2D^d$) expressing NK cell subset and these mice had enhanced ability to reject $H2^d$ BMC [153]. These data demonstrated that the removal of a subset of NK cells possessing an inhibitory receptor causes increased NK cell function of the remaining subset, indicating that the removed subset was possibly inhibitory. This study highlights the complex interactions that NK cells could have with each other, and suggests that simple blockade of inhibitory receptors or receptor–ligand mismatches may not be enough to obtain optimal NK cell activation.

## 6. Clinical applications with Natural Killer cells

There has been tremendous interest in clinical exploitation of NK cells in cancer. NK cells may be of particular benefit in blood-borne cancers, such as leukemias/lymphomas, due to the predominant presence of NK cells in the peripheral blood and spleen. Therefore, it is not surprising that the major emphasis in the clinical exploitation of NK cells was for these types of cancers. Similarly, as BMT is primarily used for the treatment of leukemias and lymphomas, and there have been some intriguing studies suggesting that immunotherapy involving NK cells may be used in combination with BMT.

Myeloablative therapy followed by BMT offers patients with cancer a potential curative treatment. However, the complications of BMT often restrict the number of patients who can receive the therapy. Allogeneic transplants are associated with a beneficial graft-*versus*-tumor effect but, unfortunately, graft-*versus*-host disease (GVHD), in which donor T cells attack solid organs such as gut, liver, and skin, remains a significant cause of morbidity. Autologous bone marrow rescue is an alternative source of hematopoietic stem cells following myeloablative therapy. Although specific complications related to allogeneic transplantation, such as GVHD, are avoided or markedly less with autografts, this procedure is associated with an increased risk of relapse related to leukemic contamination of re-infused marrow and the absence of a graft-*versus*-leukemia effect.

NK cells, unlike T cells, can be transferred in an allogeneic setting without risk of initiating GVHD since they do not reject solid tissue allografts, and this has made NK cells attractive for use in BMT therapies in the treatment of hematologic malignancies. In pre-clinical murine studies, the administration of activated allogeneic NK cells following BMT has been shown to provide a superior GVL effect compared to autologous NK cells [154]. In addition, inhibitory receptor blockade using $F(ab')_2$ fragments to Ly49 inhibitory receptors has been shown to enhance the anti-tumor function of activated NK cells in a murine BMT model [155]. In these studies, leukemia-bearing $H\text{-}2^b$ mice were given syngeneic, IL-2 activated NK cells that had been treated *ex vivo* with a $F(ab')_2$ fragment of the 5E6 monoclonal antibody which is directed against Ly49C/I, the molecule that recognizes $H\text{-}2^b$ resulting and results in an inhibitory signal. Blockade of this inhibitory signal prevented NK cell inhibition, leading to increased tumor lysis and anti-tumor effects [155]. More work is needed to determine if even greater anti-tumor effects can be achieved if blockade of other inhibitory pathways (NKG2A, 2B4) is applied. Furthermore, adoptively transferred NK cells produce cytokines, such as GM-CSF, which aid in engraftment and myeloid recovery following BMT and this may also be augmented by either triggering activating or blocking inhibitory receptors [156].

Prior studies using adoptively transferred NK cells in clinical BMT has often been limited due to the limitation of using autologous NK cells and autologous BMT, which has not been effective in the treatment for cancer [157–159]. In murine models, adoptively transferred IL-2-activated allogeneic NK cells, or adherent lymphokine activated killers (ALAKs) have been shown to suppress GVHD [87,160]. Importantly, the suppression of GVHD by activated allogeneic NK cells was coupled with greater anti-tumor responses in mice with advanced tumor burdens [87]. These studies suggested that the adoptive transfer of allogeneic NK cells could not only inhibit lethal GHVD but also cause a reduction in tumor burden and increased survival.

In recent elegant studies by Miller, et al. [161], adoptively transferred haploidentical NK cells resulted in expanded NK cells *in vivo*. This study found that a high-intensity conditioning regimen was associated with an increase in endogenous IL-15 as well as an increase in NK cell expansion. Lesser conditioning regimens had significantly lower endogenous IL-15 levels and persistence, but not expansion, of adoptively transferred NK cells. Though not a primary goal of this study, 5 of the 19 patients in this study with poor prognosis AML had complete remissions, suggesting that this therapy may work for cancer therapies [161].

Ruggeri et al. [130] showed in a retrospective study that haploidential hematopoietic stem cell transplantation with KIR–ligand mismatches in a GVH direction led to significant graft-*versus*-leukemia (GVL) effects in AML but not ALL. It is important to note that in these studies no NK cells were transferred and were not even definitely shown to be responsible for the GVL effects. This prompted other researchers to attempt to repeat these clinical observations, though this has resulted in mixed results [162,163]. The different methods used for hematopoietic transplantation may have been a complicating factor. The hematopoietic stem cell transplant used in the Ruggeri study involved the transfer of large amounts of hematopoietic stem cells generated from donor G-CSF-mobilized peripheral blood cells, collected by leukapheresis, extensively depleted of T-cells, and positively selected for $CD34^+$ cells [164]. This regimen can allow for rapid NK cell recovery with delayed T cell recovery [163]. Importantly, no post-transplant immunosuppressive regimens were given to prevent GVHD. Other studies [163,165] used transplants with reduced T cell depletion, and these patients underwent

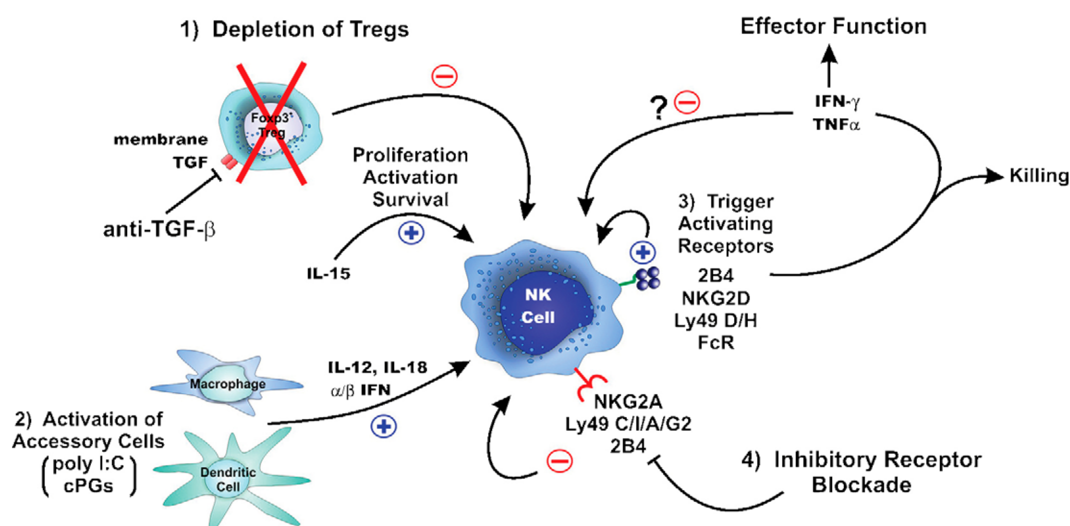


Fig. 2. Potential interventions to optimally activate NK cells. Optimal NK cell activation may require augmentation through engagement of activating stimuli and concurrent elimination of inhibitory stimuli. Stimulation of NK cells through cytokines or engagement of activating receptors alone may not generate adequate NK cell activation. Simultaneous treatment to remove suppressor cells, in addition to inhibitory receptor blockade, will potentially result in greater NK cell activations.

immunosuppressive regimens to prevent GVHD. Although the role of additional T cells in the graft has been shown to attribute to reduced IFN-γ production and KIR expression, the immunosuppressants used may also alter NK cell function [165]. One of the most common immunosuppressants used following BMT is cyclosporin A (CsA). Following BMT, many regimens use CsA to prevent or treat GVHD [166,167]. However, many studies have suggested that CsA treatment may reduce the effectiveness of NK cell therapies. Studies involving hybrid resistance models showed that treating F1 mice with CsA nine days prior to transplantation inhibited NK cell-mediated graft rejection [168]. The inhibitive effect of CsA, and other immunosuppressive drugs, on NK cells may need to be taken into consideration when NK cell-mediated effects are being desired.

NK cells are the first of the lymphoid cells to repopulate following a bone marrow transplant, generally making up 90% of peripheral blood lymphocytes for the first 20–60 days in humans [169]. If these early populating NK cells could be further expanded and activated in the post-transplant environment, more extensive elimination of residual tumor or prevention of viral infection could result. A recent study has shown that administration of IL-15 to mice following administration of T cell-depleted bone marrow allografts could increase the recovery of NK cells as well as boost the GVL effects in murine models [170]. Clinically, subcutaneous administration of low-dose IL-2 resulted in a 10-fold increase the number of $CD56^{bright}$, $CD3^{-}$ NK cells following autologous transplantation [171]. Further studies with patients with relapsed leukemia or metastatic breast cancer have shown that administration of IL-2 led not only to increased NK cell numbers, but also increased cytotoxic activity for NK cell-sensitive targets [159]. Unfortunately, the increase in NK cell number and activation in this study did not lead to an improvement in disease outcome [159]. It is possible that while administration of cytokines known to activate NK cells could be beneficial, other inhibitory signals may counterbalance any NK cell activation. Future studies with NK activation in a transplant setting should consider the removal of an inhibitory signal in addition to simply activating the NK cells. Further studies optimizing NK cell recovery post-BMT are needed to help patients recover from complication associated with BMT, including opportunistic infections and relapse of tumor.

## 7. Concluding remarks

One of the challenges facing clinicians in using NK cells is understanding the myriad of activating and inhibiting signals which affect NK cells. While our understanding of Ly49 and KIR receptor signaling has markedly increased our understanding of NK cell biology, it seems that these receptors are only partially responsible for stimulating/inhibiting NK cells. Therapies designed around single modes of NK cell activation, such as activating cytokine administration, use of KIR–ligand mismatches in BMT, or depletion of Tregs or other down-regulatory cells, may need to be augmented with multiple approaches in order to attain truly heightened activation status of NK cells (Fig. 2). NK cells have a potentially high therapeutic value based on their role as a clear link between innate and adaptive immune responses, but optimal function will require an understanding of the complex interactions between NK cells and other cells of the immune system.

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