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Developmental and Functional Control of Natural Killer Cells by Cytokines

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Natural killer (NK) cells are effective in combating infections and tumors and as such are tempting for adoptive transfer therapy. However, they are not homogeneous but can be divided into three main subsets, including cytotoxic, tolerant, and regulatory NK cells, with disparate phenotypes and functions in diverse tissues. The development and functions of such NK cells are controlled by various cytokines, such as fms-like tyrosine kinase 3 ligand (FL), kit ligand (KL), interleukin (IL)-3, IL-10, IL-12, IL-18, transforming growth factor-β, and common-γ chain family cytokines, which operate at different stages by regulating distinct signaling pathways. Nevertheless, the specific roles of each cytokine that regulates NK cell development or that shapes different NK cell functions remain unclear. In this review, we attempt to describe the characteristics of each cytokine and the existing protocols to expand NK cells using different combinations of cytokines and feeder cells. A comprehensive understanding of the role of cytokines in NK cell development and function will aid the generation of better efficacy for adoptive NK cell treatment.

Keywords: natural killer cells, cytokines, development, cytotoxicity, expansion

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INTRODUCTION

Natural killer (NK) cells were first identified as "natural killer cells" in the mid-1970s and were characterized by their vital roles in controlling cancer and viral infection (1–3). They are widely distributed in diverse tissues, such as the peripheral blood (PB), spleen, lungs, liver, and uterus (4). In human PB, NK cells are primarily divided into two subtypes: $CD3^-CD56^{dim}CD16^+$ and $CD3^-CD56^{bright}CD16^-$ cells. $CD56^{dim}$ NK cells have potent cytotoxicity and high CD16 expression, allowing them to induce antibody-dependent cell-mediated cytotoxicity (ADCC) toward target cells, whereas $CD56^{bright}$ NK cells are best known for producing diverse types of cytokines (5–7). Different from PB NK cells, NK cells in diverse tissues have distinct phenotypes. Through experimental parabiosis (8), researchers have found that, with the exception of circulating NK cells, the identification of several markers, such as CD69, CD103, and CD49a, can affirm the phenotype of tissue-resident NK cells in the liver, skin, and uterus (4, 9–14). Functions of NK cells vary depending on the cellular microenvironment, mainly due to the cytokine signals of various tissues. For instance, NK cells can regulate the outcome of pregnancy (15, 16) through the regulation of transforming growth factor (TGF)-β and interleukin (IL)-15 in the uterus (17–19) or tolerate plentiful food-derived antigens or bacterial products through the regulation of abundant TGF-β and IL-10 in the liver (20–23) (**Figure 1**).

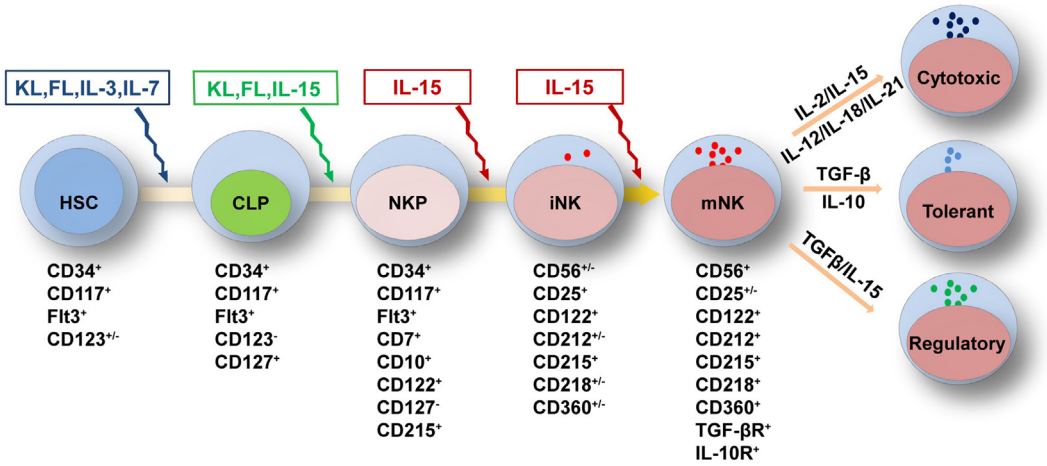

FIGURE 1 | Cytokine requirements for natural killer (NK) cell development and function. NK cell development from HSCs is regulated by multiple cytokines in the fetal liver, bone marrow, and thymus. The sequential expression of receptors for different cytokines implies the functional maturation of NK cells. The proliferation and differentiation of HSCs requires FL, KL, IL-3, and IL-7, which interact with their respective receptors. The acquisition of CD122 expression is indicative of the commitment of NK cells. IL-15 is indispensable for NK cell differentiation from CLPs to mature NK cells. Mature NK cells are shaped by cytokine signals from the diverse tissue environments in which they reside. In the peripheral blood or spleen, the abundance of stimulatory cytokines, such as IL-2, IL-12, IL-15, IL-18, and IL-21, may maintain NK cells in a cytotoxic state to combat infections. Tolerant NK cells that reside in the liver, and regulatory NK cells that reside in the uterus, are primarily regulated by TGF-β and IL-10 or by TGF-β and IL-15, respectively. Abbreviations: FL, fms-like tyrosine kinase 3 ligand; KL, kit ligand; IL, interleukin; HSC, hematological stem cell; CLP, common lymphoid progenitor; TGF-β, transforming growth factor-β.

REGULATION OF NK CELL DEVELOPMENT BY CYTOKINES

Natural killer cells can develop in many sites, including the fetal liver, bone marrow (BM), and thymus (24–27). The developmental hierarchy of NK cells has been depicted as a linear process that involves multiple stages, developing from hematopoietic stem cells (HSCs) through common lymphoid progenitors (CLPs) and NK lineage-restricted progenitors (NKPs) to mature NK cells (28). In contrast to the mouse, the human NK cell development hierarchy is less well characterized (29). Recently, human NKPs, which can exclusively differentiate into NK cells, have been clearly defined as $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ in umbilical cord blood (UCB), BM, and tonsils (30). The development of NK cells is regulated by cell-intrinsic signals through an array of transcription factors (TFs) and extrinsic signals from multiple cytokines (31–35). The TFs, including E4BP4, T-bet, Eomes, and GATA3 can regulate NK cell differentiation and maturation (26, 36–39). However, the requirement for TFs in murine NK cells may differ in different tissues. For instance, tissue-resident liver NK cells critically require the regulation of T-bet, whereas circulating NK cells are less affected by its depletion (12, 39, 40). Thymic NK cells depend on the regulation of GATA3, whereas circulating NK cells and tissue-resident liver NK cells do not (26). Nevertheless, further study of transcriptional regulation in the development of human NK cells is still required for a greater understanding of these processes. The extrinsic cytokine signals that are crucial for regulating NK cell development have been well characterized. Cytokines, such as fms-like tyrosine kinase 3 ligand (FL), kit ligand (KL), and IL-3, influence the survival, and proliferation of HSC, and are important for normal NK cell development (41, 42). In addition, NK cells are nearly absent in IL-$15^{-/-}$ or IL-$15R\alpha^{-/-}$ mice, which implies an indispensable role for IL-15 during NK cell differentiation (43, 44). Furthermore, previous reports have shown that IL-$2^{-/-}$, IL-$2R\alpha^{-/-}$, IL-$7^{-/-}$, IL-$7R\alpha^{-/-}$, and IL-$21R^{-/-}$ mice have normal numbers of mature NK cells in PB, suggesting that IL-2, IL-7, and IL-21 are redundant for peripheral NK cell development (45–48). However, IL-2 and IL-21 participate in promoting NK cell activation with enhanced cytotoxicity (49–51). Additionally, IL-12, IL-18, IL-10, and TGF-β also have roles in NK cell development or function (33). In this review, we illustrate when and how each cytokine regulates NK cell development and function (**Table 1**). Based on a thorough understanding of relative cytokines, NK cells can be further generated in large quantities using diverse cytokine cocktails for *in vitro* expansion and induction in culture systems for adoptive transfer therapy.

Natural killer cells belong to the innate immune system due to their roles in directly combating hematopoietic and non-hematopoietic cells to maintain homeostasis throughout the body (91). Previous reports have divided the developmental pattern of NK cells into four stages based on expression of the cell surface markers CD34, CD117 and CD94:$CD34^{+}CD117^{-}CD94^{-}$, $CD34^{+}CD117^{+}CD94^{-}$, $CD34^{-}CD117^{+}CD94^{-}$, and $CD34^{-}CD117^{+/-}CD94^{+}$ (92). NK cell development and function are characterized by the gradual acquisition of specific receptors. As shown above, the extrinsic signals from cytokines are vital important to regulate NK cell development and function (31–33). According to the expression pattern of cytokine receptors, we have divided NK cell development into five grading stages, including HSCs: $CD34^{+}CD117^{+}FLT3^{+}CD123^{+/-}$, CLPs:

TABLE 1 | The receptors, signaling pathways, and knockout phenotypes of cytokines.

Cytokine	Receptors	Signaling pathways	Knockout phenotypes	Reference
SCF (KL)	KIT	PI3K-AKT JAK-STAT1/3/5 Ras-MEK-ERK	Deficiency of HSCs, mast cells, and NKPs	(52–56)
FLT3L (FL)	FLT3	PI3K-AKT JAK-STAT5 Ras-MEK-ERK	Deficiency of CLPs, DCs, and NK cells	(57–60)
IL-3	IL-3Rα/βc	PI3K-AKT JAK2-STAT1/3/5/6 Ras-MEK-ERK	Deficiency of mast cells, basophil cells, and embryonic HSCs	(61–63)
IL-7	IL-7Rα/γc	JAK1/3-STAT5 PI3K-AKT	Normal NK cell number Deficiency of B cells and T cells	(46, 64, 65)
IL-15	IL-15Rα/β/γc	JAK1/3-STAT5 PI3K-AKT-mTOR Ras-MEK-MAPK	Deficiency of NK, NKT, IEL, and memory $CD8^+$ T cells	(43, 44, 66)
IL-2	IL-2Rα/β/γc	JAK1/3-STAT5 PI3K-AKT Ras-MEK-MAPK	Normal NK cell number Deficiency of Treg cells Accumulation of activated T/B cells	(45, 67–70)
IL-12	IL-12Rβ1/2	TYK/JAK2-STAT4	Failure of Th1 cell polarization Reduced autoimmune diseases	(71–74)
IL-18	IL-18R1/Rap	MyD88-IRAK4-NF-κb	Impairment of Th1 cell polarization and NK cell cytotoxicity	(75–78)
IL-21	IL-21R/γc	JAK1/3-STAT3 PI3K-AKT Ras-MEK-MAPK	Normal NK cell number Decreased Th17 cells with reduced progression of EAE Impaired secretion of IgG	(48, 79–82)
IL-10	IL-10R1/2	TYK2/JAK1-STAT3/1/5	Activated CD4 T cells accumulation Dysfunction of Treg cells	(83–86)
TGF-β	TGF-βRI/II	Smad	Auto-reactivity of immune cells Die at early age	(87–90)

SCF, stem cell factor; KL, kit ligand; FLT3L (FL), fms-like tyrosine kinase 3 ligand; IL, interleukin; TGF-β, transforming growth factor β; βc, common β chain; γc, common γ chain; Rap, receptor accessory protein; PI3K, phosphoinositide 3 kinase; AKT, protein kinase B; JAK, janus kinases; STAT, signal transduction and activation of transcription; ERK, extracellular regulated protein kinases; MAPK, mitogen-activated protein kinase; TYK, tyrosine kinase; MyD88, myeloid differentiation primary response protein 88; IRAK4, IL-1R-associated kinase 4; NF-κb, nuclear factor kappa-light-chain-enhancer of activated B cells; HSC, hematopoietic stem cell; DC, dendritic cell; NK, natural killer; NKT, natural killer T; IEL, intraepithelial lymphocytes; Treg, regulatory T cell; Th, T helper; EAE, experimental autoimmune encephalomyelitis.

$CD34^+CD117^+FLT3^+CD123^-CD127^+$, NKPs: $CD34^+CD117^+FLT3^+CD7^+CD10^+CD122^+CD127^-CD215^+$, immature NK cells (iNK cells): $CD56^{+/-}CD25^+CD122^+CD212^{+/-}CD215^+CD218^{+/-}CD360^{+/-}$, and mature NK cells (mNK cells): $CD56^+CD25^{+/-}CD122^+CD212^+CD215^+CD218^+CD360^+TGF\text{-}\beta R^+IL\text{-}10R^+$ (**Figure 1**). The differential expression of cytokine receptors implies that there are various demands for the relevant cytokines during NK cell development.

FL, KL, IL-3, and IL-7 Promote the Transition of HSCs to CLPs

Hematological stem cells are the major source of multilineage myeloid cells and lymphocytes which are vital for maintaining normal numbers and functions of immune cells (93). It is well established that the differentiation of NK cells is a step-wise process that is driven by the regulation of TFs and coordinated cytokine signals from HSCs (31–35). FL and KL, discovered in the early 1990s, have overlapping yet distinct effects to promote HSC survival and proliferation (41). Their receptors, flt3 and c-kit, belong to the family of tyrosine kinase receptors expressed primarily on cells in the very early stages of hematopoiesis (54, 57, 94). KL, also known as stem cell factor (SCF), is produced in two forms: membrane-bound and soluble, through differential splicing and proteolytic cleavage (53, 95). *Sl*/*Sl*^{d} mutant mice expressing only soluble SCF are deficient in HSCs, indicating that membrane-bound SCF is critical for HSC maintenance (52, 55, 96, 97).

Previous reports have shown that flt3 or c-kit deficiency in mice induces a reduction in the number of CLPs (58, 98). In addition, the cytokine FL deficiency in mice can also induce a sharp reduction of CLPs but has no effect on the HSC pool or on common myeloid progenitors (59, 60). Recently, it is revealed that FL can act synergistically with Hoxa9 signaling to regulate an early checkpoint of lymphopoiesis by affecting CLPs, LMPPs, and $flt3^+$ multipotent hematopoietic progenitors in the BM (99). Furthermore, FL and SCF can act synergistically to promote $CD34^+$ cell proliferation and are important for NK cell differentiation by inducing the expression of CD122 and increasing *IL15R*α expression to increase their sensitivity to IL-15 (100). The number of NKPs is reduced in mice that are deficient in either flt3 or c-kit, implying that either of these cytokines is essential for NK-cell differentiation (56). These findings imply that FL and SCF have important roles in the development of lymphoid

progenitor cells and are critical for the commitment of NK-cell lineage.

Interleukin-3 is a member of the beta common (βc) family of cytokines, which also includes granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 (101). IL-3 is a well-known hematologic growth factor that can promote HSC survival, proliferation and differentiation (62). Previous research has demonstrated that IL-3 alone can rescue low HSC activity in *Runx1*$^{+/-}$ AGMs (Aorta-Gonad-Mesonephros) that have a reduced HSC population (63, 102). Receptors for IL-3, members of the gp140 family, are composed of an IL-3 receptor-specific α subunit (IL-3Rα or CD123) and a homo-dimeric βc subunit (61, 103). Both CD123 and βc subunits are detected on the surface of hematopoietic tissues and HSCs (42). After binding with the receptors, it can activate janus kinases (JAK) 2-signal transduction and activation of transcription (STAT) 5/1/3/6, phosphoinositide 3 kinase (PI3K)-protein kinase B (AKT), and Ras-extracellular regulated protein kinases (ERK) pathways (62, 104). In the *in vitro* differentiation system of human primitive progenitors, IL-3 has been reported to maintain lymphoid progenitor development and promote NK cell or B cell differentiation (105–107). Moreover, IL-3 can also preserve the engraftment and lymphoid reconstitution capacity *in vivo* of the transduced CD34^{+} cells in severe combined immunodeficiency (SCID)-hu mice (108). Therefore, IL-3 may primarily facilitate the survival and proliferation of HSCs and the differentiation of CLPs, and further promote NK cell development.

CXCR4 signaling has been shown to regulate quiescence and long-term maintenance of HSCs upon interaction with the chemokine CXCL12 (109, 110). Recently, a group of researchers found that CXCR4 can provide lineage-instructive signals to control progenitor cell differentiation (111). They showed that signals from CXCR4-CXCL12 interactions regulate multipotent progenitor (MPP) differentiation into CLP subsets in the BM and further affect lymphoid lineage production. Moreover, a deficiency of CXCR4 signaling resulted in a profound reduction in the number of T, B, and NK cells which suggests that the addition of CXCL12 may be helpful to promote *in vitro* NK cell differentiation from HSCs.

Interleukin-7 is another important cytokine for the differentiation of lymphoid lineages, mainly for the differentiation of T and B cells (46, 64). It induces the differentiation of HSCs into lymphoid progenitor cells and facilitates their expansion and survival. The IL-7 receptor is a heterodimeric complex composed of IL-7Rα (CD127) and the common γ chain subunit (CD132) (112). The IL-7-IL-7R interaction primarily activates JAK1/3-STAT5 and PI3K-AKT pathways to induce prosurvival, cell cycle, and metabolism regulation signals (65, 113). Previous reports have shown that knockouts of IL-7 and IL-7Rα do not induce significant defects in mouse NK cells from the PB or spleen (46, 47). Thus, IL-7 may contribute in a redundant way and may not be essential for circulatory NK cell development. However, NK cells in the thymus, characterized by IL-7Rα^{+}, require IL-7 for their homeostasis (26). Whether other NK cell subsets in different tissues require IL-7 for their effector functions or homeostasis is unknown.

IL-15 Directs CLPs toward Mature NK Cells

Important cytokines for the development and function of immune cells are highlighted in X-SCID, characterized by mutations of *IL-2RG,* which encodes the common γ chain (γc), a common receptor for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (114, 115). X-SCID is characterized by extreme vulnerability to viruses and pathogens due to the developmental and functional deficiency of T, B, and NK cells, which indicates that γc family cytokines play vital roles in normal immune responses.

IL-15$^{-/-}$ and IL-15R$^{-/-}$ mice have dramatically reduced populations of NK cells (43, 44); however, IL-2$^{-/-}$Rag1$^{-/-}$, IL-7$^{-/-}$, and IL-21R$^{-/-}$ mice have normal NK cell numbers (45–48), suggesting that IL-15 is essential for NK cell commitment and maturation. Furthermore, mature NK cells fail to be maintained when transferred to IL-15$^{-/-}$ mice (116, 117). The prosurvival ability of IL-15 is potentially mediated by upregulated expression of antiapoptotic B cell lymphoma 2 (BCL-2) family members and downregulated expression of proapoptotic proteins (116). IL-15 has extensive roles in immune cells. For example, it can regulate NKT cell development and maintain normal memory phenotypes of CD8^{+} cells (43, 44, 117–120).

Interleukin-15 can be produced by hematopoietic and non-hematopoietic cells, such as activated DCs, macrophages, monocytes, and stromal cells (121). IL-15R is a heterotrimeric receptor including a unique IL-15Rα (CD215) subunit, a shared β chain with IL-2 (CD122), and CD132 (122, 123). The expression of CD122 is a major phenotypic marker of NKPs, which allows the cells to respond to IL-15 (124, 125). IL-15 has a special manner of transducing its signals through trans-presentation, whereby the IL-15-producing cells support IL-15 by binding IL-15Rα, and then present it to activate neighboring cells expressing CD122/CD132 (126–130). Enlightened by these findings, researchers have designed NK cells expressing membrane-bound IL-15 that have autonomous growth and increased cytotoxicity toward tumor cells, which can help to enhance the antitumor effects of NK cells and avoid the side effects of cytokine administration (131). The activation of IL-15R induces the autophosphorylation and activation of JAK1/3 and downstream cascades, including the JAK-STAT, the mitogen-activated protein kinase (MAPK), and PI3K/AKT-mTOR signaling pathways, in order to fulfill its different functions (66, 132). The IL-15-STAT5 signaling pathway is indispensable for NK cell development and homeostasis, as mice that are deficient in this pathway have dramatically reduced mature NK cell numbers. In addition, both *Stat5*-deficient and NK cell-specific *Stat5*-deficient mice have an absence of NK cells in peripheral blood and tissues (133–135). A patient with a *STAT5b* mutation also showed a severe reduction in NK cell numbers (136). The PI3K/AKT-mTOR pathway also plays a role in NK cell development. A recently published paper has shown that PDK1, a kinase upstream of mTOR, is a critical component that connects IL-15 signaling to E4BP4, an indispensable TF for NK cell development (137). The early depletion of PDK1 induces a severe loss of NK cells with much weaker mTOR activation, E4BP4 induction after IL-15 stimulation and the reduced expression of CD122 (137). These findings underscore the importance of the IL-15-PI3K-PDK1-mTOR-E4BP4-CD122 positive feedback loop in the development of NK cells. Other factors can also

affect NK cell development by influencing their responsiveness to IL-15. The TF ID2 can affect NK cell development by antagonizing E-protein function and altering lineage fate (138, 139). Recently, researchers have found that ID2 can suppress E-protein target gene SOCS3 expression to maintain IL-15 receptor signaling for normal NK cells development, and strong IL-15 receptor stimulation can overcome this requirement for ID2 (140). The abovementioned findings strengthen the roles of IL-15 in NK cell development and explain how IL-15 induces its effects.

POLARIZATION OF NK CELL FUNCTION BY CYTOKINES

Natural killer cells have diverse functions in different tissues, which can be divided into three subsets: cytotoxic, regulatory, and tolerant NK cells (141). Different NK cell subsets are primarily affected by signals from diverse cellular microenvironments and signals from cytokines are important in shaping their unique functions. In an inflammatory or virus-infected situation, activated T cells and dendritic cells (DCs) secrete abundant IL-2, IL-12, IL-18, and IL-21, which drive NK cell activation with enhanced secretion of cytotoxic cytokines and the ability to directly kill transformed targets (142–144). As mentioned above, NK cells in their physical environment play leading roles in maintaining normal pregnancy in the uterus and tolerating a significant amount of food-derived or gut-derived antigens in the liver, which are mainly determined by the regulation of different cytokine cocktails.

IL-2 and IL-15 Induce NK Cell "Priming"

Natural killer cells serve as the first line of defense during viral infection or the elimination of transformed cells, which is characterized by the production of cytokines, such as IFN-γ and TNF-α, and granzyme-mediated cytotoxicity (3). Comprehensive activation is necessary for NK cells to fulfill their roles as sentinels (145). Previous reports have demonstrated that IL-2, IL-15, IL-12, IL-18, and IL-21 all play roles in activating NK cells. Therefore, we discuss how these cytokines function and how to achieve better efficacy in adoptive NK cell therapy.

Interleukin-2 is an immune-stimulatory cytokine that was first identified as a "T cell growth factor" (67–69, 146). Subsequently, it has been shown that activated T cell-derived IL-2 can enhance NK cell responses toward infection *in vivo* and can activate NK cells *in vitro* (142, 147). Furthermore, researchers found that NK cell responses are impaired in IL-2β$^{-/-}$ mice suggesting that IL-2 may have an important role in maintaining NK cell activity (148). However, IL-2-deficient mice have normal NK cell development and numbers, which implies that it is needed for NK cell effector functions but is not indispensable for their development (45). IL-2 achieves its functions predominantly through the JAK1/3-STAT5 signaling pathway by binding with the heterotrimeric receptor, which is composed of IL-2Rα (CD25), CD122, and CD132 (70). Interestingly, IL-2 has different affinities for the different receptors. IL-2 binding to a single CD25 (Kd = 10^{-8} M) or CD122 (Kd = 10^{-6} M) has a low affinity. There is an intermediate binding affinity with the CD25/CD122 heterodimer (Kd = 10^{-9} M), and the greatest binding affinity is seen with the IL-2Rαβγ trimeric complex (Kd = 10^{-11} M) (149–151). NK cells primarily express CD122 and CD132 receptor components that can respond to high concentrations of IL-2. To achieve better IL-2 application efficacy, one group of researchers developed an IL-2 "superkine," also called super-2, with increased affinity for CD122 (152). Super-2 has a vigorous role in promoting activation and proliferation irrespective of CD25 expression. In addition, previous reports have shown that preactivation with IL-12, IL-15, and IL-18 induces CD25 expression on NK cells, which suggests that pretreatment of NK cells with the above cytokines can enhance NK cell responsiveness to IL-2 and may lead to better IL-2 treatment efficacy (153, 154). However, high-dose IL-2 induces a selective expansion of regulatory T (Treg) cells, which limits the activity of NK cells, resulting in poor clinical responses to IL-2 therapy (155). The depletion of Treg cells leads to increased IL-2 availability for NK cells to increase IFN-γ production and cytotoxicity (155, 156). To overcome Treg cell inhibition, researchers have produced a mutant form of IL-2 that preferentially binds to CD122/CD132 and that has reduced binding to CD25 (157). In contrast to wild-type IL-2, the mutant form efficiently induces NK cell proliferation and activation with a dramatic reduction in Treg cells, and achieves better responses to tumors.

Interleukin-15 is discovered by its "IL-2-like" stimulatory role and is important for NK cell development and function (158–160). IL-15 and IL-2 share the common β and γ chain receptor subunits and only differ due to an α chain, as IL-2Rα (CD25) binds to IL-2 and IL-15Rα binds to IL-15 (122, 123, 161, 162). However, IL-15Rα alone binds to IL-15 with a high affinity (Kd = 10^{-11} M), which is comparable to that of the binding of IL-2Rαβγ to IL-2 (162). The high affinity between IL-15 and IL-15Rα makes it possible, when compared with IL-2, to activate NK cells with relatively lower concentrations.

Prior exposure to IL-15 sensitizes NK cells to secondary stimuli, referred to as "priming," thereby resulting in exaggerated responses (163, 164). Previous studies have shown that IL-12 can induce elevated IFN-γ production in IL-15-primed NK cells. Other cytokines, such as IL-2, IL-4, IL-21, and type I IFN, can also induce heightened functions (165). As mentioned above, the JAK-STAT5 signaling pathway is important for IL-15-mediated NK cell development and viability (133–136). However, the PI3K-AKT-mTOR signaling pathway is critical for the IL-15-induced priming effect (132, 165). Inhibition of the PI3K-mTOR signal would abrogate enhanced IL-12- and IL-21-induced phosphorylation of STATs and stronger responses in IL-15-primed NK cells (165). Nevertheless, the inhibited mTOR pathway does not affect the phosphorylation of STATs in naïve NK cells, suggesting that this pathway specifically functions in IL-15-primed NK cells (165). The crosstalk between the PI3K-mTOR signaling pathway and the STAT pathway is critical for efficient NK cell priming; however, the mechanism by which this occurs remains unclear.

A previous report has shown that IL-15 promotes NK cells to be fully equipped to respond to infections through the rapid induction of granzymes and perforin (166). However, the induced production of granzymes/perforin is reduced in NK-mTOR$^{-/-}$ cells, suggesting that mTOR is significant for mediating NK cell cytotoxicity (167). Researchers have also shown that IL-15, but

not IL-2, can maintain NK cell cytolytic functions after cytokine withdrawal to simulate postinfusion performance (168). The IL-15-induced functional advantages are dependent on activated mTOR-regulated signaling (168). The mTOR-regulated IL-15-induced maintenance of NK cell functions suggests a beneficial implementation of IL-15 in adoptive NK-cell clinical therapy.

IL-12, IL-18, and IL-21 Strengthen NK Cell Cytotoxicity

Interleukin-12 was first named NK cell stimulating factor based on its ability to induce NK cells to secrete high levels of IFN-γ (169). It is composed of two subunits, IL-12p35 and IL-12p40, and these two subunits must be coexpressed in the same cell to form the disulfide-linked bioactive IL-12p70 (170). The cytokine is mainly produced by antigen-presenting cells, such as DCs, monocytes and macrophages (171–173). NK cells and activated T cells express the high-affinity heterodimeric receptor (IL-12Rβ1/β2) for interaction with IL-12, which can activate tyrosine kinase 2 (TYK2) and JAK2, leading to phosphorylation of STATs (mainly STAT4) and the eventual promotion of IFN-γ production and other biological reactions (71–74, 174).

One previous report suggested that NK cells can mediate a long-lived contact hypersensitivity response to haptens in mice devoid of T and B cells; thus, the concept of "memory NK cells" was established (175). However, the mechanism by which memory NK cells develop is unknown. The group of Lewis L. Lanier observed that IL-12-STAT4-dependent-IFN-γ-independent signaling is indispensable for the generation of mouse cytomegalovirus-specific memory NK cells (176). As memory NK cells are long-lived and have relatively higher responses compared with naïve NK cells (175, 177–179), developing memory NK cells for clinical therapy is an attractive approach. Wayne M. Yokoyama's group first showed that preactivation of mice NK cells with IL-12 and IL-18, along with low-dose IL-15 to maintain survival, could produce cytokine-induced memory-like NK cells (180). The memory-like cells could respond more robustly to reactivation without inducing enhanced cytotoxicity toward tumor cells (180). However, Adelheid Cerwenka laboratory observed that IL-12/15/18 preactivated NK cells, when combined with irradiation, could achieve greater efficacy, as determined by reduced growth of established mouse tumors (153). They also showed that the antitumor effect is mediated by IL-2 produced by $CD4^+$ T cells. As the abovementioned memory-like NK cells were all established in mice, Romee et al. showed that human NK cells can also display memory characteristics after short-term preactivation with IL-12/15/18 (181). Subsequently, they also found that the memory-like NK cells induce prolonged expression of CD25, forming a high affinity IL-2R complex to respond to IL-2 at picomolar concentrations (154). However, single cytokine, like IL-12, IL-15, or IL-18 preactivation cannot induce a higher expression of CD25. Moreover, prior treatment with low-dose IL-2 before adoptive transfer can enhance proliferation and effector function of memory-like NK cells, which supports an additional immunotherapy strategy. Recently, this group performed a phase 1 study to treat active rel/ref AML patients with memory-like NK cells (182). They found that donor memory-like NK cells exhibited enhanced IFN-γ production and yielded an overall response rate of 55%. This result reminds us that preactivation with cytokines can indeed strengthen NK cell antitumor functionality and can be used as a therapeutic method to treat tumors. Nevertheless, understanding the appropriate cell doses still requires further study.

The NK cells without expressing any inhibitory MHC-I-specific receptors, such as killer cell immunoglobulin-like receptors (KIRs) in humans and Ly49 receptors in mice, are hyporesponsive. The signals from KIRs and Ly49 receptors are critical for NK cells to be functionally competent (183, 184). This process is also termed "NK cell licensing." A recent report has shown that the unlicensed NK cells display enhanced functionality after preactivation with IL-12, IL-15, and IL-18 (185). It has also been reported that human $CD56^{bright}KIR^-$ and $CD56^{dim}KIR^-$ NK cells can acquire KIR expression upon stimulation with IL-15 in the presence of stromal cells (186). Furthermore, the developed KIR^+ NK cells display enhanced cytotoxicity and cytokine-producing potential compared to the KIR^- NK cells. Later, another group has identified that activation with cytokines such as IL-2, IL-15, or IL-12 can induce the *de novo* expression of KIR and/or NKG2A on KIR^-NKG2A^- NK cells without feeder cells (187, 188). Similar to human NK cells, the responsive capacity of unlicensed murine NK cells can be restored as licensed cells when stimulated *in vitro* with high doses of IL-12 and IL-18 or IL-2 (183). These findings suggest that cytokine stimulation can induce the hypo-responsive unlicensed NK cells to be re-educated and acquire stronger responses toward target cells.

Interleukin-12 has also been shown to promote further maturation of *in vitro*-differentiated NK cells with enhanced cytotoxicity. One group has shown that low-dose IL-12 can decrease the fluorescence intensity of CD56 and can induce the expansion of more mature $CD56^{dim}CD16^+$ and $CD56^{dim}KIR^+$ NK cells (189). Subsequently, another group identified that *in vitro*-derived NK cells display enhanced cytotoxicity toward primary AML cells and have improved antileukemic responses in MHC class I-positive AML mice after IL-12 culturing (190). These findings suggest that IL-12 can not only regulate the functions of mature NK cells but also promote the differentiation level and function of developing NK cells.

Interleukin-18 is a proinflammatory cytokine belonging to the IL-1 cytokine family and was originally defined as an IFN-γ-inducing-factor (191, 192). It is constitutively produced by hematopoietic cells, such as DCs, macrophages and neutrophils (191, 193–195), and non-hematopoietic cells, such as microglial cells and epithelial cells (196). IL-18 is initially produced as an inactive precursor, pro-IL-18, which requires cleavage by caspase-1 of the N-terminal fragment to become the mature, biologically active form (197, 198). Mature IL-18 binds to its receptor, composed of IL-18R1 and IL-18R accessory protein in a heterodimeric receptor complex, to initiate signal transduction by myeloid differentiation primary response protein 88 (MyD88). Then, IL-1R-associated kinase 4 (IRAK4) and TNFR-associated factor 6 (TRAF6) are recruited, leading to the activation of the nuclear factor (NF) kappa-light-chain-enhancer of activated B cells (κB) and MAPK pathways to promote IFN-γ transcription and stabilization of *IFNG* mRNA (78, 199).

Interleukin-18 is important for promoting the production of IFN-γ by NK cells against viral, fungal, bacterial, and parasitic infections (200–203). NK cells display reduced secretion of IFN-γ and compromised cytotoxicity in IL-18-deficient, IL-18R1-deficient, or IRAK4-deficient mice (75–77). Furthermore, IL-18 is essential for upregulating CD25 expression in NK cells, and the increased production of IFN-γ is enhanced by IL-2 during *Plasmodium yoelii* infection in mice (204). Similarly, low-dose IL-18, through synergistic interactions with IL-2, IL-12, IL-15, or IL-21, can induce potent CD25 and IFN-γ upregulation to strengthen human NK cell effector function (205). A previous report has shown that T and B cells can upregulate IL-18R expression upon IL-12 treatment, and the combination of IL-18 and IL-12 synergistically induces their IFN-γ production (206). Recently, researchers also found that the combination of these two cytokines can reverse NK cell anergy and increase the survival rate of mice bearing MHC-deficient tumors (207). These findings imply that IL-18 can play stronger roles when combined with other stimulatory cytokines; therefore, combining cytokines together is better for achieving enhanced efficacy.

Interleukin-21 is a pleiotropic cytokine mainly produced by T follicular helper cells, Th17 cells, and NKT cells (208). It acts through a receptor complex, including IL-21R and γc (209, 210), to activate JAK1 and JAK3, which leads to recruitment and phosphorylation of STAT (predominantly STAT3 but also STAT1 and STAT5) to promote the expression of IFN-γ and other factors (81). The IL-21 signal can also be transduced by the MAPK and PI3K/AKT pathways (81).

Interleukin-21 has diverse effects in immune cells, which can affect the differentiation of inflammatory T cells, immunoglobulin production of B cells, and development and functions of NK cells (48, 79, 80, 82, 211). IL-21, combined with FL and IL-15, can specifically promote the differentiation and expansion of $CD16^+CD56^+$ cytotoxic NK cells from BM progenitors *in vitro* (210). It also promotes rapid differentiation and acquisition of killer Ig-like receptors of NK cells from cord blood CD34-positive cells (212). Additionally, it induces mature mouse NK cells to develop a large granular lymphocyte phenotype with increased production of cytokines, such as IFN-γ, and perforin through coactivation with IL-2 or IL-15, resulting in enhanced cytotoxicity (213). Similarly, human NK cells cocultured with IL-21 and therapeutic antibody-coated breast cancer cells secrete higher levels of IFN-γ, TNF-α, IL-8, CCL3, and CCL5, and the supernatants are able to drive the migration of naïve and activated T cells *in vitro* (214). The administration of IL-21 and antibody-coated tumor cells leads to synergistic cytotoxic effects of NK cells toward tumor cells, which suggests that IL-21 may be an effective adjuvant for antibody treatment (214). Interestingly, IL-21 limits IL-15-mediated NK cell expansion and viability; nevertheless, it can stimulate IFN-γ production and cytotoxicity in NK cells previously activated with poly I:C or IL-15 (48). This finding reminds us that it may be important to apply cytokine cocktails sequentially.

Insulin-like growth factor 1 (IGF-1) is an important growth factor to regulate longevity and immunity (215). And it was also shown to promote NK cell development from $CD34^+$ cells and increase NK cell cytotoxicity by promoting the production of perforin by STAT3 activation (216). Furthermore, foxO1, which negatively regulates NK cell maturation and function, is a key molecule of IGF-1 signaling pathway (217, 218). Therefore, IGF-1 may also strengthen NK cell function through IGF-1-induced foxO1 inactivation, which is mediated by increased phosphorylation of foxO1 at Ser256 and Thr24 (219). Such information about IGF-1 may present new opportunities to boost NK cell cytotoxicity therapeutically.

TGF-β and IL-10 Shape Tolerant NK Cells

Natural killer cells are not homogenous, and cells with low cytotoxicity, residing in the liver or a chronic pathogenic microenvironment, are termed tolerant NK cells (141). The liver is an important immune-tolerant organ, as no severe inflammation occurs despite constant stimulation by bacterial products and antigens from the gut (20, 220, 221). NK cells, composed of $CD56^{dim}$ and $CD56^{bright}$ subtypes, occupy up to 30–40% of all hepatic lymphocytes located in human liver sinusoids (222, 223). $CD56^{dim}$ hepatic NK cells share many similarities with PB $CD56^{dim}$ NK cells that may frequently circulate throughout the body. By contrast, $CD56^{bright}$ cells with CD69 and CD49a expression are more specifically retained in the liver (13, 224). Similarly, one subset of $DX5^-CD49a^+$ mouse NK cells specifically resides in the liver with adaptive-like properties (10). However, the specific roles of $CD56^{bright}$ and $CD56^{dim}$ human intrahepatic NK cells still require further study. One important function of intrahepatic NK cells is to tolerate harmless antigens that can help maintain liver homeostasis (225). IL-10 and TGF-β, which are produced by DCs, Kupffer cells, hepatic sinusoidal endothelial cells, and stellate cells, are important for shaping tolerant NK cells.

Interleukin-10, first recognized for its ability to inhibit the activation and cytokine secretion of Th1 cells, is described as a cytokine synthesis inhibitory factor (83–85, 226). It is an important immune-regulatory cytokine that is produced by T cells, B cells, NK cells, DCs and macrophages (227, 228). The receptors for IL-10 mainly contain two subunits, IL-10R1 and IL-10R2, and are expressed on many hematopoietic and non-hematopoietic cells (229). Once IL-10 and IL-10R bind together, JAK1 and TYK2 are activated, which leads to the phosphorylation and activation of STAT3, STAT1, and STAT5 (86). As mentioned above, IL-10 is important for allowing liver NK cells to maintain immune-tolerant states. Indeed, one study demonstrated that intrahepatic IL-10 can maintain NK cells in a functionally hypo-responsive state with a phenotype of $NKG2A^+Ly49^-$ cells (22). NKG2A is critical for NK cells, when cocultured with non-transformed hepatocytes, to prime DC cells. Furthermore, the primed-DCs can result in the induction of regulatory $CD4^+CD25^+$ T cells, a subset responsible for inhibiting excessive immune activation (230, 231). Even transferred splenic NK cells migrating into the liver show changes in phenotype and function, suggesting that the liver environment reshapes NK cells to maintain a steady state (22).

Transforming growth factor-β includes three isoforms, TGF-β1, 2, and 3, which are highly homologous (232). TGF-β1 is the primary isoform expressed in the immune system (233). If deficient, mice die of systemic inflammation by 3–4 weeks of age, suggesting that TGF-β plays important roles in maintaining

normal immune responses (87–89). TGF-β binds to its receptors, primarily TGF-βRI and TGF-βRII, to activate a downstream cascade (234). First, the intracellular receptor Smad (R-Smad) proteins Smad2/3 are recruited and phosphorylated, and the phosphorylated Smad2/3 then combine with Smad4 or TIF1γ to form a trimeric complex. The trimeric complex translocates to the nucleus to regulate relative gene expression by binding to the responsive regulatory regions (90). TGF-β can also activate Smad-independent pathways, such as small GTPases, MAPK, and PI3K pathways (235).

Tolerant NK cells can mediate benign effects to maintain physiological homeostasis. However, cells induced in the context of chronic infection or the cancer microenvironment are harmful for the treatment of related diseases (236, 237). For example, TGF-β upregulation induces reduced secretion of IFN-γ by repressing the normal expression of T-bet in the pathologic niche (238). High levels of TGF-β induce a weak NKG2D/DAP10 and CD244/SAP signal, leading to NK cells that are unable to eliminate hepatitis B virus (HBV) infection in chronic hepatitis B (CHB) (239, 240). Later, another report showed that TGF-β and IL-10 in CHB-infected and hepatic carcinoma patients induce the expression of microRNA (miR)-146a, which causes reduced IFN-γ production and cytotoxicity, resulting in a poorer prognosis (241). Furthermore, TGF-β-induced miR-183 represses DAP12 transcription and translation (242), or reduces the expression of NKp30 and NKG2D (243), to further weaken NK cell cytotoxic functions in the tumor microenvironment. The abnormal tolerant NK cells cannot eliminate infected or transformed cells, which leads to immune evasion by these cells.

TGF-β and IL-15 Develop Regulatory NK Cells

Decidual NK cells (dNK) are distinguishable from PB NK cells because more than 90% of these cells are characterized by a $CD56^{bright}CD16^{-}CD49a^{+}CD9^{+}$ phenotype with a low cytotoxic effect and high cytokine secretion ability (15, 244, 245). Previous reports have shown that they constitute 50–90% of decidual lymphocytes during the first trimester of pregnancy (244, 246, 247). The accumulated NK cells can control extravillous trophoblast invasion and vascular remodeling by secreting different amounts of molecules, such as GM-CSF, colony-stimulating factor 1, angiopoietin-2, vascular endothelial cell growth factor, and placental growth factor (248, 249). Furthermore, dNK cell-derived IFN-γ has been shown to be critical for vessel modification and decidual cellularity in mice (250). Inflammatory responses in decidual, caused by allogenic fetal cell invasion and other factors, can result in abortion (246, 251). To ensure a normal pregnancy, researchers have determined that regulatory T cells can mediate an inhibitory response to the aggressive alloantigen (252), and CD86 blockage (253) and PD-1 (254) involvement can both play protective roles in pregnancy outcomes. As the dominant member of decidual lymphocytes, dNK cells can provide immune-regulatory mechanisms to maintain a regular uterine environment. Indeed, IFN-γ derived from $CD27^{+}CD56^{bright}$ dNK cells dampens Th17-induced inflammation to maintain a normal pregnancy (255). The abnormalities in the proportion and IFN-γ secretion of dNK cells in patients with recurrent spontaneous abortions result in long-term Th17-induced inflammation and eventual pregnancy failure (255). Another study subsequently showed that the crosstalk between dNK cells and $CD14^{+}$ myelomonocytic cells results in the generation of regulatory T cells for the inhibition of abnormal inflammation in the uterus (256).

The above findings demonstrate the important regulatory roles of dNK cells; however, whether dNK cells are derived from the differentiation of local NKPs or the migration of PB NK cells or both still needs to be studied. Nevertheless, under hypoxic conditions, endometrium-derived TGF-β and stromal cell-derived IL-15 may function to shape unique dNK cells (16, 19, 257). Indeed, TGF-β promotes the conversion of $CD16^{+}$ PB NK cells to $CD16^{-}$ cells and inhibits the expression of NKp30 to further inhibit cytotoxic functions, both of which lead to cells with similarities to dNK cells (17). Furthermore, TGF-β can induce the expression of CD103 and CD49a to increase the likelihood of the cells residing in the uterus (9, 258). Moreover, one study applied TGF-β1, IL-15 and a demethylating agent under hypoxic conditions to successfully transform PB NK cells into dNK-like cells (18). These findings imply that the specific hypoxic state of the uterus, combined with the help of TGF-β and IL-15, shapes particular regulatory NK cells.

CYTOKINE COCKTAILS PROMOTE *IN VITRO* EXPANSION OF NK CELLS

As mentioned above, multiple cytokines can regulate NK cell development, proliferation and activation. Previous studies have observed that NK cells can exert graft-versus-leukemia reactions without causing graft-versus-host disease (GVHD) in allogeneic hematopoietic transplantation (259). Furthermore, pioneering work by the Miller group showed that infusion of haplo-identical NK cell infusions activated by IL-2 can induce remission in AML patients (260). NK cells, therefore, are promising candidates for the treatment of hematological malignancies. However, the generation of sufficient NK cell quantities with robust effectiveness remains challenging. Therefore, strategies to expand or induce NK cells from primary NK cells or $CD34^{+}$ cells with high cytotoxicity using different combinations of cytokines are actively being developed (261, 262) (**Figure 2**).

Expansion of NK Cells from PB or UCB NK Cells

The most effective protocols to expand NK cells from primary NK cells depend on the presence of feeder cells, such as genetically modified K562 cells, Epstein-Barr virus-transformed lymphoblastoid B cell lines (EBV-BLCL) and irradiated autologous cells (263–265) (**Figure 2**). One group has utilized double-transduced K562 cells with membrane-bound IL-15 and costimulating ligand 4-1BBL (K562-mbIL15-41BBL) as feeder cells along with low concentrations of IL-2, which results in dramatic PB NK cell expansion with negligible T cell expansion (264, 266, 267). K562-mbIL15-41BBL cell-stimulated NK cells can continually

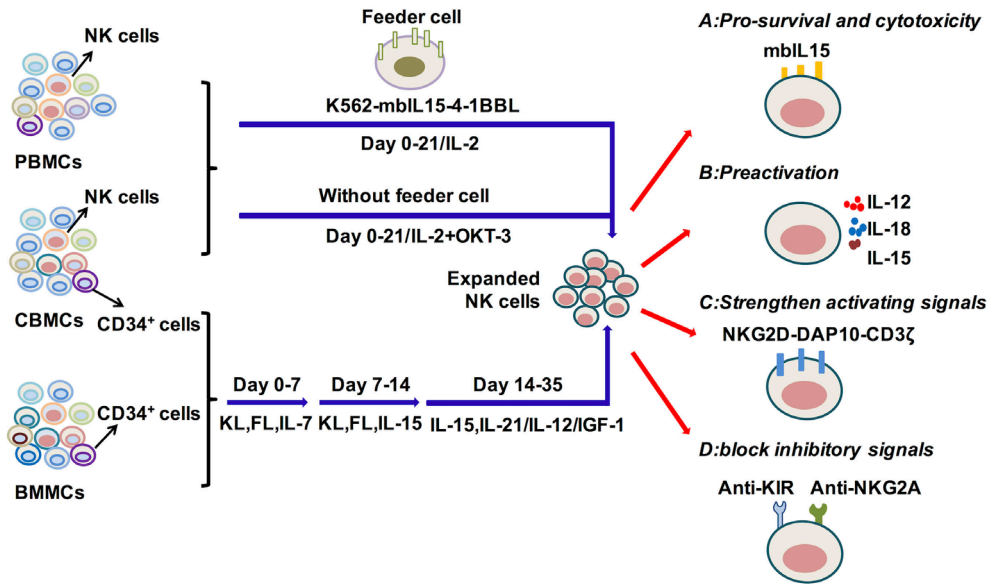

FIGURE 2 | Cytokine regulation of natural killer (NK) cell expansion and cytotoxicity. Genetically modified K562 cells and IL-2 or IL-2 and OKT-3 without feeder cells, applied for the expansion of primary NK cells, can generate significant amounts of functional NK cells. The differentiation and expansion of NK cells from $CD34^+$ HSCs are regulated by early activating cytokines, such as FL, KL, and IL-7, to promote HSC proliferation and differentiation, as well as by cytokines to activate NK cells, such as IL-15, IL-12, IL-21, and IGF-1. To improve NK cell survival or antitumor function, relative signals, such as the expression of mbIL-15 or preactivation with IL-12/15/18, strengthen activating or block inhibitory signals. These are vital for improving NK cell efficacy in adoptive cell therapy. Abbreviations: PBMC, peripheral blood mononuclear cell; CBMC, cord blood mononuclear cell; BMMC, bone marrow mononuclear cell; IL, interleukin; mbIL-15, membrane-bound IL-15; 4-1BBL, 4-1BB ligand; IGF-1, insulin-like growth factor 1; NKG2D, natural killer group 2D; DAP10, DNAX-activating protein 10; KIR, killer cell immunoglobulin-like receptors; NKG2A, natural killer group 2A.

proliferate for 8–12 weeks, acquire up to 10^8 percent of the number of originally seeded NK cells and maintain high cytotoxic capacity (268). The K562-mbIL15-4-1BBL-based expansion method has been adapted for use under Good Manufacturing Practice conditions that can be used in the clinic (264, 269). K562-mbIL-15-4-1BBL cells can also be used to expand UCB NK cells with enhanced proliferation and cytotoxicity (270). Other gene-modified K562 cells are also used in expansion systems. K562-IL-15Rα-4-1BBL feeder cells, in combination with soluble IL-15, stimulate NK cell expansion with increased expression of natural cytotoxicity receptors, correlating with enhanced cytolytic functions (271, 272). Furthermore, K562-mbIL-21 cells can be developed to act as feeders and are reported to promote more vigorous NK cell expansion compared with K562-mbIL-15 cells (273). EBV-BLCL and autologous cells are also used as feeder cells, combined with the addition of IL-2, IL-15 and OKT3 (anti-CD3 antibody to inhibit T cell expansion) (263, 274). The protocols that expand NK cells without feeder cells are also applied for NK cell treatment. The PB mononuclear cells from healthy donors or leukemia patients are cultured in stem cell growth medium supplemented with 5% human serum with the addition of IL-2 and OKT3 (275–277). Moreover, the expanded NK cells show significant cytotoxicity toward primary leukemia cells and K562 cells. Overall, primary NK cell expansion is primarily based on the help of feeder cells to provide appropriate signals from cytokines and activating ligands and can also be expanded merely with cytokines.

Differentiation and Expansion of NK Cells from BM or UCB $CD34^+$ Cells

Initially, BM $CD34^+$ cells were widely used for the generation of NK cells, but UCB $CD34^+$ cells have become more frequently utilized as UCB is easier to obtain and is rich in HSCs (2, 278–282) (**Figure 2**). An efficient protocol to expand NK cells from $CD34^+$ cells was established by the group of Spanholtz et al. (283, 284). They separated UCB $CD34^+$ cells and then cultured them in a clinical-grade culture medium with human serum and a mixture of cytokines, such as FL, KL, IL-7, GM-CSF, TPO, IL-15, and IL-2, and heparin in the absence of feeder cells. They can acquire up to 10^9 $CD34^+$cell-derived NK cells in fully closed static cell culture bags and automated bioreactors that can effectively kill leukemia cells *in vitro* or *in vivo*. These NK cell products have been used in a phase I trial to treat elderly AML patients (CCMO nr. NL31699 and Dutch Trial Register nr. 2818). Other protocols that improve NK cell development and function by adding methylprednisolone to induce preferential differentiation of HSCs toward NK cells or by adding IL-12 or IL-21 to promote NK cell maturation and cytotoxicity to tumor cells are developing (189, 190, 285). The improvement of protocols to acquire functional NK cells with high quantity can be efficiently applied in the clinic.

Improving NK Cell Survival and Function in Clinical Treatments

Natural killer cells, which are derived and expanded from autologous or allogeneic blood samples, can be applied in adoptive therapy. However, the low concentration or absence of cytokines in the body has often limited NK cell persistence postinfusion. To improve *in vivo* expansion, the Dario Campana group linked the human *IL15* gene to the gene encoding the transmembrane domain of *CD8*α (mbIL15) (131). The mbIL-15-NK cells can survive and proliferate *in vitro* or *in vivo* without exogenous cytokines. They have superior cytotoxicity against solid tumors and leukemia cells *in vitro* and against leukemia cells in xenograft models, indicating that the expression of mbIL15 may improve the postinfusion cytotoxic capacity of NK cells. Similarly, we have noted that IL-15 can induce prolonged NK cell antitumor effects after cytokine withdrawal, which suggests that IL-15 can be widely used in adoptive NK cell therapy (168). Moreover, the super-agonist IL-15-IL-15Rα-Sushi-Fc fusion protein (ALT-803) potently stimulates NK cell cytotoxic activity than native IL-15 (286) and has been used in the clinical trail to evaluate its safety and efficacy (NCT02099539). Additionally, preactivation of NK cells with IL-12/15/18 can induce memory-like NK cells with enhanced cytotoxicity toward tumors. The cells have been used in the clinical treatment with AML patients (NCT01898793). IL-12 and IL-21 can promote NK cell maturation with improved functions, which are good candidates to be applied in NK cell adoptive therapy (189, 190, 212).

Chimeric antigen receptor (CAR)-modified NK cells display a new possibility for the application of adoptive NK cell-based therapy (287). Preclinical studies to utilize CAR-expressing NK cells targeting CD19 or CD20 in B cell leukemia show effective killing toward tumor cells (267). In addition, CD19-CAR NK cells have been applied in treating B-ALL (NCT01974479) or ALL and CLL (NCT03056339) in clinical trails. To improve the efficacy of CAR-NK cells, the efforts to add genes that can elicit IL-15 production or other activating signals are now underway (288). However, new strategies still need to be developed to overcome the low transfection efficiency of NK cells.

Negative regulators can be treated as immune-checkpoints to shape immune responses. KIR and NKG2A are well-studied immune-checkpoints of NK cells, which can be blocked to gain better NK cell efficacy (289, 290) (**Figure 2**). The combination of anti-KIR mAbs lirilumab and lenalidomide has been used in a Phase I clinical trial (NCT01217203) with multiple myeloma patients. However, the outcomes need further study. Furthermore, anti-NKG2A antibody has also been applied in multiple clinical trials for patients with chronic lymphocytic leukemia (NCT02557516), squamous cell carcinoma of the head and neck (NCT02643550), gynecologic malignancies (NCT02459301), and squamous cell carcinoma of the oral cavity (NCT02331875). Other strategies are developing to upregulate activating signals that can significantly prolong antitumor activity of NK cells, such as retroviral transduction of NKG2D-DAP10-CD3ζ in NK cells (291) (**Figure 2**). The design of bi-specific antibodies that link the antigens on tumor cells, such as CD33, CD20, and CD19, together with CD16 on NK cells direct NK cells toward tumors and elicit efficient tumor cell killing(292). Additionally, the tri-specific antibody that integrates IL-15 in the existing bi-specific antibody further promotes NK cell activation to facilitate NK cell cytotoxicity (293). Overall, the developments to improve the efficacy of NK cell adoptive therapy are ongoing and may result in broader clinical applications in the near future.

CONCLUSION

The development and functional maturation of NK cells are controlled by diverse cytokines. Different cytokine cocktails are needed for distinct NK cell developmental stages that are guided by the expression pattern of relative cytokine receptors. NK cells are heterogeneous and can be divided into cytotoxic, tolerant and regulatory NK cells. They distribute throughout the body in different tissues and can be shaped by their specific tissue environment via diverse combinations of cytokines. Given a robust understanding of each cytokine in NK cell development and function, NK cells can be differentiated and expanded *in vitro* to generate sufficient numbers for clinical treatment. NK cells derived from primary NK cells mainly require cytokines to promote NK cell expansion and function, such as IL-2, IL-12, and IL-15. Cells from HSC differentiation need cytokines to promote the survival and proliferation of HSCs, such as FL, KL, and IL-3, and to specify differentiation to NK cells with high cytotoxicity, such as IL-15, IL-2, IL-12, and IL-21. The application of IL-15 or IL-12/15/18 can further enhance NK cell cytotoxicity to induce greater efficacy for adoptive transfer therapy. Overall, understanding the primary roles and modes of action of each cytokine is critical to apply them more effectively in the clinic.

AUTHOR CONTRIBUTIONS

YW wrote the manuscript. ZT and HW designed the review and revised the manuscript.

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