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Review Article

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Cytokines in Focus: IL-2 and IL-15 in NK Adoptive Cell Cancer Immunotherapy

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ABSTRACT

NK cell adoptive cell therapy (ACT) has emerged as a promising strategy for cancer immunotherapy, offering advantages in scalability, accessibility, efficacy, and safety. *Ex vivo* activation and expansion protocols, incorporating feeder cells and cytokine cocktails, have enabled the production of highly functional NK cells in clinically relevant quantities. Advances in NK cell engineering, including CRISPR-mediated gene editing and chimeric Ag receptor technologies, have further enhanced cytotoxicity, persistence, and tumor targeting. Cytokine support post-adoptive transfer, particularly with IL-2 and IL-15, remains critical for promoting NK cell survival, proliferation, and anti-tumor activity despite persistent challenges such as regulatory T cell expansion and cytokine-related toxicities. This review explores the evolving roles of IL-2 and IL-15 in NK cell-based ACT, evaluating their potential and limitations, and highlights strategies to optimize these cytokines for effective cancer immunotherapy.

Keywords: Natural killer cell; Cancer immunotherapy; Cancer immunotherapy; IL-2; IL-15; Cytokine

INTRODUCTION TO NK ADOPTIVE CELL THERAPY (ACT)

NK cells, a subset of innate lymphocytes, were first identified for their cytotoxic activity against tumor cells and have since become a cornerstone in cancer immunotherapy research. When interacting with ligands on target cells, the integration of signals received from their diverse repertoire of germline-encoded receptors allows NK cells to distinguish between normal cells, which require immunological tolerance, and aberrant cells, which necessitate cytolytic intervention. It is the balance between inhibitory signals, triggered by ligands on healthy cells, and activating signals, induced by ligands associated with cellular stress, infection, or malignant transformation, that is essential for triggering NK cell cytotoxicity. Once activated, NK cells execute their cytotoxicity through exocytosis of granules containing perforin and granzyme or through death receptor engagement. Beyond their cytolytic role, NK cells produce cytokines, including IFN-γ, TNF-α, and GM-CSF. NK cell contributions to

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Abbreviations

ACT, adoptive cell therapy; ADCC, Ab-dependent cellular cytotoxicity; ANKET, Ab-based NK cell engager therapeutic; ASCT, sodium-dependent neutral amino acid transporter; BaEV-G, baboon retroviral envelope glycoprotein; BSA, body surface area; CAR, chimeric Ag receptor; CDK, cyclin-dependent kinase; CIML, cytokine-induced memory-like; CRS, cytokine release syndrome; DC, dendritic cell; HCMV, human cytomegalovirus; iPSC, induced pluripotent stem cell; KIR, killer-cell immunoglobulin-like receptor; LAK, lymphokine-activated killer; mbIL-15, membrane-bound IL-15; NKint, intermediate NK; NKP, NK cell precursor; sbIL-15, soluble IL-15; scRNA-seq, single-cell RNA-sequencing; SLT, secondary lymphoid tissue; SOCS, suppressor of cytokine signaling.

Author Contributions

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immunity are highlighted by rare cases of patients with NK cell-specific deficiencies, which are associated with heightened susceptibility to viral infections (1).

In the 1980s, studies on lymphokine-activated killer (LAK) cells—a heterogeneous population of cytotoxic immune cells generated by culturing peripheral blood lymphocytes with interleukin-2 (IL-2), ignited significant interest in adoptive cell therapy for cancer (2). Subsequent studies revealed that NK cells were critical drivers of the anti-tumor effects of LAK cells, setting the stage for more focused research on NK cell-based adoptive cell therapies (3). However, early attempts to use enriched autologous NK cells for cancer treatment yielded disappointing outcomes (4).

A breakthrough came with the realization that allogeneic NK cells could elicit responses in cancer patients without triggering severe reactions such as graft-versus-host disease, cytokine release syndrome (CRS), or immune effector cell-associated neurotoxicity syndrome (**Fig. 1**) (5). This pivotal finding catalyzed a paradigm shift, opening the door for the development of "off-the-shelf" NK cell therapies, where donor NK cells are isolated, manipulated, stored, and eventually used without the need for patient-specific modifications (**Fig. 2**). In most applications, patients receive lymphodepleting therapy prior to allogeneic NK ACT to reduce tumor burden, prevent rejection of the administered NK cells and to create a receptive host milieu (5). In conjunction with hematopoietic stem cell transplantation, NK ACT is being explored both pre- and post-transplant as strategies to reduce relapse risk by targeting residual disease. In non-transplant settings, NK ACT is being tested as a standalone therapeutic approach, aimed at targeting and eliminating malignant cells. Importantly, allogeneic NK cells may demonstrate heightened efficacy due to the killer-cell immunoglobulin-like receptor (KIR)-HLA mismatch phenomenon, where the absence of matching HLA molecules on the recipient's cells prevents inhibitory KIR signaling in donor NK cells, thereby enhancing tumor cytotoxicity (6). A wide range of NK cell sources are being explored, including NK cell lines, induced pluripotent stem cell (iPSC) derived NK cells, cord blood-derived NK cells, and most frequently, peripheral blood-derived NK cells. Each source provides unique advantages and challenges in terms of availability, cytotoxic potential, and scalability, making them crucial components of ongoing research and development.

Advances in cell engineering have significantly broadened the potential of NK cell therapies. For example, genetic engineering approaches, such as CRISPR-mediated knockout of key

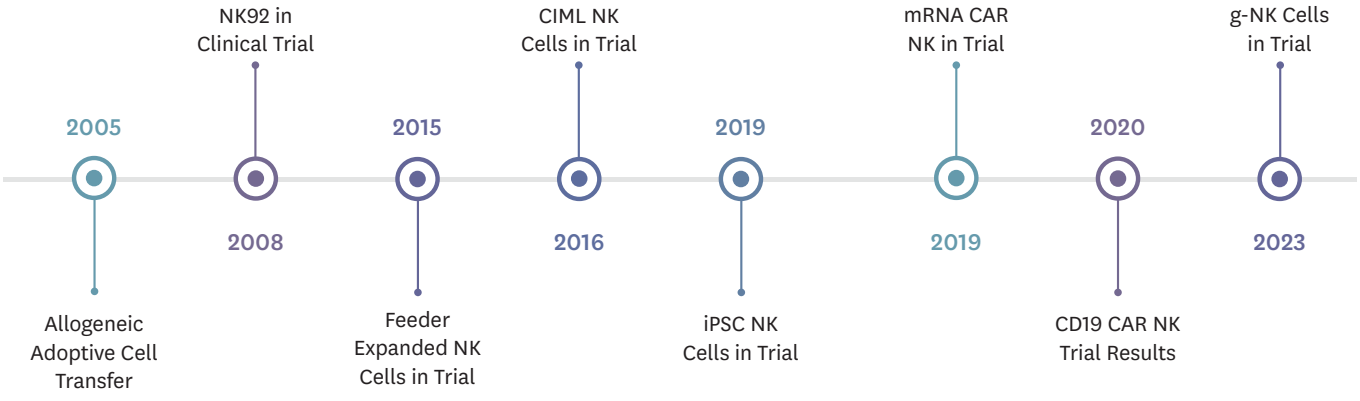

Figure 1. Twenty years of innovation in NK cell therapy. Timeline highlighting milestones in the advancement of NK cell adoptive cell cancer immunotherapy between 2005 and 2025. Created in BioRender.

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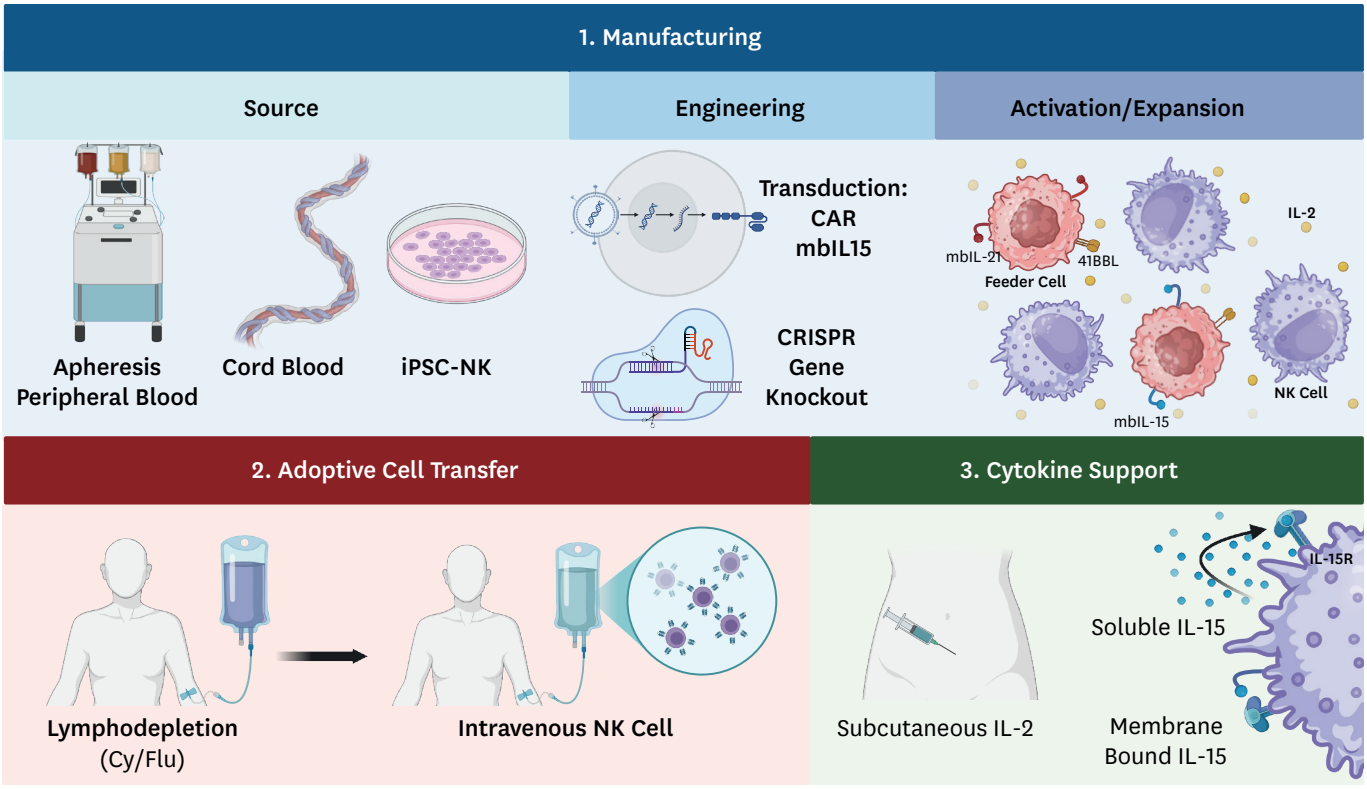

Figure 2. Typical phases of allogeneic NK adoptive cell therapy. The process consists of three key stages: (A) Manufacturing, where NK cells are sourced from apheresis of peripheral blood, cord blood, or iPSC-NK, potentially followed by genetic engineering steps such as CAR NK transduction or CRISPR-mediated gene knockout. Subsequently, NK cells are activated and expanded using feeder cells (e.g., expressing mbIL-21, 41BBL) and cytokines (e.g., IL-2, IL-15, IL-21). (B) Adoptive cell transfer, where patients undergo lymphodepletion (e.g., cyclophosphamide and fludarabine) before receiving infusion of NK cells. (C) *In vivo* support, which provides continued stimulation for NK cell survival and function through cytokine treatment like subcutaneous IL-2 or by engineering NK cells to express soluble IL-15 or membrane-bound IL-15. Created in BioRender.

regulatory genes, are being explored to enhance NK cell activity against tumors. Building on the success of chimeric Ag receptor (CAR) T cell therapy, there is growing interest in leveraging NK cells as a platform for CAR-based therapies (7). Most promisingly, a single-arm trial at MD Anderson demonstrated that CAR NK cells targeting CD19+ hematologic malignancies could achieve significant tumor reduction with minimal adverse effects (**Fig. 1**) (8). Recently, our group developed a one-step strategy for CRISPR-Cas9-mediated gene knockout and CAR transgenesis in NK cells using retroviral particles (9).

NK ACT is being investigated not only as a stand-alone therapy but also in combination with complementary tools to enhance its efficacy. One such approach involves leveraging the potent Ab-dependent cellular cytotoxicity (ADCC) mediated by CD16 (FcγRIII) on NK cells. For example, labeling tumor cells with monoclonal Abs such as rituximab prior to NK cell adoptive transfer has shown enhanced anti-tumor efficacy in non-Hodgkin's lymphoma (10,11). Similarly, trastuzumab, targeting human epidermal growth factor receptor 2-expressing breast cancers, has been used to attempt to boost NK cell cytotoxicity (12). These approaches demonstrate the potential of leveraging ADCC to augment the clinical impact of NK cell therapies. NK cells are also being combined with engagers, molecules that simultaneously bind to tumor-associated Ags and activating receptors on NK cells, such as CD16, boosting their ability to target and destroy malignant cells (13,14).

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Despite rapid progress, no NK cell-based cancer therapy has yet to achieve regulatory approval for cancer immunotherapy, underscoring the need for continued optimization. Previous studies exhibit high response rates, but there remains a need to push towards durable remissions. Achieving this goal will likely involve enhancing NK cell manufacturing, engineering, and performance and developing combination therapies that synergize NK cells with other immunomodulatory agents or treatments. The efficacy of NK cell therapy hinges on several critical factors: the NK cell source, the administered NK cell dose, *in vivo* persistence, the host immune environment, and the intrinsic lytic capacity of the NK cells. Treatment efficacy differs depending on the NK cell source and the quality of the donor product (8). For example, receiving a high-quality cord-blood product was the most significant predictor of positive outcomes in a CAR NK cell trial (8). Additionally, an ideal patient microenvironment, free from immunosuppressive influences, is crucial for maximizing NK cell functionality and persistence (5,11). Persistence is pivotal, as patients with prolonged NK cell persistence exhibit the most favorable outcomes, highlighting it as a key determinant in driving effective responses (8,15).

There is a long history of utilizing IL-2 both *ex vivo* and *in vivo* to stimulate NK cells for immunotherapy, reflecting its pivotal role in enhancing the cytotoxic potential, expansion, and survival of these innate immune cells. These approaches, spanning decades of research and clinical trials, have demonstrated significant promise but also revealed critical limitations, such as dose-limiting toxicities and unintended expansion of regulatory T cells, which can hinder therapeutic outcomes. In response to these challenges, innovative strategies have emerged to mitigate the drawbacks of IL-2 in NK cell therapy. Alternatively, there is a trend toward using IL-15, a cytokine with overlapping signaling pathways but distinct differences, such as avoiding regulatory T cell expansion and promoting enhanced NK cell survival and persistence. This review will delve into the mechanisms of IL-2 and IL-15 receptor signaling in NK cells, tracing the history of its application in immunotherapy and discussing the latest advances aimed at optimizing this pathway for enhanced NK cell-based cancer treatments.

IL-2 AND IL-15 RECEPTOR SIGNALING

IL-2 and IL-15 are cytokines that play pivotal roles in the development, survival, proliferation, and effector functions of NK cells. IL-2, discovered in 1976, is a well-known pro-inflammatory cytokine central to activating lymphocytes during an immune response (16,17). It is widely recognized for its pivotal role in driving clonal T cell expansion; however, IL-2 can also stimulate NK cells. IL-15, discovered in 1994, is now recognized as a critical driver of NK cell homeostasis and function (18).

Most resting NK cells respond to IL-2 using an intermediate affinity receptor composed of the IL2/15Rβ (CD122) and the common γc chain (CD132). This receptor is signaling competent, but its relevancy in driving IL-2 signaling *in vivo* is disputed because of its low affinity for IL-2 (Kd=0.5–2 nM) (19,20). Following activation, NK cells can upregulate IL-2Rα (CD25), forming a high affinity heterotrimeric IL-2αβγ with 100-fold increased affinity for IL-2 (Kd=10–80 pM) (20). The IL-15R shares the IL2/15Rβ and γc subunits with the IL-2R but does not rely on IL-2Rα. Instead, IL-15 is primarily delivered through trans-presentation by accessory cells expressing IL-15 bound to IL-15Rα (CD215); however, NK cells can also express IL-15Rα upon activation for cis-signaling (21).

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Engagement of the IL-2/IL-15 receptor induces dimerization of the cytoplasmic tails of IL-2Rβ and γc, bringing JAK1 and JAK3, which are associated with IL-2Rβ and γc respectively, into close proximity (20,22). This proximity enables cross-phosphorylation of the JAKs, leading to their activation and the phosphorylation of key tyrosine residues on the cytoplasmic domains of IL-2Rβ and γc. These phosphorylated tyrosines serve as docking sites for STAT proteins, specifically STAT5 and STAT3 (20,23,24). Upon recruitment, STAT5 and STAT3 are phosphorylated, dimerized, and translocated to the nucleus, mediating transcriptional regulation. Negative feedback mechanisms tightly regulate this signaling cascade. Suppressor of cytokine signaling (SOCS) proteins, such as SOCS-2 and CIS repress mediators of IL-2R-JAK-STAT signalling (25-28). Additionally, the PI3K-AKT-mTOR pathway is initiated downstream of IL-2/15 receptor activation via the binding of adaptor protein Shc to a phosphotyrosine on IL-2Rβ (29-31). Shc facilitates the recruitment of Grb2 and Gab2 to activate PI3K, which phosphorylates PIP2 to generate PIP3, leading to the activation of AKT (20,32,33). Activated AKT phosphorylates and regulates multiple downstream targets, including mTOR. The RAS-RAF-MAPK pathway is also activated downstream of IL-2 and IL-15 receptor engagement. Grb2, in conjunction with SOS, activates RAS, initiating a phosphorylation cascade involving RAF, MEK, and ERK (20,33). Sequential activation of these kinases culminates in the phosphorylation and activation of transcription factors such as c-Fos and c-Jun, which propagate the signaling response. In summary, IL-2/IL-15 receptor signaling is propagated mainly via the JAK-STAT, PI3K-AKT-mTOR, and RAS-RAF-MAPK pathways.

ROLES OF IL-2 & IL-15 IN NK CELL BIOLOGY

NK cells are a heterogeneous population of lymphocytes distributed throughout the blood, spleen, bone marrow, liver, tonsils, lymph nodes, lungs, skin, uterus, and gut (34). Using surface marker expression, NK cells can be defined as $CD3^-$ and $CD56^+$ and are often categorized into two differentially functional groups based on their level of CD56 expression. The predominant subset, constituting most circulating NK cells, is routinely categorized as $CD56^{dim}$ $CD16^+$ and noted for its profound cytotoxic capabilities and secretion of pro-inflammatory cytokines. Conversely, the $CD56^{bright}$ $CD16^-$ NK subset possesses less cytolytic capacity, but potently produces cytokines and is enriched in certain secondary lymphoid tissues (SLTs) (35,36). Data generated via single-cell RNA-sequencing (scRNA-seq) can further refine NK cell heterogeneity (37,38). Recent work by Rebuffet et al. (37) integrated scRNA-seq datasets to analyze around 225,000 NK cells from 718 donors. Their analysis of peripheral blood NK cells categorized them into three major groups: NK1, enriched in $CD56^{dim}$ $CD16^+$ NK cells; NK2, enriched in $CD56^{bright}$ $CD16^-$ NK cells; and NK3 encompassing both terminally differentiated and adaptive NK cells. Further sub-clustering of these populations revealed 6 distinct NK cell subsets differing in their expression of cytotoxic molecules, expression of activating and inhibitory receptors, production and sensitivity to cytokine and chemokine, and metabolic engagement. The NK2 subset, constituting only an average 6% of PB NK cells expresses less cytotoxic molecules but exhibits high proliferative and tissue homing signatures. Accordingly, NK2 cells ($CD56^{bright}$) are enriched in a variety of tissues such as the tonsils or lymph nodes (34). RNA-velocity shows that NK2 cells can maintain a distinct NK2 identity or progress into an intermediate NK (NKint) population and subsequently into NK1 cell subsets. Overall, NK1 subsets, collectively making up an average 53% of circulating NK cells, exhibit pronounced cytotoxic molecule expression, cell-adhesion properties, cytoskeletal activity, activation markers and augmented metabolic engagement. The NK3 cluster comprises mature terminally differentiated $CD56^{dim}$ NK cells which increase

in frequency with age, are enriched in various organs, and may specialize in unique effector functions (34,37). In human cytomegalovirus (HCMV) seropositive individuals, this cluster also contains NK cells with an adaptive gene expression signature. The persistently HCMV-expanded adaptive NK cell population is epigenetically modified to have lengthened lifespan and various enhanced effector functions such as enhanced CD16-mediated ADCC and IFN-γ production (37,39-44). The heterogeneity of NK cells is significantly influenced by tissue residency, as NK cells residing in different tissues exhibit variation in phenotype and function (34). While $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets found in different tissues share many features with their circulating counterparts and can be classified accordingly, they also exhibit tissue-specific variations that shape their functional potential.

NK cells basally express the heterodimeric IL-2Rβγ, rendering them responsive to trans-presented IL-15 and higher concentrations of IL-2. In contrast, $CD56^{bright}$ NK cells constitutively express the high-affinity IL-2Rαβγ receptor, allowing them to respond to low concentrations of IL-2 (35,45). Comparing IL-15/2R expression across NK subsets, scRNA-seq showed that IL-2Rβ mRNA was most abundant in NK2 cells followed by NKint, NK1B, NK1C and NK3 cells (37). IL-2Rγ was expressed higher amongst NK1 subsets compared to the other NK cell clusters. These findings highlight that NK cell subsets differ in their intrinsic sensitivity to IL-2 and IL-15. Furthermore, IL-2/15 receptor subunit expression is dynamically regulated in response to activation, further modulating NK cell sensitivity to these cytokines. For example, IL-2Rα can be increased on NK cells following cytokine stimulation or target cell stimulation (21,46-50). Viral infections provide another example of this modulation. For instance, in patients infected with COVID-19, $Ki67^{+}$ $CD56^{bright}$ NK cells upregulated IL-2Rα compared $Ki67^{-}$ NKs or healthy controls (51). Additionally, $CD25^{+}$ $CD56^{dim}$ NK cells are enriched in advanced hepatitis C virus-driven liver cirrhosis (52). These examples illustrate how IL-2/15 receptor expression is frequently altered in response to diverse stimuli, shaping NK cell function. In summary, NK cells are a heterogeneous population that differentially express IL-2/15 receptor subunits across subsets and activation states, enabling context-dependent responsiveness to cytokine signaling.

IL-15 plays a crucial and nuanced role in the development of NK cells. NK cell development commences in the bone marrow, differentiating from the common lymphoid progenitor into pre-NK cell precursors (NKPs) that will then commit to the NK cell lineage, becoming NKPs. Further maturation occurs either within the bone marrow or in peripheral tissues, leading to the development of $CD56^{bright}$ and later, $CD56^{dim}$ NK cells. Genetic knockout of STAT5, IL-2Rβ, IL-15, or IL15Rα in mice results in a dramatic loss of NK cells, implicating IL-15 as necessary for their development (53-56). Evidence suggests that IL-15 is not required for initial NK lineage commitment as pre-NKPs do not express IL-2Rβ and are maintained in γc knockout mice (57,58). Instead, IL-3, IL-7, kit ligand, and Flt3 have been implicated in driving the differentiation of stem cell precursors to NKP (59-61). The earliest committed NKP in the bone marrow expresses IL-2Rβ and requires IL-15R signaling to mature. $IL\text{-}2R\beta^{+}$ NKPs can also migrate from the bone marrow to SLTs where they can develop into more mature NK cells (62,63). The functional maturation of $CD56^{bright}$ NK cells to $CD56^{dim}$ cells has been implicated as an IL-15-dependent process (64). Together, evidence suggests that the commitment of progenitor cells to the NK cell lineage is IL-15 independent, but IL-15 responsiveness of NKP and intermediates is a central process in development. Recently, it was shown that IL-15 derived from dendritic cells (DC) and monocytes, but not stromal cells, was indispensable for NK cell development in the bone marrow (65).

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A critical study by Koka et al. (66) demonstrated that the trans-presentation of IL-15 is essential for maintaining mature peripheral NK cells. In adoptive transfer experiments, transferred NK cells could not survive in IL-15Rα-deficient mice. Furthermore, IL-15Rα–deficient NK cells survived in normal but not IL-15Rα–deficient mice. Therefore, IL-15Rα expression on non-NK cells is critical for maintaining peripheral NK cells, while its expression on NK cells themselves is dispensable. Conditional knockout of IL-15 in DCs and monocytes caused a reduction in NK cell populations of the spleen and blood, emphasizing the significance of hematologic sources of IL-15 for survival in the periphery (65). However, non-hematological sources of IL-15 also likely contribute to maintaining NK cell survival around the body (67-69).

In contrast to IL-15, whose major roles include facilitating NK cell development and survival, IL-2 functions predominantly as a secondary signal enhancing the activation of NK cells during an immune response. IL-2 is primarily produced by activated T cells for autocrine signaling and clonal expansion. However, T cell-derived IL-2 also diffuses to activate nearby NK cells, effectively "bridging" the adaptive immune system with the innate immune system (70-73). DC-derived IL-2 can also stimulate NK cells, as evidenced in a bacterial infection model where NK cells continued to produce IFN-γ in an IL-2-dependent mechanism even in the absence of T cells, pinpointing DCs, rather than T cells, as the dominant source of IL-2 (74). Importantly, NK cells have not been shown to produce IL-2; therefore, they rely exclusively on external sources rather than autocrine signaling. Research indicates that NK cell development and maintenance largely operate independently of IL-2 signaling. This is evidenced by mice lacking IL-2-producing T cells or with IL-2 or IL-2Rα gene knockouts which still maintain NK cells (75-77). This independence is not due to an inherent inability of IL-2 to support NK cell survival, as the shared receptor subunits for these cytokines lead to the engagement of survival genes following either IL-2 or IL-15 ligand binding, a point elaborated on below. Indeed, NK cells can be maintained in culture with IL-2 alone (78). Instead, the limited role of IL-2 in NK cell development and maintenance arises from the restricted expression of IL-2 in the body and IL-2Rα on NK cells. By contrast, trans-presented IL-15 is abundantly available to NK cells, playing a key homeostatic role in their development and maintenance. In the absence of IL-2, IL-15 remains sufficient to support these processes, explaining why IL-2 knockout does not disrupt NK cell survival. Nonetheless, NKPs express the high-affinity IL-2Rαβγ, so IL-2 may still play a role in NK cell development through overlapping or redundant pathways (62,63). Despite IL-2's traditionally understood role, emerging studies suggest additional, previously unrecognized functions. For instance, Wang et al. (79) demonstrated for the first time that epithelial-derived IL-2 in the mammary gland is critical for maintaining NK cell homeostasis. When IL-2 was deleted from epithelial cells, NK cell numbers were reduced and their function was altered, which correlated with tumor development. This study marks a significant expansion in our idea of how non-hematopoietic sources of IL-2 may be involved in regulating NK cell responses.

Considering the extensive roles of IL-2 and IL-15 signaling, encompassing NK cell development, functional maturation, and polarization, it is to be expected that these cytokines exert significant and broad-ranging effects on NK cells. Critically, IL-15 and IL-2 have chief effects on NK cell survival signalling. As previously mentioned, IL-15 and IL-15Rα deficient mice cannot sustain mature NK cells, suggesting that continued IL-15 signaling is vital for NK cell survival (66). Signaling downstream of IL-15 and IL-2 upregulates the expression of anti-apoptotic protein Bcl-2, thereby preventing regulated cell death (80,81). In fact, NK cell development in IL-2Rβ deficient mice can be rescued by forcing the expression

of Bcl-2 (82). Furthermore, STAT5 signaling forces the expression of Mcl1, a protein that is continuously required for supporting NK cell survival (83). Conditional murine knockout of Mcl1 in NK cells results in complete loss of NK cells, emphasizing the importance of Mcl1 for survival. IL-15 signaling, and to a lesser extent IL-2, enhances TERT expression, potentially extending the lifespan and proliferative capacity of NK cells by preserving telomere integrity (84). Interestingly, Felices et al. (85) showed that in an IL-2 free system, the expression of IL-2Rα was inversely related with cell survival. While multiple mechanisms were at play in that model, knockout of IL-2Rα decreased cell death by 1.4-fold, indicating that the IL-2Rα chain modulated survival in an IL-2-independent mechanism. The authors hypothesized that IL-2Rα might influence survival by competing for common signaling subunits with the IL-15R, whose signaling was necessary for survival in that experiment.

In vitro, both IL-2 and IL-15 promote the proliferation of NK cell cultures. RNA-seq of IL-2-activated NK cells shows stimulation of cell cycle progression by upregulating cyclin-dependent kinases (CDKs), downregulating inhibitory regulators of CDKs, and upregulating DNA replication and repair factors (86). Knockout of regulatory protein CIS causes hypersensitivity to IL-2 and IL-15, resulting in increased proliferation in response to these cytokines (25,87,88). Activation of NK cells can promote increased expression of IL-2Rα, making them more sensitive to low-dose IL-2 expansion *ex vivo* (46,47). *In vivo*, STAT5, IL-2, and IL-15 are required in a non-redundant mechanism to support NK cell expansion post-murine cytomegalovirus infection (89). Specifically, inducible NK-specific knockout of STAT5, IL-2Rα, or IL-2Rβ and neutralization of IL-2 or IL-15 cytokines all resulted in an impaired increase in NK cell numbers and a reduction in proliferation as measured by Ki67. This study highlights the imperative role both IL-2 and IL-15 signaling play in NK cell proliferation *in vivo*.

Another critical aspect of IL-15 and IL-2 stimulation is enhancing NK cell cytolytic activity. IL-2 and IL-15-activated peripheral blood-derived NK cells have higher cytotoxicity in co-culture assays (90). Activation by these cytokines triggers upregulation of perforin, granzyme B, FASL, and TRAIL which mediate cytotoxic enhancement (86). Furthermore, IL-2 and IL-15 activation can modulate NK cell activating and inhibitory receptors which tune NK engagement with a target cell (86). For instance, IL-2 signaling was shown to be essential for the sustained expression of NKp30 in NK cells. IL-2 deprivation reduces NK cell cytotoxicity by selectively downregulating NKp30, without similarly affecting other receptors, such as NKp46 (91). Moreover, IL-15 signaling during development is critical for acquiring cytolytic function. Forced expression of Bcl-2 rescues NK cell deficiency in IL-2Rβ knockout mice but fails to restore their cytotoxic function, highlighting the role of IL-15 signaling in developing and maintaining NK cell cytotoxicity (82). These molecular changes underscore the role of IL-2/IL-15 in sustaining and enhancing NK cell cytotoxicity, making them important cytokines in immunotherapies to boost NK cell activity against cancer.

Enhancing proliferation, survival, and cytotoxicity poses energetic demands. Therefore, IL-2 and IL-15 stimulation engage NK cell metabolism which at resting state is mostly quiescent. *Ex vivo* cytokine stimulation significantly increases NK cell size, enhances the expression of nutrient transporters, and upregulates both glycolysis and oxidative phosphorylation (90,92-95). Interestingly, mTOR, a key regulator in NK metabolism, is differentially upregulated by both IL-15 and IL-2 stimulation. IL-15 induces greater mTOR activation than IL-2, underpinning IL-15's ability to uniquely modulate metabolic processes and mitochondrial functions essential for sustained NK cell survival and antitumor responses

(90). Inhibition of mTOR exerts a more pronounced impact on IL-15-mediated stimulation than IL-2-mediated effects, reflecting distinct dependencies on mTOR for downstream functions. This finding is particularly interesting because, in the *ex vivo* culture context, IL-15 signaling occurs in the absence of trans-presentation, where NK cells can receive secondary signals at an immunological synapse. Mechanistically, the differences in signaling are not yet well described but may be shaped by the unique properties of the cytokine receptor subunits. IL-15Rα, has been reported to facilitate signal transduction, while IL-2Rα lacks a signaling-competent cytoplasmic domain (96). Crucially, the delivery of IL-15 through trans-endocytosis or shedding of IL-15Rα–IL-15 complexes plays a critical role in tailoring the downstream signaling outputs (97). In trans-endocytosis, intact, membrane-bound IL-15 (mbIL15) Rα–IL-15 complexes from presenting cells are engulfed into NK cells. This mechanism directs the IL-15Rα–IL-15 complex into endosomes, allowing sustained signaling within intracellular compartments. Trans-endocytosis preferentially activates mTOR. This localized signaling in endosomes is thought to create a distinct spatial dynamic for signal amplification that is not accessible to IL-2, which lacks trans-endocytosis as a mechanism. In contrast, the shedding of IL-15Rα–IL-15 complexes generates soluble cytokine-receptor complexes that primarily engage the IL-2Rβγ receptor subunits on NK cells. These complexes are highly effective at inducing STAT5 phosphorylation, which drives survival and transcriptional responses but does not robustly activate mTOR signaling. The ability of IL-15 to engage in both membrane-bound and soluble signaling modes allows it to fine-tune NK cell responses depending on the mode of presentation and local cytokine concentrations. Therefore, despite sharing receptor subunits, soluble IL-2 and IL-15 stimulation differentially engage mTOR. Importantly, there are likely more unique pathways influenced by these cytokines as RNA-sequencing has revealed differential gene expression profiles for NK cells stimulated with soluble IL-2 versus IL-15 (90).

IL-15 and IL-2 signaling also converge with other pathways to shape NK cell responses. Their stimulation significantly alters cytokine sensitivity, upregulating interleukin and chemokine receptors such as IL-2Rα, IL-2Rβ, IL-2Rγ, IL-15Rα, and components of the IL-18R, IL-12R, and IL-17R complexes (86,98). These changes collectively modulate NK cell cytokine responsiveness. DC are known for co-localizing with NK cells and producing a variety of cytokines that modulate them, including IL-15 and IL-2 (99-101). NK cells and DCs can interact through a specialized immunological synapse that spatially organizes IL-15R along with IL-12/IL-12R, CD94, KIRs, MHC-I (102,103). The importance of this interaction is highlighted by DC knockout models in which NK cells are hyporesponsive to various microbial stimuli. Therefore, the interaction of NK cells with DCs in secondary lymphoid organs is a key stimulus of NK cell activity that can combine IL-15 and IL-2 with other signals (99). DC cross-talk serves as a well-characterized example, but consider that IL-15 and IL-2 can be produced by various cells during inflammation, where diverse stimuli may interact to shape signaling pathways and drive distinct outcomes.

In summary, IL-15 and IL-2 contribute to NK cell biology in both distinct and overlapping ways. Trans-presentation of IL-15 from a diverse array of cellular sources is crucial for the development and homeostasis of NK cells, ensuring their continuous presence and readiness. In contrast, IL-2 expression is severely restricted in the body, emerging predominantly from activated T-cells, with its receptor expression modulated on NK cells to precisely calibrate receptor affinity, optimizing the response during immune challenges. In general, IL-2 acts as a transient yet potent trigger during immune activation, whereas IL-15 provides a sustained signal essential for NK cell survival and development; however, this description

is not all-encompassing. At the cellular level, IL-15 and IL-2 exhibit overlapping pleiotropic effects, influencing survival, proliferation, and cytolytic function. However, the end result of these cytokines' effect on the NK cell depends on various factors, including the NK cell subset, the environmental context of the NK cell, and the concentration of the cytokine. Moreover, the IL-15R and IL-2R complexes may initiate distinct signaling cascades, allowing for precise modulation of functions. Altogether, IL-15 and IL-2 are pivotal modulators of NK cells, offering immense potential to unlock and harness the power of NK cells in cancer immunotherapy.

EX VIVO ACTIVATION OF NKs FOR ADOPTIVE CELL THERAPY

The discovery of IL-2 was pivotal in initiating the field of ACT, particularly for the *ex vivo* propagation of immune cells. It provided a powerful tool to maintain lymphocytes in culture, including T cells and NK cells, enabling researchers to study immune cell biology in unprecedented detail. In 1987, Rosenberg et al. (2) demonstrated that of 106 patients receiving LAK cells and IL-2, 30% had at least a limited response. Subsequent studies identified NK cells as the primary mediators of the anti-tumor effects in LAK therapy, underscoring their potent cytotoxic activity when activated by IL-2. This finding shifted focus toward developing IL-2-activated NK cells for cell therapy, with the first ACT of an enriched NK cell population occurring in 1990 (104).

Since then, many studies have investigated ACT of NK cells to treat both hematological and solid malignancies. Most have used IL-2 to activate NK cells *ex vivo* before transplant (**Table 1**). Often, NK cells are isolated from peripheral blood and cultured in around 100–1,000 U/ml of IL-2 prior to treatment. However, dose manufacturing strategies vary significantly in terms of NK cell source, enrichment, time post-leukopoiesis to transplant, and use or omission of cryopreservation. There have not been many clinical NK ACT studies evaluating the differences in IL-2 activation regimens on patient response. However, in a small sample size, Szmania et al. (105) noted that patients had better post-transplant NK cell expansion when the NK dose was manufactured in 500 U/ml IL-2 as opposed to 10 U/ml.

Table 1. Use of cytokines in reviewed NK adoptive cell transfer clinical studies

Study	NK source	Combination	*Ex vivo* activation	Activation/ expansion duration	NK cell dose	Exogenous cytokine
Marin et al. (8) (2024)	Allo PB	CD19 CAR NK w/ IL-15	IL-2, K562-mbIL21-41BBL	15–22 days	8×10^8 cells/kg	No exogenous cytokine
Naik et al. (166) (2024)	Allo PB		Not reported	No activation/ expansion	Median 10.88 (1.26–99.19)$\times10^6$ cells/kg	No exogenous cytokine
Mohseni et al. (167) (2024)	Allo PB		IL-2 (500 U/mL), IL-15(140 U/ml), IL-21 (1 U/ml)	19–25 days	1 & 5×10^7 cells/kg	No exogenous cytokine
Burger et al. (168) (2023)	NK92	HER2 CAR	IL-2 (500 U/ml)	Not reported	(0.1–1)$\times10^8$ cells	IL-2 (formulated in 100 U/ml)
Ahmadvand et al. (169) (2023)	Allo PB		IL-2 (500 U/ml), OKT3, irradiated PBMC	21 days	2–10$\times10^6$ cells/kg, 2 doses	No exogenous cytokine
Yoon et al. (170) (2023)	Allo PB	Rituximab	IL-2 (500 U/ml), OKT3, irradiated PBMC	21 days	(1–9)$\times10^7$ cells/kg, 12–16 doses	IL-2 (1×10^6 U/m^2), 1 dose
Lee et al. (171) (2023)	Allo PB		IL-15 (10 ng/ml), IL-21 (10 ng/ml), hydrocortisone (10–6M)	2–3 wk	(0.5–4)$\times10^8$ cells/kg, 2 doses	No exogenous cytokine
Ciurea et al. (113) (2022)	Allo PB		IL-2 (50 U/ml), K562-mbIL21-41BBL	14 days	(0.0001–1)$\times10^8$ cells/kg, 1–3 doses	No exogenous cytokine

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Table 1. (Continued) Use of cytokines in reviewed NK adoptive cell transfer clinical studies

Study	NK source	Combination	*Ex vivo* activation	Activation/ expansion duration	NK cell dose	Exogenous cytokine
Bednarski et al. (172) (2022)	Allo PB		IL-12 (10 ng/ml), IL-15(50 ng/ml), IL-18(50 ng/ml)	12–16 h	2.81 (1.17–8.69)×10^8 cells/kg	No exogenous cytokine
Shapiro et al. (173) (2022)	Allo PB		IL-12 (10 ng/ml), IL-15(50 ng/ml), IL-18(50 ng/ml)	12–16 h	5–10×10^6 cells/kg	IL-2 (1×10^6 U/m^2) for 7 doses
Jia et al. (174) (2022)	Auto PB	Sintilimab	IL-2 (600 U/ml) & IL-15 (10 ng/ml) & OK432	14 days	3×10^9 cells, 2–36 doses	No exogenous cytokine
Bae et al. (175) (2022)	Auto PB		IL-2 (10–100 U/ml), IL-15 (10 U/ml), K562	14–18 days	(2.5–10)×10^8 cells, 5 doses	No exogenous cytokine
Otegbeye et al. (176) (2022)	Allo PB		IL-2 (100 U/ml), feeder cell	14–21 days	(1–5)×10^7 cells	No exogenous cytokine
Lim et al. (177) (2022)	Auto PB	Cetuximab	IL-2 (40 U/ml), K562-mb15-41BBL	10 days	(1–10)×10^6 cells	IL-2 (1×10^6 U/m^2), 6 doses
Kim et al. (178) (2022)	Auto PB	Pembrolizumab	IL-2 (500 U/ml), IL-21 (50 ng/ml), KL-1, LCL feeders	17–18 days	(2–4)×10^9 cells, 6 doses	No exogenous cytokine
Devillier et al. (179) (2021)	Allo PB		IL-2 (1,000 U/ml)	7 days	1–10×10^6 cells/kg	No exogenous cytokine
Silla et al. (180) (2021)	Allo PB		IL-2 (50 U/ml), K562-mbIL21-41BBL	8–21 days	7.86±3.82 (1–14)×10^6 cells/kg, 6 doses	No exogenous cytokine
Berrien-Elliott et al. (139) (2020)	Allo PB		IL-12 (10 ng/ml), IL-15 (50 ng/ml), IL-15 (50 ng/ml)	12 h	(0.5–10)×10^6 cells/kg	IL-2, 6 doses
Nagai et al. (181) (2020)	Auto PB		IL-2 (2,813 U/ml)	14 days	(1–71)×10^7 cells total	No exogenous cytokine
Lee et al. (12) (2020)	Auto PB	Trastuzumab & bnevacizumab	IL-2 (40 U/ml), K562-mb15-41BBL cells	10 days	(0.01–1)×10^8, 1–4 doses	IL-2 (1×10^6 U/m^2), 6 doses
Khatua et al. (182) (2020)	Auto PB		IL-2 (100 U/ml), K562-mbIL21-41BBL	Not reported	(0.09–9)×10^8 cells/m^2	No exogenous cytokine
Lin et al. (183) (2020)	Allo PB	Pembrolizumab	IL-2 (10 U/ml), Undisclosed Synergist	12 days	Not reported	No exogenous cytokine
Khatua et al. (182) (2020)	Auto PB		IL-2 (100 U/ml), K562-mbIL21-41BBL		(0.03–3)×10^8 cells/dose, 27 doses	No exogenous cytokine
Multhoff et al. (184) (2020)	Auto PB		IL-2 (100 U/ml), TKD peptide	3–5 days	(1.04–5.63)×10^8 cells, 4 doses	No exogenous cytokine
Cooley et al. (152) (2019)	Allo PB		IL-15 (10 ng/ml)	Overnight	Mean 1.2-1.9×10^7 cells/kg	No exogenous cytokine
Nguyen et al. (185) (2019)	Allo PB		No activation	No activation/ expansion	Median 12.5 (3.6–66.2)×10^6 cells/kg	IL-2 (1×10^6 U/m^2) for 6 doses
Tanaka et al. (186) (2019)	Auto PB	Rituximab	IL-15 (10 ng/ml), IL-2 (5 ng/ml), OKT3	21 days	1–10×10^6 cells/kg	No exogenous cytokine
Yang et al. (187) (2019)	Allo PB	IRE	Undisclosed synergist	14 days	Not reported	No exogenous cytokine
Xiao et al. (188) (2019)	Auto or Allo PB	NKG2D CAR	IL-2 (50 U/ml), K562-mbIL21-41BBL	18 days	(0.5–2)×10^9 cells/dose, 2–6 doses	No exogenous cytokine
Björklund et al. (107) (2018)	Allo PB		IL-2 (1,000 U/ml)	Overnight	Median 6.7 (1.3–17.6)×10^6 cells/kg	No exogenous cytokine
Bachanova et al. (135) (2018)	Allo PB	Rituximab	IL-2 (1,000 U/ml)	8–16 h	Median 1.9 (0.5–3.33)×10^7 cells/kg	IL-2 (9×10^6 U) for 6 doses
Modak et al. (189) (2018)	Allo PB	anti-GD2	IL-2 (500–1,000 U/ml)	Overnight	Mean 0.4–32.6×10^6 cells/kg, 1-3 Doses	No exogenous cytokine
Tang et al. (190) (2018)	NK92	CD33 CAR	NK92 expresses IL-2	Not reported	0.3, 0.6, 1×10^8 cells, doses 1–3 respectively	No exogenous cytokine
Fehniger et al. (191) (2018)	Allo PB		CNDO-109 lysate	16 h	0.3–3×10^6 cells/kg	No exogenous cytokine
Ishikawa et al. (192) (2018)	Auto PB		IL-2, T cell feeder	18–24 days	(0.5–3)×10^9, 3 doses	No exogenous cytokine
Liang et al. (193) (2018)	Allo PB	Cetuximab	K562-mb15-41BBL, undisclosed synergists	12 days		No exogenous cytokine
Alnaggar et al. (194) (2018)	Allo PB	IRE	IL-2 (10 U/ml), K562-mb15-41BBL, undisclosed synergist	12 days	(2.6–3.3)×10^9 cells, 3 doses	No exogenous cytokine
Dolstra et al. (195) (2017)	Allo HSC Differentiation		Differentiation from HSC	42 days	(1–15)×10^6 cells/kg total	No exogenous cytokine
Federico et al. (196) (2017)	Allo PB	Anti-GD2	No activation	Not reported	15.5 (4.7–59.5)×10^6 cells/kg, 1–3 doses	IL-2 (1×10^6 U/m^2) for 6 doses & GM-CSF

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Table 1. (Continued) Use of cytokines in reviewed NK adoptive cell transfer clinical studies

Study	NK source	Combination	*Ex vivo* activation	Activation/ expansion duration	NK cell dose	Exogenous cytokine
Boyiadzis et al. (197) (2017)	NK92		IL-2 (450 U/ml)	20–28 days	(1–3)×10^9 cells/m^2, 2–6 doses	No exogenous cytokine
Lin et al. (198) (2017)	Allo PB	Cryoablation	IL-2, K562-mb15-41BBL	12 days	8–10×10^9 cells	No exogenous cytokine
Shah et al. (199) (2017)	Allo CB		IL-2 (100 U/ml), K562-mbIL21-41BBL	14 days	(0.05–1)×10^8 cells/kg	No exogenous cytokine
Romee et al. (124) (2016)	Allo PB		IL-12 (10 ng/ml), IL-15 (50 ng/ml), IL-18 (50 ng/ml)	12–16 h	(0.5–10)×10^6 cells/kg	IL-2 (1×10^6 U/m^2) for 6 doses
Shaffer et al. (200) (2016)	Allo PB		No activation	No activation/ expansion	Median 11 (4.3–22.4)×10^6 cells/kg	IL-2 (1×10^6 U/m^2) for 6 doses
Lee et al. (201) (2016)	Allo PB		IL-2 (1,000 U/ml)	16 h	1–5×10^6 cells/kg	IL-2 (0.5×10^6 U/m^2) for 5 doses
Yang et al. (202) (2016)	Allo PB		IL-2 (500 U/ml), OKT3, irradiated PBMC	14 days	(1–30)×10^6 cells/kg, 1–3 doses	No exogenous cytokine
Sakamoto et al. (203) (2015)	Auto PB		IL-2 and T cell feeders	21–22 days	(0.5–2)×10^9 cells/dose, 3 doses	No exogenous cytokine
Kottaridis et al. (204) (2015)	Allo NK		Tumour lysate	20 days	(0.1–1)×10^7 cells/kg	No exogenous cytokine
Choi et al. (205) (2014)	Allo PB		IL-15 (10 ng/ml), IL-21 (10 ng/ml), hydrocortisone (10^{-6}M)	13–20 days	Median 0.5 (0.2–0.5)–2.0 (0.9–5) ×10^8 cells/kg, 4 doses	No exogenous cytokine
Szmania et al. (105) (2015)	Auto or Allo PB		IL-2 (10 or 500 U/ml), K562-mbIL15-41BBL	8–9 days	6.6±3.3 (2.2–10)×10^7 cells/kg	IL-2 (3×10^6 U) for 13 doses
Shah et al. (206) (2015)	Allo PB		IL-15 (10 ng/ml), KT32.A2.41BBL.64	9–11 days	1 or 10×10^5 cells/kg, 1–2 doses	No exogenous cytokine
Bachanova et al. (11) (2014)	Allo PB		IL-2 (1,000 U/ml)	Overnight	Mean 0.34±0.05–2.6±1.5×10^7 cells/kg	IL-2 (9×10^6 U) for 6 doses
Killig et al. (207) (2014)	Allo PB		No activation	1 day	Mean 9.79×10^6/kg, 1 dose	No exogenous cytokine
Tonn et al. (208) (2013)	NK92		IL-2 (1,000 U/ml)	–12 days	1–10×10^{10} cells /m^2, 2 Doses	No exogenous cytokine
Stern et al. (209) (2013)	Allo PB		No activation	No activation/ expansion	1.21 (0.3–3.8) 10^7/kg, 1–3 doses	No exogenous cytokine
Geller et al. (134) (2011)	Allo PB		IL-2 (1,000 U/ml)	Overnight	Mean 2.16 (0.833–3.94)×10^7 cells/kg	IL-2 (10×10^6 U) for 6 doses
Parkhurst et al. (132) (2011)	Auto PB		IL-2 (600 U/ml) with PBMC feeder	21 days	Mean 4.7±2.1 (1.9–7.6)×10^{10} cells	IL-2 (720,000 U/kg) every 8 h to tolerance
Brehm et al. (210) (2011)	Allo PB		IL-2 (1,000 U/ml)	10 days	Median 14.6×10^3 cells/dose, 1–2 doses	No exogenous cytokine
Iliopoulou et al. (211) (2010)	Allo PB		IL-15 (20 ng/ml)	21–23 days	Median 4.5 (0.2–29)×10^6 cells/kg, 2–4 doses	No exogenous cytokine
Bachanova et al. (10) (2010)	Allo PB	Rituximab	IL-2 (1,000 U/ml)	8–16 h	Mean 21±19 (2.2–15)×10^6 cells/kg	IL-2 (10×10^6 U) for 6 doses
Rubnitz et al. (212) (2010)	Allo PB		No activation	No activation/ expansion	Median 29 (5–81)×10^6 cells/kg	IL-2 (1×10^6 U/m^2) for 6 doses
Yoon et al. (213) (2010)	Allo PB		Differentiation from CD34+ cells	42 days	9.28 (90.33–24.5)×10^6 cells/kg	No exogenous cytokine
Arai et al. (214) (2008)	NK92		IL-2 (500 U/ml)	15–17 days	(0.1–3×10^9) cells/m^2, 3 doses	No exogenous cytokine
Shi et al. (215) (2008)	Allo PB		IL-2 (300 U/ml)	0–1 day	(2.7–92)×10^6 cells/kg total	IL-2 (3×10^6 U/m^2) for 11 doses
Miller et al. (5) (2005)	Allo PB		IL-2 (1,000 U/ml)	Overnight	Mean 8.5±0.5 (2.2–15)×10^6 cells/kg total	IL-2 (1.75×10^6 U/m^2) for 14 doses
Ishikawa et al. (216) (2004)	Auto PB		IL-2 (200 U/ml) with HFWT	14 days	(0.6–6.5)×10^9 cells, 1–3 courses	IL-2 (<100 U/kg) with NK ACT & IFN-β
Passweg et al. (217) (2004)	Allo PB		No activation	Overnight	(2.1–14.1)×10^6 cells/kg	No exogenous cytokine
Hercend et al. (103) (1990)	Auto PB		IL-2 (50 U/ml) with LAZ and PBMC feeder	32 days	Mean 45.1 (7–125)×10^9 cells total	IL-2 (3×10^6 U/m^2) for 18 doses

NK cell doses reported as range (X–Y), mean or median and reported as either per dose or cumulative dose.
Blank entries represent information not reported.
Allo, allogeneic; PB, peripheral blood; Auto, autologous; CB, cord blood.

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Given the role of IL-2 as a strong driver of NK cell proliferation, *ex vivo* IL-2 stimulation also plays a practical role in facilitating NK cell expansion for therapeutic applications. In peripheral blood, NK cells represent a small population, usually between 5%–15%. Therefore, *ex vivo* expansion of NK cells from a donation product is needed to produce large doses. IL-2 alone can only modestly expand NK cells in culture. After testing various IL-2 concentrations ranging from 10 to 6,000 U m^{-1}, Fujisaki et al. (106) achieved only four-fold expansion of peripheral blood-derived NK cells over 7 days (78). Given this limited expansion, most studies that use IL-2 activation of NK cells alone inject limited cells per kg (**Table 1**). For example, Miller et al. (5) administered a mean 8.5±0.5 (2.2–15)×10^6 cells/kg. Years later, Björklund et al. (107) administered a median NK-cell dose of 6.7 (1.3–17.6 E6)×10^6 cells/kg. In a 6-patient cohort, Bachanova et al. (10) reported a mean dose of 21±19 (2.2–15)×10^6 cells/kg. Furthermore, the interval between leukapheresis and ACT typically is minimal, with administration commonly occurring after a brief overnight incubation period.

The HLA-1 deficient K562 cell line has been instrumental in promoting the expansion of NK cells in culture (**Fig. 1**). K562 cells have been genetically engineered to express potent NK cell activators, irradiated to prevent proliferation, and then combined with cytokines in culture to stimulate NK cell expansion. In 2005, K562 transduced to express mbIL-15 and 41BBL were generated and specifically expanded NK cells from PBMC a median 23-fold in 7 days, and over 1,000-fold in 3 wk (108). Expansion with K562-mbIL-15-41BBL was far superior to K562 expressing either mbIL-15 or 41BBL alone. In a subsequent study, expansion exhibited remarkable donor variability, with one week NK expansion from PBMCs ranging 5.1 to 86.6-fold (106). The authors also demonstrated that feeder cell-expanded NK cells had significant differential gene expression compared to NK cells activated by IL-2 alone, including increased expression of IL-2Rα. Furthermore, feeder cell-expanded NK cells demonstrated enhanced cytotoxicity in co-culture assays compared to NK cells stimulated with IL-2 alone. However, continued proliferation using this method was shown to be limited by telomere shortening and senescence (109).

Later, in 2012, Denman et al. (110) engineered K562 expressing CD64 (FccRI), CD86 (B7-2), CD137L (41BBL), truncated CD19 and mbIL-21, leading to a protocol which generated 47,967-fold expansion in 3 wk. Importantly, this level of expansion was achieved without driving cell senescence or loss of cytotoxic activity. In fact, NK cells expanded by mbIL-21 had longer telomeres post-expansion than initially (110). In this protocol, IL-2 is supplemented in the growth medium, complementing NK cell activation alongside the feeder cells. In a clinical study using peripheral blood-derived NK cells expanded by K562-mbIL-21-41BBL feeder cells, up to 1×10^8 cells/kg were administered, well beyond what was achieved using IL-2 activation alone (111). Recently, using rhesus macaque models, researchers demonstrated that K562-mbIL-21-41BBL *ex vivo*-expanded NK cells maintain clonal diversity during expansion (112). Using these feeder cells, Ciurea et al. (113) could administer three doses of 1×10^8 cells/kg. Similarly, the Rezvani group generated K562 feeder cells expressing mbIL-21, 41BBL, and CD48, which in combination with soluble IL-2, achieved over 900-fold expansion of cord blood-derived NK cells in 2 wk (114). Furthermore, Ojo et al. (115) engineered the leukemia cell line OCI-AML3 with mbIL-21, creating a feeder cell capable of expanding NK cells over 10,000-fold in 5 wk. Their study highlighted the ability of mbIL-21 to engage the IL-21-STAT3-cMyc pathway responsible for stimulating glycolysis and cell cycle progression.

Creating different feeder cells to optimize NK cell expansion is an ongoing area of research. For instance, Thangaraj et al. (116) generated K562-OX40L-mbIL-18/-21 cells that could

expand NK cells 9,860-fold over 28 days in culture with IL-2 and IL-15. As mentioned, HCMV seropositivity drives the persistent formation of adaptive NK cells that are endowed with various enhanced functions and an extended lifespan (39,40). To selectively expand these potent cells *ex vivo* for therapeutic applications, feeder cells expressing HLA-E, the activating ligand for NKG2C, are employed alongside IL-15 (117). Since NKG2C is highly expressed on adaptive NK cells, as it facilitates the recognition of HLA-E-HCMV peptide complexes, this approach efficiently enriches the adaptive NK cell subset (43). To mitigate the risk associated with using whole feeder cells, membrane particles have been produced by lysing engineered K562 feeder cells through nitrogen cavitation, followed by ultracentrifugation and sucrose gradient purification to isolate closed plasma membrane vesicles (118). These particles expanded NK cells a mean 825-fold in 14 days. Others, like Johnson et al., are developing feeder-cell-free NK cell expansion. In their protocol, NK cells were expanded 387-fold in 10 days using dissolvable hydrogel microspheres functionalized with humanized anti-CD2 and anti-NKp46 Abs in media supplemented with platelet lysate, IL-2, IL-12, IL-18, and IL-21 (119).

Sequential stimulation with exogenous IL-21 followed by IL-15 or IL-2 has been shown to enhance NK cell expansion and function (120). This sequential approach synergistically combines the advantage conferred by IL-21 with necessary IL-15 or IL-2 signaling, yielding NK cells that are both abundant and highly effective. However, prolonged exposure to IL-21 alone can result in suboptimal functional characteristics. Specifically, when exogenous IL-21 was maintained in culture alongside IL-15/IL-2, it limited expansion compared to the sequential method, which involves removing IL-21 following initial stimulations of NK cell product (121).

In 2009, Cooper et al. (123) first reported that treating NK cells with IL-18, IL-12 and IL-15 creates a cytokine-induced memory-like (CIML) phenotype (122). These pre-activated NK cells have increased proliferation, persistence, cytokine secretion, and cytotoxicity following repeated stimulation, hence their "memory" phenotype. Given the functional advantage of CIML NKs, they continue to be considered in clinical trials (**Fig. 1**) (124). Notably, the cytokine pre-treatment induces sustained upregulation of IL-2Rα on CIML NK cells, forming the high-affinity heterotrimeric IL-2Rαβγ. The increased responsiveness to IL-2 is thought to be responsible for their enhanced proliferative capacity and *in vivo* persistence (124). A fusion protein complex that combines IL-12, IL-15, and IL-18 signaling has been created and efficiently generates CIML NK cells with enhanced functionality and scalability for clinical application (125).

In addition to their crucial role in NK cell expansion, IL-2 and IL-15 are essential for enhancing NK cell modification through viral vectors, playing a key role in engineered NK cell immunotherapy. In 2019, Bari et al. (129) introduced baboon retroviral envelope glycoprotein (BaEV-G)-pseudotyped lentiviral vectors for NK cells, which have since become one of the most effective and widely utilized tools for NK cell engineering (126-128). IL-2 and IL-15-mediated NK cell activation upregulates the ligands for BaEV-G, sodium-dependent neutral amino acid transporter (ASCT)-1, and ASCT-2, increasing their susceptibility to lentiviral entry and genetic modification (128,129). Currently, most NK cell transduction protocols rely on IL-2 or IL-15 pre-activation, underscoring the critical role of IL-2 and IL-15 in NK cell engineering (126,129-131). This dual function of IL-2 and IL-15, driving both NK cell proliferation and transduction, establishes the foundation for generating genetically modified NK cells equipped with CARs or other therapeutic modifications.

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EXOGENOUS IL-2 POST ADOPTIVE TRANSFER OF NK CELLS

IL-2 is frequently administered post-ACT to support NK cell activation and persistence during treatment. Most often, IL-2 is administered in rather low doses, owing to the more toxic side effects of high-dose IL-2. The maximum tolerated dose of IL-2 is around 600,000–720,000 IU/kg every 8 h until toxicity limits are reached. This treatment induces flu-like symptoms, neurologic symptoms, cytokine storm, excessive inflammation, capillary leak syndrome, organ dysfunction, elevated creatine and bilirubin, and requires intensive care unit care (2). These side effects are transient and return to baseline following withdrawal of the IL-2. Despite these toxicities, high-dose IL-2 has continued to be tested in various clinical trials, such as Parkhurst et al. (132), who treated patients with autologous NK cells and 720,000 IU/kg IL-2 in 2011.

Low-dose IL-2 regiments are usually administered in weighted (per kg) or body surface area (BSA) based (per m^2) sub-cutaneous doses and are typically administered in repeated injections over a few weeks. In a review of published clinical studies, subcutaneous IL-2 is often administered at 1E6U/m^2 a dose in the days and weeks following NK ACT. In some studies, IL-2 was administered without adjustment for weight or BSA, applying low doses ranging from 3E6U to 9E6U per day. Studies performed in mice provide evidence for the ability of exogenous IL-2 to support NK cells post-ACT. In Miller et al. (133), mice that received feeder cell-expanded NK cells without IL-2 exhibited largely undetectable levels of NK cells in the blood 7 days post-transfer, indicating poor survival in the absence of cytokine support. Conversely, mice treated with IL-2 displayed significantly higher levels of circulating NK cells, peaking at 14 days post-transfer. Notably, NK cell levels in IL-2-treated mice remained elevated compared to untreated counterparts, sustaining above baseline levels up to 28 days post-transfer. These findings underscore the critical role of exogenous IL-2 in promoting the survival, expansion, and persistence of NK cells following ACT.

Unfortunately, administration of IL-2 to patients post-NK ACT is complicated by the presence of Tregs, which themselves abundantly express the high-affinity IL-2Rα chain. Therefore, upon administration of IL-2, any Tregs that remain post-lymphodepletion may sequester the IL-2 and expand. Low-dose IL-2 expansion of Tregs has been documented in multiple NK ACT studies (10,105,106,134). When allogeneic NK cells were used to treat refractory lymphoma by Bachanova et al. (10) with subcutaneous IL-2 post-ACT, the authors found a significant expansion of Tregs in all patients. They suspected that inadequate depletion of host T cells prior to IL-2 therapy permitted expansion of residual Tregs, causing immunosuppression and inhibition of NK cell expansion. Later, the same authors showed that selectively depleting Tregs with an IL-2 diphtheria toxin fusion protein pre-ACT led to improved NK cell expansion and higher complete response rates (10). Specifically, NK cell expansion post-ACT was only observed in 10% of patients with no Treg depletion. However, 27% of patients with Treg depletion experienced NK cell expansion, demonstrating the profound limiting effect of Treg expansion on NK cell therapy. Furthermore, in a trial testing NK ACT with rituximab for non-Hodgkin's lymphoma, the authors found that responding patients had lower levels of Tregs at baseline and post-ACT (135). Tregs are characterized by their potent immunosuppressive capabilities and can be associated with poorer prognosis when infiltrated in the tumor microenvironment (136). Thus, their expansion represents a serious limitation in the application of IL-2 to support adoptively transferred NK cells. Studies have shown that Tregs use their high affinity for IL-2 to deprive NK cells of the cytokine and limit their activation

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(137,138). Moreover, Tregs are capable of secreting immunosuppressive cytokines such as TGFβ and IL-10, further inhibiting NK cell cytotoxicity within the tumor. Notably, the study by Bachanova et al. (10) reported a threefold increase in Tregs following administration of low-dose IL-2, coinciding with elevated serum levels of IL-10.

MANIPULATING THE IL-2 RECEPTOR AND ITS EXPRESSION

Several strategies have been explored to enhance IL-2 activation of NK cells while minimizing the expansion of Tregs. These include modulating IL-2R subunit expression to improve NK cell responsiveness to IL-2, creating receptor-cytokine fusion proteins and designing cytokine variants with selective binding properties that preferentially activate NK cells over Tregs. Collectively, these approaches aim to harness the therapeutic potential of IL-2 while addressing its limitations.

While priming NK cells with IL-12, IL-18, and IL-21 to generate CIML NK cells has far-reaching effects on NK cell phenotype, one discernable advantage in the context of ACT is the sustained upregulation of IL-2Rα. *In vitro*, the increased high-affinity IL-2Rαβγ expression promotes heightened proliferation and cytotoxicity in response to low-dose IL-2 (47). In a mouse model of adoptive transfer, CIML NK cells exhibited preferential expansion in response to exogenous IL-2 support. However, in a clinical study where CIML NK cells were administered to patients in conjunction with subcutaneous low-dose IL-2, CIML NK cells did not maintain IL-2Rα expression post-transfer (139). This phenomenon may result from receptor internalization following signaling events rather than being attributed to downregulation at either the transcriptional or translational level; however, this mechanism warrants further investigation. In summary, pre-activation with IL-12, IL-18, and IL-21 may improve responsiveness to exogenous low-dose IL-2 post-ACT.

Super-2, an engineered IL-2 variant with increased preference for binding to the heterodimeric IL-2Rβγ has been shown to be potent at activating NK cells without activating Treg responses (140). Furthermore, Super-2 treatment was shown to cause less pulmonary edema than IL-2 in a mouse model. Ardolino et al. (141) showed that Super-2 extended the survival of tumor-bearing mice in an endogenous NK cell-dependent mechanism. Similarly, NKTR-214 is a PEGylated variant of IL-2 that masks binding the binding region for IL-2Rα, making it IL-2Rβ/IL-2Rγ-biased (142). Studies support its role in activating endogenous NK cells while limiting Tregs. In a mouse model of T cell adoptive cell therapy, NKTR-214 improved the persistence, proliferation, and homing of transferred T cells (143). Furthermore, NKTR-214 was well tolerated in the clinic and induced tumor shrinkage while promoting Ki67 expression amongst NK cells (144). OMCP-mutIL-2, a fusion protein combining a mutated IL-2 with reduced IL-2Rα binding and OMCP, a high-affinity NKG2D ligand, was created to selectively activate NKG2D-expressing NK cells without stimulating Tregs (145). In comparison to wild-type IL-2, OMCP-mutIL-2 more potently activated NK cell expansion and tumor control in an *in vivo* model, while offering a more favorable safety profile. Ab-sumIL-2 is another reported IL-2 variant that combines a mutated IL-2 with a tumor-targeting Ab, designed to selectively deliver the cytokine to the tumor site (146). The IL-2 variant features mutations that reduce IL-2Rα binding while enhancing IL-2Rβbinding, minimizing Treg stimulation. In an *in vivo* model, sumIL-2 demonstrated potent immune activation of CD8 T cells without inducing pulmonary edema; however, it was not directly compared to wild-type IL-2 in the study. Notably, NK cells were dispensable for the

therapeutic efficacy of anti-Erb-sumIL-2 in a B16 tumor model, suggesting either a lack of NK cell responsiveness in this specific context or an inherent limitation of the IL-2 variant in engaging NK cells. Future studies should assess whether NK cell unresponsiveness is model-dependent or if further optimization of the IL-2 variant could enhance NK cell activation and contribute to therapeutic efficacy. In summary, IL-2 cytokine variants represent potential NK exogenous activators that minimize Treg activation and toxicity following ACT.

Receptor engineering may also ameliorate IL-2 signaling for NK ACT. For instance, IL-2 tethered directly to the IL-2Rβ subunit bypasses the requirement for IL-2Rα and has been explored to enhance NK cell activation (147). When this fusion protein was transduced into NK92 cells, it promoted proliferation without exogenous IL-2 and conferred increased antitumor cytotoxicity and resistance to TGF-β, IL-10, and IL-4. Similarly, NK92 transduced with a membrane-bound IL-2 by fusing IL-2 to IL-2Rα with a linker supports NK-92 cell survival and proliferation in the absence of exogenous IL-2 but also enhances *in vitro* and *in vivo* antitumor activity (148).

NK cell engagers are engineered immunotherapeutic molecules designed to bridge NK cells to tumor cells, facilitating immune synapse formation and promoting targeted cytotoxicity. By promoting receptor engagement, these engagers enhance endogenous NK cell anti-tumor responses but also hold promise as adjuncts to NK ACT by potentiating the anti-tumor activity of infused NK cells. Developing tetraspecific Ab-based NK cell engager therapeutics (ANKETs) represents an interesting technological advancement in harnessing NK cells for cancer immunotherapy (149). ANKETs incorporate a modified IL-2 variant engineered to bypass IL-2Rα binding, thereby reducing Treg activation and systemic toxicity, along with Ab fragments that target NK cell receptors (NKp46, CD16a) and tumor-associated Ags. This platform enhances endogenous NK cell activation and cytotoxicity through cooperative receptor engagement and selective IL-2 signaling. In non-human primates, a CD20 targeting tetraspecific ANKET led to $CD20^+$ B cell depletion with no sign of toxicity. These findings underscore the translational potential of ANKETs as a next-generation immunotherapy, which could also be investigated as a complementary tool for adoptive NK cell therapy.

Another interesting strategy that was investigated by Kagoya et al. (150) involved a novel CAR construct which included a truncated IL-2Rβ chain and a STAT3-binding motif in addition to the conventional CD3ζ and co-stimulatory CD28 domains. This unique design enabled the CAR-T cells to activate the JAK-STAT signaling pathway upon Ag engagement, promoting robust T cell proliferation, persistence, and effector functions. To the best of our knowledge, CARs containing IL-2R signaling domains have yet to be tested in NK cells.

Constitutively active STAT5 (CASTAT5) has been investigated for its potential to enhance T cell function in immunotherapy (151). Engineering CASTAT5 into CD19 CAR T cells significantly boosted cytokine production, improved persistence, enhanced antitumor efficacy, and reduced exhaustion in a preclinical mouse model, demonstrating its promise in overcoming key limitations of CAR T cell therapy. However, we failed to find any examples of CASTAT5 use in NK cell-based cancer immunotherapy studies. If tested in NK cells, it may help circumvent limitations of extracellular ligand-receptor interactions, keeping NK cells highly active without activating Tregs.

TRANSITIONING TO IL-15 FOR NK CELL THERAPY

Due to the significant overlap in signaling pathways between IL-2 and IL-15, some research groups have shifted from IL-2 to IL-15 for stimulating NK cells during adoptive cell therapy. This transition addresses limitations associated with IL-2, particularly its ability to inadvertently drive Treg proliferation. In contrast, IL-15's mechanism sidesteps this issue, selectively engaging NK cells without promoting Treg expansion. Furthermore, some evidence argues that IL-15 may promote enhanced proliferation and cytotoxicity compared to NK cells stimulated with IL-2.

Following adoptive cell transfer, NK cells activated with cytokines in culture will experience cytokine withdrawal, as the cytokine concentration in their post-ACT environment will be limited or absent compared to conditions during dose manufacturing. *Ex vivo* IL-15 activation has been shown to provide cytotoxicity and survival advantages in the withdrawal phase compared to IL-2 (90). IL-15 stimulation upregulated genes involved with mitochondrial function and cell cycle progression compared to IL-2-stimulated NK cells. This corresponded with a higher basal and maximal cellular respiratory function of IL-15-stimulated NK cells than those treated with IL-2. Mechanistically, IL-15 more robustly activates mTOR signaling compared with IL-2. The functional advantages conferred by IL-15 could be abrogated by inhibiting mTOR. Therefore, this study highlighted the potential benefit of increased activation of the IL-15-mTOR axis in the post-ACT setting.

In the landmark 2005 trial led by Miller et al. (5), allogeneic NK cells were administered following lymphodepletion, which created a surplus of endogenous IL-15 that supported NK cell persistence. The study proposed a mechanism where robust lymphodepletion induced lymphopenia, reducing competition for IL-15 and increasing its availability to NK cells. In contrast, patients who received less extensive lymphodepletion exhibited limited IL-15 surpluses, resulting in suboptimal NK cell expansion. This trial is frequently cited as foundational evidence for the critical role of IL-15 in enhancing NK cell persistence and proliferation post-ACT. Later, Miller et al. (133) demonstrated that IL-15 is far superior to IL-2, driving a significant improvement in expansion post-ACT in a mouse model. Specifically, mice receiving feeder cell expanded NK received 6 doses of IL-2 or IL-15 post-adoptive NK cell transfer. When measured 14 days post-ACT, mice receiving IL-15 had 3-fold more NK cells measured in peripheral blood compared to mice receiving IL-2.

Later, another study led by Miller first used exogenous IL-15 post-NK cell transfer to support transferred NK cells (152). The authors reported that "rhIL-15 induced better rates of *in vivo* NK-cell expansion and remission compared with previous trials with IL-2, but it was associated with previously unreported CRS after (subcutaneous) but not IV dosing." Specifically, intravenous IL-15 supported NK cells that achieved remissions in 32% of patients, while subcutaneous IL-15 achieved a slightly higher remission rate of 40%. However, subcutaneous IL-15 caused CRS in 56% of patients and was associated with neurotoxicity. These side effects, particularly with subcutaneous IL-15, were attributed to slower cytokine clearance. This led to prolonged IL-15 exposure and heightened systemic inflammation. Elevated IL-6 levels, linked to the subcutaneous administration route, were identified as a key driver of CRS. Therefore, while exogenous IL-15 effectively supports NK cells post-ACT, the route of administration requires careful consideration to balance efficacy and toxicity.

Recently, a clinical study directly compared IL-2 and N-803, a long-acting IL-15R agonist, for cytokine support in NK ACT (153). Interestingly, treatment with N-803 was associated with a significant reduction in clinical response rates compared to IL-2. Further analysis revealed that while N-803 supported greater NK cell expansion during the early post-infusion period (days 7–14), the persistence of NK cells was markedly reduced relative to IL-2 support. Mass cytometry confirmed that the NK cells from both treatment groups exhibited comparable phenotypes, suggesting that differences in persistence were not due to inherent NK cell characteristics. Crucially, N-803 was found to drive robust recipient T-cell expansion and activation. This T-cell activation, particularly of CD8 T cells, was associated with allo-rejection of the infused NK cells, thereby limiting their persistence and therapeutic efficacy. These findings highlight a dual effect of exogenous IL-15: promoting NK cell expansion while simultaneously activating endogenous T cells, which creates a competitive immune environment that impairs the persistence of adoptively transferred NK cells.

To optimize IL-15's therapeutic potential for NK cell-specific activation, researchers have taken to engineering the expression of IL-15 variants in NK cells. Transducing NK cells with the protein-coding sequence of IL-15 forces the expression of soluble IL-15 (sbIL-15). In a mouse model, Liu et al. (154) showed that the CD19 CAR NK cells engineered to express sbIL15 significantly extended survival and more effectively controlled tumor progression compared to CAR NK cells lacking IL-15 expression. Although the administration of exogenous IL-15 augmented the response of CD19 CAR NK cells relative to CAR NK cells alone, it was associated with increased toxicity and treatment-related mortality, highlighting the advantage of integrating IL-15 expression directly into NK cells. In a clinical trial, the Rezvani group administered CD19-CAR NK cells expressing sbIL-15 (8,155). Astonishingly, they showed persistence of IL-15 expressing CAR NK for over a year in patients and achieved objective responses in 68% of patients at 30 days and 32% at 100 days. Nonetheless, it is imperative to monitor the production of sbIL-15 by NK cells, as in one model, sbIL-15 NK cells elevated serum IL-15 concentration and correlated with treatment-induced mortality (156). However, most other preclinical studies using sbIL-15 in NK cells demonstrate improved efficacy without toxicity.

mbIL-15 variants have been generated by fusing the cytokine with a linker and transmembrane domain. For example, Imamura et al. (157) fused IL-15 to the transmembrane domain and signaling peptide of CD8α, ensuring its delivery and anchoring to the plasma membrane where it is available for IL-15R binding. Other groups have created an IL-15/IL-15R fusion protein to drive autonomous IL-15 signaling (158). Using mbIL-15 could rescue NK cell dysfunction following tumor cell engagement by enhancing their metabolic fitness, maintaining functional phenotypes, and delaying the onset of exhaustion (159). Trials using CAR NKs manufactured from iPSCs have applied mbIL-15 (158,160,161). Importantly, these types of NK cell-bound IL-15 strategies may limit toxicity and endogenous T cell expansion observed in studies using exogenous or NK cell-expressing sbIL-15 (153).

Caution should be exercised when using IL-15 to stimulate NK cells, as prolonged exposure to this cytokine can paradoxically impair their functionality through a process of exhaustion. A study by Felices et al. (85) demonstrated that continuous IL-15 treatment, while initially promoting NK cell proliferation, ultimately leads to decreased viability, diminished cytotoxicity, and reduced cytokine production. These effects are driven by metabolic dysregulation, particularly a defect in fatty acid oxidation and mitochondrial respiration. Moreover, continuous IL-15 exposure altered cell cycle gene expression, inducing checkpoint

arrest and increasing susceptibility to activation-induced cell death. Intermittent IL-15 dosing preserved NK cell functionality, suggesting that dosing strategies are crucial for optimizing therapeutic outcomes. This research highlights the need for precise modulation of IL-15 signaling in NK cell therapies, either through adjusted dosing regimens or adjunctive treatments.

Recently, a direct comparison was made between NK cells engineered to express IL-15 and IL-21 in a mouse patient-derived xenograft model testing NK ACT against glioblastoma (162). Specifically, mice with established glioblastoma that received an intertumoral injection of IL-21-expressing NK cells exhibited sustained tumor control and long-term survival. In contrast, mice injected with IL-15-expressing NK cells exhibited poor tumor control and treatment-related toxicity. Adverse effects were apparent even at minimal NK cell dosages or with NK cells exhibiting lower IL-15 transduction efficiency. The authors postulated that the treatment-related toxicity was due to high IL-15Rα expression in the brain. Co-culture experiments revealed that IL-15-expressing NK cells failed to maintain their responsiveness to repeated glioblastoma cell challenges, potentially indicating the onset of NK cell exhaustion driven by sustained IL-15 signaling, as suggested by prior research (85).

TARGETING NEGATIVE REGULATORS OF IL-2 AND IL-15 SIGNALING

In ACT, the potent anti-tumor activity of NK cells is often limited by intrinsic and extrinsic mechanisms of immune suppression. While the cytokines IL-2 and IL-15 are critical for NK cell proliferation, survival, and cytotoxicity, their signaling pathways are tightly regulated by negative feedback mechanisms. One key intracellular regulator is the CIS, encoded by the CISH gene. CIS and other members of the SOCS family act as intracellular checkpoints, attenuating signalling downstream of the IL-2 and IL-15 receptors, thereby limiting NK cell activation. To date, there have been no published clinical study results utilizing knockout of CIS or SOCS in NK cells for ACT; however, preclinical studies exist.

Deletion of the CISH gene has emerged as a promising strategy to enhance NK cell functionality. The development of a conditional NK cell CISH knockout created an advantageous NK phenotype (25,87). Specifically, these cells demonstrated enhanced proliferation in response to IL-15 and IL-2 stimulation, increased cytotoxicity, and improved tumor control, as evidenced by reduced metastasis following intravenous injection of cancer cells as well as suppressed growth of tumors implanted in the mammary fat pad. Moreover, CISH-deficient NK cells exhibit increased tumor infiltration and reduced expression of the inhibitory receptor TIGIT, supporting their enhanced anti-tumor activity. Zhu et al. (88) developed CISH knockout NK cells using an iPSC-derived platform. These CISH knockout NK cells demonstrated heightened IL-15-mediated JAK-STAT signaling, improved expansion, and cytotoxicity. Promisingly, CISH-deficient iPSC-derived NK cells demonstrate significantly enhanced *in vivo* persistence and greater inhibition of tumor progression in a leukemia xenograft model. Furthermore, CISH knockout improved the metabolic fitness of iPSC NK cells, characterized by increased glycolytic capacity and mitochondrial respiration via mTOR signaling. Recently, Nakazawa et al. (163) performed CRISPR-Cas9 knock-out of CISH in primary NK cells and found enhanced killing of allogeneic glioblastoma cells in a mouse xenograft model. Daher et al. (164) combined CISH knockout with CAR engineering. Specifically, cord blood-derived NK cells were modified to express an IL-15-secreting CAR

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construct targeting CD19. CRISPR-Cas9-mediated CISH knockout boosted Akt/mTORC1 and c-MYC signaling, increasing aerobic glycolysis and improving metabolic fitness. In an ACT experiment using an aggressive NK-resistant lymphoma xenograft model, mice treated with CISH-deficient IL-15-secreting CAR-NK cells exhibited complete tumor eradication, whereas mice receiving non-edited CAR-NK cells showed only partial tumor regression.

Deletion of SOCS1, another critical member of this family, has also been explored as a strategy to enhance NK cell functionality for ACT (165). In an abstract from Chen et al. (165), SOCS1 knockout NK cells were generated and co-engineered with a CD19-targeting CAR and an IL-15-secreting construct. SOCS1 knockout CAR-NK cells exhibited increased production of IFN-γ and TNF-α, greater degranulation, and improved cytotoxicity against CD19-positive lymphoma cells compared to control CAR-NK cells. They also exhibited faster growth rates than non-edited controls. These preliminary findings highlight SOCS1 as a promising target for genetic modification to boost NK cell functionality in CAR-based ACT settings.

In 2021, PRDM1 was identified as a transcriptional repressor of the IL2RA gene (19). Ectopic expression of PRDM1 forces the downregulation of IL-2Rα mRNA and protein, which limits low-dose IL-2 expansion in an NK cell line. By engineering increased expression of IL-2Rα, NK cells demonstrated a growth advantage in low-dose IL-2 when competing with cells expressing standard IL-2Rα levels. These findings suggest that IL-2Rα overexpression or PRDM1 knockout could represent a novel axis for modulating NK cell functionality and expansion in ACT.

PERSPECTIVES

IL-2 signaling has a significant history of application in NK ACT. Crucially, it is a potent *ex vivo* stimulator of NK cells, especially in conjunction with feeder cell expansion pre-ACT. Subcutaneous IL-2 is frequently administered in clinical trials post-NK cell transfer to support the persistence and function of transferred NK cells. However, there remains a significant gap in clinical research directly comparing the effects of exogenous IL-2 versus its omission, as well as studies optimizing its dosing to enhance NK cell efficacy. Unfortunately, low-dose IL-2 administration post-NK cell transfer carries the risk of expanding Tregs, underscoring the need for strategies to mitigate Treg activation in this context.

In line with this understanding, researchers are investigating the utilization of exogenous IL-15 or genetic engineering of IL-15 signaling in NK cells for support post-NK cell transfer. While IL-15 treatment has shown promise in promoting robust NK cell expansion and function, recent data highlights potential challenges, including toxicity and the expansion of allo-reactive T cells, which can impair the persistence of transferred NK cells and reduce therapeutic efficacy. To address these issues, multiple groups are investigating mbIL-15 to restrict IL-15 signaling to transferred NK cells, minimizing systemic side effects. However, prolonged IL-15 exposure poses the risk of inducing NK cell exhaustion, thereby limiting their functionality post-NK cell transfer. Notably, the mb-IL-15 strategy demonstrated promising outcomes in the MD Anderson phase I/II trial of CD19-targeting CAR NK cells, achieving high response rates with persistence observed for over a year (8).

There is substantial evidence demonstrating that the IL-2 and IL-15 signaling cascades have immense potential to modulate NK cell activity for therapeutic purposes. However, further

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research is needed to better understand their effects and side effects when utilized post-NK cell transfer to support NK cells. Ongoing efforts to manipulate and engineer this axis, such as receptor engineering and CRISPR mediated knockout of regulatory genes in NK cells, will be critical for maximizing their therapeutic efficacy while minimizing associated risks.

Of course, optimizing *ex vivo* cytokine activation and post-NK cell transfer cytokine support are just two strategic moves in the larger and more complex chess game against tumors. Future research must prioritize discovering ways to sensitize tumors to immune-mediated destruction, refine the tumor microenvironment to better support ACT, further enhance the functional phenotype, persistence, and durability of NK cells, and optimize combination treatments. The remarkable progress made in this field over just a few decades underscores the potential of NK cell therapy, but there is still a long journey ahead. With every innovation, we edge closer to unlocking the full therapeutic potential of NK cells. The urgency is clear as countless current and future patients are depending on these advancements to turn experimental treatments into accessible and effective lifesaving therapies.

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