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ARTICLE

Differential IL-12 signaling induces human natural killer cell activating receptor-mediated ligand-specific expansion

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IL-12 is an essential cytokine involved in the generation of memory or memory-like NK cells. Mouse cytomegalovirus infection triggers NK receptor-induced, ligand-specific IL-12–dependent NK cell expansion, yet specific IL-12 stimulation ex vivo leading to NK cell proliferation and expansion is not established. Here, we show that IL-12 alone can sustain human primary NK cell survival without providing IL-2 or IL-15 but was insufficient to promote human NK cell proliferation. IL-12 signaling analysis revealed STAT5 phosphorylation and weak mTOR activation, which was enhanced by activating NK receptor upregulation and crosslinking leading to STAT5-dependent, rapamycin-sensitive, or TGFβ-sensitive NK cell IL-12–dependent expansion, independently of IL-12 receptor upregulation. Prolonged IL-2 culture did not impair IL-12–dependent ligand-specific NK cell expansion. These findings demonstrate that activating NK receptor stimulation promotes differential IL-12 signaling, leading to human NK cell expansion, and suggest adopting strategies to provide IL-12 signaling in vivo for ligand-specific IL-2–primed NK cell–based therapies.

Introduction

IL-12 is a heterodimeric pro-inflammatory cytokine composed of p35 (IL-12A) and p40 (IL-12B) subunits that form the active p70 cytokine (Tait Wojno et al., 2019). IL-12 is secreted by myeloid cells during pathogen recognition to establish T helper 1 cell responses. The IL-12 receptor is composed of two receptor chains, IL-12Rβ1 and IL-12Rβ2, and leads to STAT4 signaling to induce IFNγ secretion by natural killer (NK) cells and T cells (Vignali and Kuchroo, 2012). Co-administration of IL-12 and IL-18 to mice induces NK cell activation and can lead to a fatal inflammatory response (Carson et al., 2000), yet IL-12 promotes tumor rejection (Ohs et al., 2016; Rademacher et al., 2021). However, IL-12 treatment is limited due to a narrow therapeutical window, short half-life, and high toxicity, restricting the clinical use of high IL-12 concentrations, leaving room for innovation (Tugues et al., 2015; Glassman et al., 2021; Propper and Balkwill, 2022; Nguyen et al., 2020).

NK cells are innate lymphocytes with cancer and viral immunosurveillance capabilities (Cerwenka and Lanier, 2016). IL-12 is a crucial positive regulator of mouse Ly49H$^+$ NK cell memory generation and expansion by Ly49H ligand-specific recognition of mouse CMV (MCMV) during infections (Sun et al., 2012). IL-2, IL-15, IL-18, or IFNα are reported to increase IL-12 sensitivity in NK cells by upregulating the IL-12 receptors, increasing STAT4 signaling, and sustaining NK cell survival (Wang et al., 2000; Madera et al., 2016; Carson et al., 2000; Wu et al., 2000; Wiedemann et al., 2021). IL-12 is also essential for the in vitro generation of cytokine-induced memory-like NK cells by IL-18 and IL-15 co-stimulation (Ni et al., 2012; Cooper et al., 2009; Romee et al., 2016). CD25 upregulation on human or mouse NK cells during IL-12 stimulation increases NK cell sensitivity to IL-2, which is known to promote a robust NK cell expansion and is used to sustain NK cell persistence in cancer patients (Ni et al., 2012; Leong et al., 2014; Pomeroy et al., 2020; Liu et al., 2021), and is essential for mouse Ly49H$^+$ or human NKG2C$^+$ memory NK cell expansion during Ly49H or NKG2C ligand recognition, respectively (Wiedemann et al., 2020; Rölle et al., 2014, Rölle et al., 2018b). In contrast, in *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice MCMV infection or exogeneous IL-12 administration drives NK cell expansion and lymphopoiesis in the absence of IL-2 or IL-15 (Sun et al., 2009; Ohs et al., 2016). However, prolonged

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IL-12 co-stimulation with IL-2, IL-15, or IL-18 inhibits or has a limited influence on NK cell proliferation and can induce NK cell apoptosis (Marçais et al., 2014; Wiedemann et al., 2021; Huang et al., 2011; Oka et al., 2020). Ex vivo expansion by IL-12 of mouse Ly49H$^+$ memory NK cells during Ly49H-ligand recognition or by other activating receptors is not reported and has not been fully addressed in human primary NK cells.

Given the re-emergence of the therapeutic use of IL-12 in oncology (Propper and Balkwill, 2022), here we examined the ability of IL-12 to mediate human NK cell proliferation and expansion without concomitant IL-2 or IL-15 stimulation. In line with previous publications, human NK cells exhibit high sensitivity to low IL-12 concentrations leading to NK cell activation and IFNγ secretion. We discovered that IL-12 sustained primary human NK cell viability without providing IL-2, IL-15, IL-18, or IFNα, and independently of Bcl2 expression while leading to STAT5 phosphorylation. However, IL-12 alone did not mediate robust primary NK cell proliferation. We found that activating NK receptor stimulation, and not the increased expression of the IL-12 receptors, is essential for IL-12–mediated NK cells expansion as demonstrated by IL-2–primed NK cells and by engineering of primary NK cells with a chimeric cytokine receptor (CCR). We discovered that IL-2/IL-15 priming-dependent upregulation of activating NK receptors and their cross-linking synergistically mediate IL-12–dependent proliferation, which is sensitive to STAT5 or mammalian target of rapamycin (mTOR) inhibition and suppressed by TGFβ. Thus, activating NK receptor stimulation orchestrates differential IL-12 signaling to promote ligand-specific proliferation and expansion of human NK cells previously reported in memory NK cells.

Results

Low IL-12 concentrations lead to human primary NK cell activation without expansion

Our objective was to evaluate the ability of IL-12 to mediate human NK cell proliferation and expansion since IL-12 is essential for mouse NK cell expansion and memory NK cell generation during MCMV infection (Sun et al., 2012). In line with the mouse model, phenotypic changes in human NK cells are associated with human CMV (HCMV) infection and elevated IL-15 levels in vivo (Pickering et al., 2021). Further analysis of IL-12 concentrations in the plasma of kidney transplant patients with reported NK cell expansion showed evidence for a long-term increase of the IL-12p40/IL-12p70 ratio in CMV PCR+ individuals, suggesting a contribution of these low IL-12 levels to NK cell expansion (Fig. 1 A; Pickering et al., 2021; Piazzolla et al., 2001; Ethuin et al., 2003). Consequently, we evaluated the efficacy of IL-12 to mediate human NK cell expansion directly by ex vivo stimulation of NK92 cells or purified primary NK cells with titrated amounts of IL-12 (Romee et al., 2012). Tracing the number of NK92 cells indicated that an IL-12 concentration of 2.5 ng/ml (IL-12^{low}) was sufficient to promote a modest NK92 cell expansion relative to IL-2 (Fig. 1 B). However, purified primary NK cells, isolated by negative selection to avoid nonspecific stimulation, did not expand during high or low IL-12 (25 or 2.5 ng/ml) stimulation and relative to IL-2 used as a positive control (Fig. 1 C). Note that NK cells were purified from healthy donors without mature adaptive (memory), FcRγ$^{-/low}$ NK cells, which are reported to experience reduced IL-12 sensitivity (Schlums et al., 2015). Additionally, IL-12 did not mediate primary NK cell expansion following IL-2 priming, reported to increase IL-12 receptor expression (Fig. 1 C; Wang et al., 2000; Glassman et al., 2021). However, IL-12^{low} alone was sufficient to upregulate IL-18 receptors (Fig. 1 D) and induce IFNγ in fresh and IL-2–primed NK cells (Fig. 3 A and Fig. S2 A) indicating these levels of IL-12 can activate IL-2–primed human primary NK cells without promoting cell expansion.

Low concentrations of IL-12 mediate NK cell survival, but not NK cell proliferation

IL-15 is essential for NK cell survival and homeostasis in mice but not in humans (Wang and Zhao, 2021; Lebrec et al., 2013). Either IL-15 or IL-2 stimulation increases human NK cells' sensitivity to IL-12 (Lebrec et al., 2013; Wang et al., 2000). To evaluate the contribution of IL-2/IL-15 priming on IL-12–mediated proliferation, we stimulated ex vivo human primary NK cells with IL-12^{low} concentrations and IL-2^{low} or IL-15^{low} concentrations (3 U/ml or 1 ng/ml, respectively). As homotypic interactions between NK cells were reported to contribute to IL-2 sensitivity, we cultured the cells in U-shaped 96-well plates (Kim et al., 2014). We used flow cytometry to evaluate cell proliferation by cell trace violet (CTV) dilution and cell viability by fixable near-infrared viability dye (Tempany et al., 2018). IL-2^{low} or IL-15^{low} concentrations sustained NK cell viability (Fig. 2 A.i) with minimal influence on cell proliferation (Fig. 2 A.ii). A significant reduction in NK cell viability was detected on days 4 and 6 of cell culture without cytokine stimulation (Fig. 2 A.i). Further, high IL-2 or IL-15 concentrations slightly reduced NK cell viability during NK cell proliferation and increased NK cell numbers, possibly due to increased cell activation or media consumption (Fig. 2, A.i and ii; and Fig. S1 A; Huang et al., 2011). IL-12^{low} and IL-2^{low} or IL-15^{low} co-stimulation induced minimal cell proliferation with some donor-specific variability (Fig. 2 B.i). However, IL-12^{low} alone significantly sustained NK cell viability relative to media without cytokines and decreased NK cell viability during co-stimulation with IL-2^{low} or IL-15^{low} or higher IL-2 or IL-15 concentrations (Fig. 2 B.i and Fig. S1 A; Huang et al., 2011). Accordingly, IL-12 stimulation showed higher levels of the anti-apoptotic proteins Mcl1 and pBad^{S112} expression, but not Bcl-X$_L$ or Bcl2 (Fig. S1 B; Kale et al., 2018). IL-12^{low} and IL-18 or IFNα co-stimulation did not promote robust human NK cell proliferation (Fig. S1 C; Madera et al., 2016; Carson et al., 2000; Wiedemann et al., 2021). However, IL-18 did enhance IL-2^{low}–mediated cell proliferation, which was inhibited by IL-12^{low}, possibly due to an increase in p21 expression as was previously reported (Fig. S1 C.i; Huang et al., 2011; Oka et al., 2020). These observations indicate that low IL-12 concentrations can sustain NK cell viability but have a limited ability to initiate NK cell proliferation even during co-stimulation by other cytokines reported to increase NK cell sensitivity to IL-12.

Low IL-12 amounts synergize with IL-2 or IL-15 and NKp30 stimulation to induce NK cell proliferation

Myeloid cells secrete IL-12 and mediate NK cell priming by IL-15 (Long, 2007). NKp30 and DNAM1 mediate NK cell–dendritic cell crosstalk, while ex vivo mass cytometry profiling of cytokine-induced memory-like NK cells showed high NKp30 levels

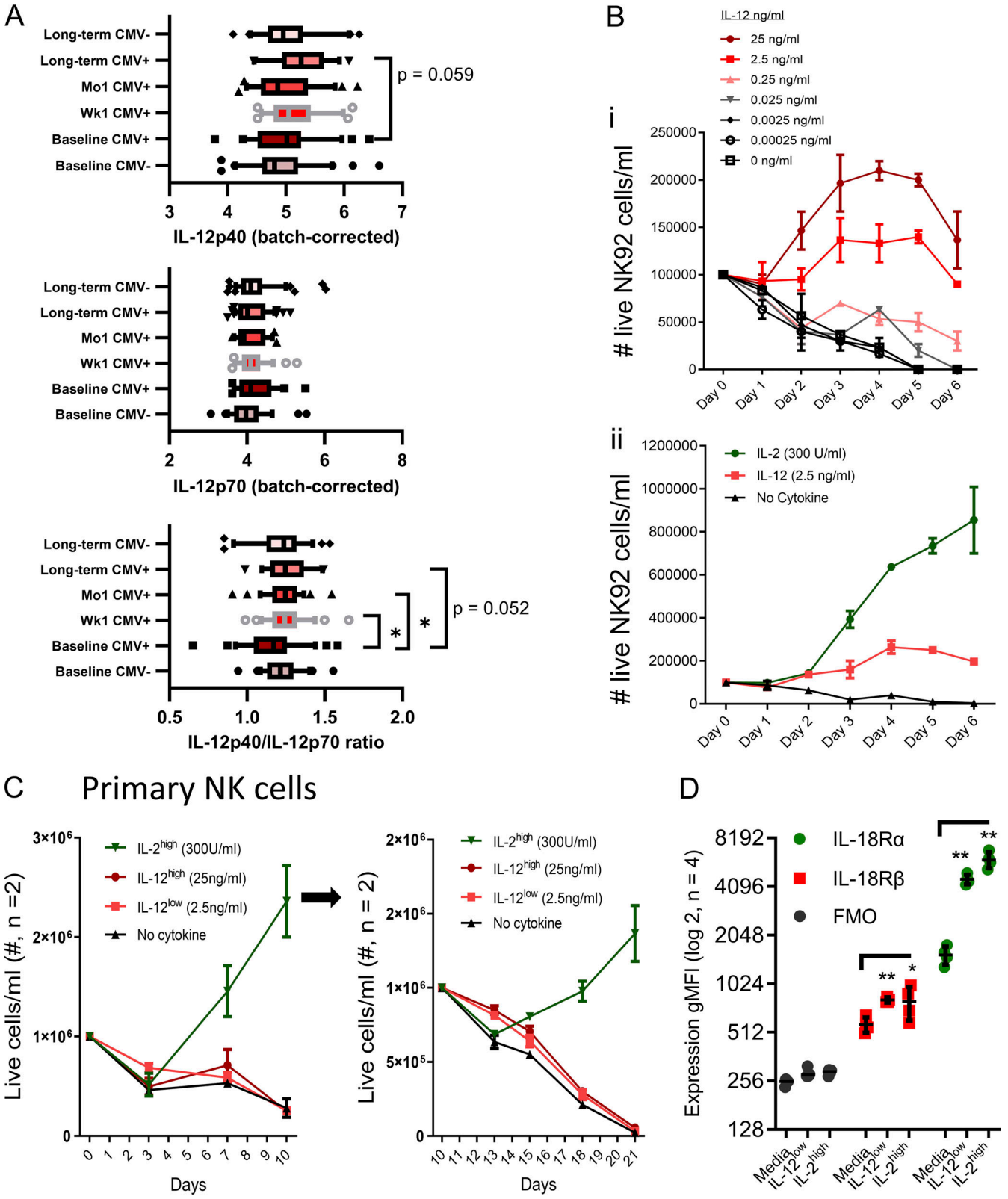

Figure 1. **Human NK cells are responsive to low IL-12 concentrations. (A)** IL-12p40 or IL-12p70 levels (batch-corrected) in plasma samples from HCMV-negative or -positive kidney transplant patients. The concentration range of both subunits is 0–64 pg/ml. Plasma was collected before the detection of viremia (baseline), at the time of viremia detection (confirmed by PCR), at 1 wk, 1 mo, or long-term (>6 mo) after HCMV infection or re-activation. Left, mean + 95% IC; right, box and whiskers 10–90th percentiles. Non-viremic transplant patients were sampled at baseline and long-term time points (CMV-positive $N = 121$; non-viremic patients $n = 33$). Unpaired t test, nonparametric, one-tail; *, $P < 0.05$. **(B)** Expansion assay. **(B.i)** Upper panel: NK92 cells were cultured in 24-well plates with decreasing IL-12 concentrations (without IL-2). **(B.ii)** Cell expansion of IL-12^{low} (2.5 ng/ml) relative to IL-2^{high} (300 U/ml). Live NK92 cell numbers were

measured by cell counting and trypan blue staining exclusion (mean ± SEM; ≥2 independent experiments). **(C)** Expansion assay. Ex vivo primary NK cells (left) or 10-d IL-2–primed NK cells (right) were stimulated with the indicated cytokine concentrations in 24-well plates. Live NK cell numbers were measured by cell counting and trypan blue staining exclusion. Mean ± SD. *n* = 2 donors (two independent experiments). **(D)** IL-18 receptor subunit (IL-18Rα and IL-18Rβ) expression on 10-d IL-2–primed NK cells re-stimulated with IL-2^{high} (300 U/ml), IL-12^{low} (2.5 ng/ml), or media without cytokines for 3 d. Mean ± SD. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01. *n* = 4 donors (two independent experiments).

(Walwyn-Brown et al., 2018; Foltz et al., 2019). Further, NKp30 ligand, B7-H6, is upregulated on pro-inflammatory monocytes (Matta et al., 2013; Trabanelli et al., 2017). Therefore, we stimulated ex vivo NK cells with IL-12^{low} with or without IL-2^{low} or IL-15^{low} and with agonist anti-NKp30–coated beads to provide prolonged NK receptor activation (Fig. 2 B.ii; Rölle et al., 2018b). NKp30 co-stimulation with IL-12^{low} and IL-2^{low} or IL-15^{low} induced robust NK cell proliferation relative to mouse IgG1 (mIgG1) control-coated beads (Fig. 2 B.ii). Anti-DNAM1–coated beads did not induce or increase NK cell proliferation during IL-12^{low} stimulation (Fig. S1 D), indicating NKp30 co-stimulation synergizes with IL-12^{low} and IL-2^{low} or IL-15^{low} to promote robust NK cell proliferation. High IL-18 concentrations significantly enhanced NK cell proliferation by IL-12 and NKp30 co-stimulation (Fig. S1 C.i), while IFNα had no influence (Fig. S1 C.ii). Note that in the presence of IL-2^{low} and NKp30, low amounts of IL-18 were sufficient to enhance cell proliferation (Fig. S1, C.i and iii), while IFNα inhibited NK cell proliferation in

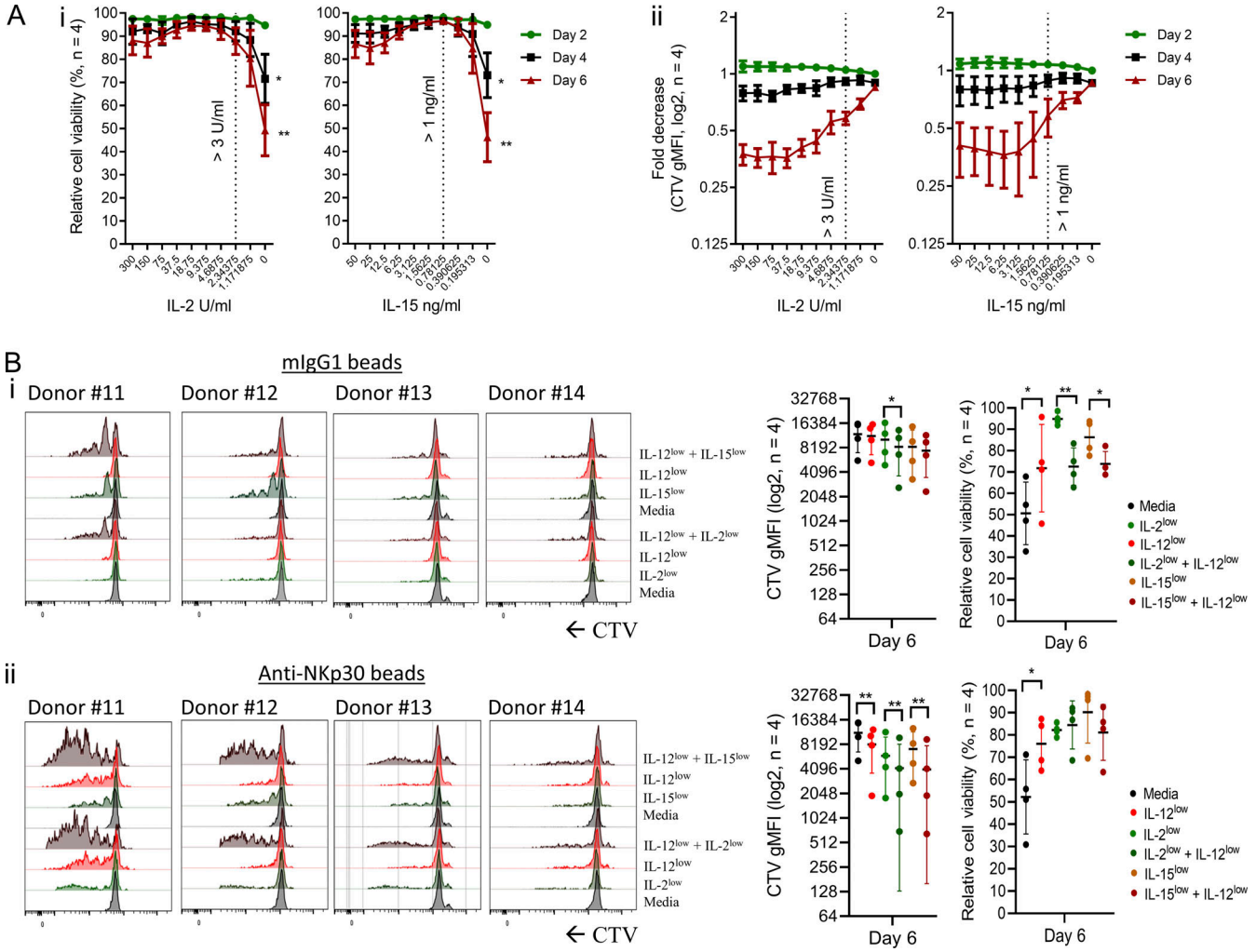

Figure 2. **Low IL-12 concentrations sustained human NK cell survival and induced NK cell proliferation during IL-2 or IL-15 and NKp30 co-stimulation.** Purified human peripheral blood NK cells were stimulated for 6 d. Cytokines were supplemented at the beginning of the cell culture in U-shaped 96-well plates. *n* = number of donors. Mean ± SD. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01. IL-12^{low} = 2.5 ng/ml, IL-2^{low} = 3 U/ml, and IL-15^{low} = 1 ng/ml. **(A)** Purified human peripheral blood NK cells were stimulated with increasing concentrations of IL-2 (U/ml) or IL-15 (ng/ml), cell proliferation was measured using cell trace violet dilution, and cell viability was measured by fixable near-infrared viability dye. *n* = number of donors. Mean ± SD (two independent experiments). **(B)** Proliferation (CTV-dilution) assay induced by IL-12^{low} (2.5 ng/ml), IL-2^{low} (3 U/ml), IL-15^{low} (1 ng/ml), and mIgG1 isotype-matched control-coated (B.i), or anti-NKp30–coated beads (B.ii). Left, CTV histograms; middle, CTV geometric MFI (gMFI); right, relative cell viability (>2 independent experiments).

the presence of IL-2 (Fig. S1, C.ii and iii), suggesting differential signaling of IL-18 and IFNα. These results demonstrate that NKp30 stimulation synergizes with low-dose IL-12 and IL-2 or IL-15 to promote NK cell proliferation.

Activating NK receptor stimulation increases early STAT phosphorylation by IL-12

STAT4, STAT5, and mTOR are essential for NK cell proliferation induced by IL-12, IL-2, or IL-15 (Sun et al., 2012; Wang and Zhao, 2021). To further examine the mechanisms involved in the contribution of IL-12 to support the proliferation of NK cells ex vivo, we measured mTOR activation by $pS6^{S235/235}$ levels and $pSTAT4^{Y693}$ or $pSTAT5^{Y694}$ levels at 48 h after stimulation before NK cell proliferation can be detected (Fig. 3 A; Marçais et al., 2014; Wang and Zhao, 2021). IFNγ secretion was used as a positive control (Fig. 3 A). STAT4 and STAT5 phosphorylation was increased during IL-12^{low} stimulation and by NKp30 co-stimulation. mTOR activity was significantly enhanced by IL-12^{low} and further enhanced during IL-2^{low} co-stimulation, as expected. IL-2^{low} stimulation significantly increased $pSTAT4^{Y693}$ (Fig. 3 A). Notably, prolonged IL-2^{low} stimulation was sufficient to increase the expression of IL-12Rβ2 and NKp30 (Fig. 3 B; Wang et al., 2000; Wu et al., 2000). These results suggested that the synergy between NKp30, IL-2, and IL-12 leading to robust NK cell proliferation might be due to an upregulation of IL-12 receptor chains by IL-2 and NKp30 stimulation or by upregulation of IL-2 receptor α (CD25) or IL-15 receptor α (CD215; Rölle et al., 2014) by IL-12 and NKp30 stimulation, and/or due to the upregulation of NKp30 by IL-2 and IL-12 stimulation.

NKp30 upregulation orchestrates differential IL-12 signaling to promote NK cell expansion

We examined whether the increase in the IL-12 receptor levels or NKp30 levels by short-term high-dose IL-2 priming (IL-2^{high}; 300 U/ml) can lead to NK cell proliferation by IL-12^{low} without concomitant IL-2 or IL-15 stimulation (Fig. 3 B). Hence, we cultured NK cells for 3 d with IL-2^{high} to upregulate the IL-12 receptors and NKp30. IL-2 was washed out before restimulation with IL-12^{low} with or without anti-NKp30– or mIgG1-coated beads (Fig. 4 A). IL-12^{low} or NKp30 stimulation alone did not promote robust NK cell proliferation, yet NKp30 and IL-12^{low} co-stimulation induced robust proliferation (Fig. 4 B). The increase in NK cell proliferation was not due to IL-2 or IL-15 production by NK cells (Fig. S2 A). These data indicate that increased NKp30 levels are likely required to promote robust cell proliferation of IL-2–primed NK cells by IL-12 alone without providing concomitant IL-2 or IL-15 stimulation. This observation aligns with the higher Ly49H levels on NK cells during MCMV infection and the generation of memory NK cells (Grassmann et al., 2019). In line with our observations, stimulation of IL-2–primed NK cells from two healthy donors with a high percentage of adaptive $NKG2C^{high}$ NK cells (>30%) demonstrated a similar robust increase in cell proliferation when engaging NKG2C, NKp30, or CD16, which are expressed in high amounts on the cell surface (Rölle et al., 2014; Vitale et al., 1998; Pazina et al., 2017; Pahl et al., 2018; Fig. S2 B). In contrast, NKp44, NKp46, or NKG2D, which exhibited lower expression, induced less cell proliferation, demonstrating the upregulation of innate-activating receptors by IL-2 priming can facilitate NK cell proliferation by IL-12.

Examination of downstream signaling revealed that IL-12 stimulation promoted STAT4 and STAT5 phosphorylation but could not induce strong mTOR activation (Fig. 4, C–E; and Fig. S3, A and B). However, NKp30 co-stimulation increased $pS6^{S235/236}$ levels (Fig. 4 E), induced proliferation (Ki-67; Fig. 4 F), and led to NK cell expansion by IL-12^{low} (Fig. 4 G). Without IL-12, NKp30 stimulation caused activation-induced cell death, indicating that IL-12 is necessary for NK cell survival or escape from apoptosis during NK cell proliferation (Fig. 4, B and G; Poggi et al., 2005; Viant et al., 2017). In line with our observations, chemical inhibition of mTOR or STAT5 activity or TGFβ stimulation, which is reported to antagonize IL-2 and IL-15 signaling, suppressed NK cell proliferation induced by IL-12 and NKp30 (Fig. S3 C; Eckelhart et al., 2011; Viel et al., 2016; Price et al., 1992). These results show that NKp30 upregulation synergizes with IL-12 to promote mTOR activation necessary for NK cell proliferation. Thus, we concluded that activating NK receptor stimulation facilitates differential IL-12 signaling to promote IL-2–primed NK cell memory-like expansion.

CC12R expression on primary NK cells increases NK cell proliferation during IL-2 co-stimulation

To further investigate if the upregulation of the IL-12 receptor chains and not activating NK receptor expression is associated with an increase in IL-12–mediated cell proliferation of IL-2–primed NK cells, we designed a chimeric cytokine IL-12 receptor (CC12R) by fusion of the extracellular domains of the human IFNγ receptor (IFNγR1 and IFNγR2) with the transmembrane and intracellular domains of the human IL-12Rβ1 and IL-12Rβ2 receptors, respectively (Fig. S4 A). IFNγR ectodomains for the construction of the chimeric receptor were used as exogenous IL-12, but not IFNγ, induces cell expansion of NK92 cells. CC12R expression in the IL-2–dependent NK92 cell line sustained cell proliferation, viability, and expansion by IFNγ stimulation without exogenous IL-2 or IL-12 (Fig. 5 A and Fig. 1 B). IFNγ alone did not promote the proliferation of primary $CC12R^{+}$-transduced NK cells (Fig. S4 B). However, $CC12R^{+}$-transduced primary NK cells exhibited a significant increase in NK cell proliferation relative to untransduced donor-matched $CC12R^{-}$ NK cells in the presence of IL-2 (Fig. 5 B). Accordingly, CC12R expression significantly increased IFNγ secretion relative to untransduced donor-matched $CC12R^{-}$ NK cells (Fig. 5 C). Thus, we concluded that other factors associated with IL-2 or IL-15 priming, besides the upregulation of the IL-12 receptor chains, are necessary to promote IL-12–mediated proliferation.

IL-12 and activating NK receptor co-stimulation are sufficient to overcome cytokine-induced exhaustion and induce NK receptor ligand–dependent NK cell expansion

Prolonged IL-2 receptor stimulation leads to NK cell exhaustion by the upregulation of the intracellular cytokine-checkpoint cytokine-inducible Src homology 2-containing (CIS) protein, associated with diminished mTOR activation (Felices et al., 2018; Daher et al., 2021). Further, human NK cells are expanded with high IL-2 concentrations before clinical administration (Marofi et al., 2021). Therefore, we investigated how long-term

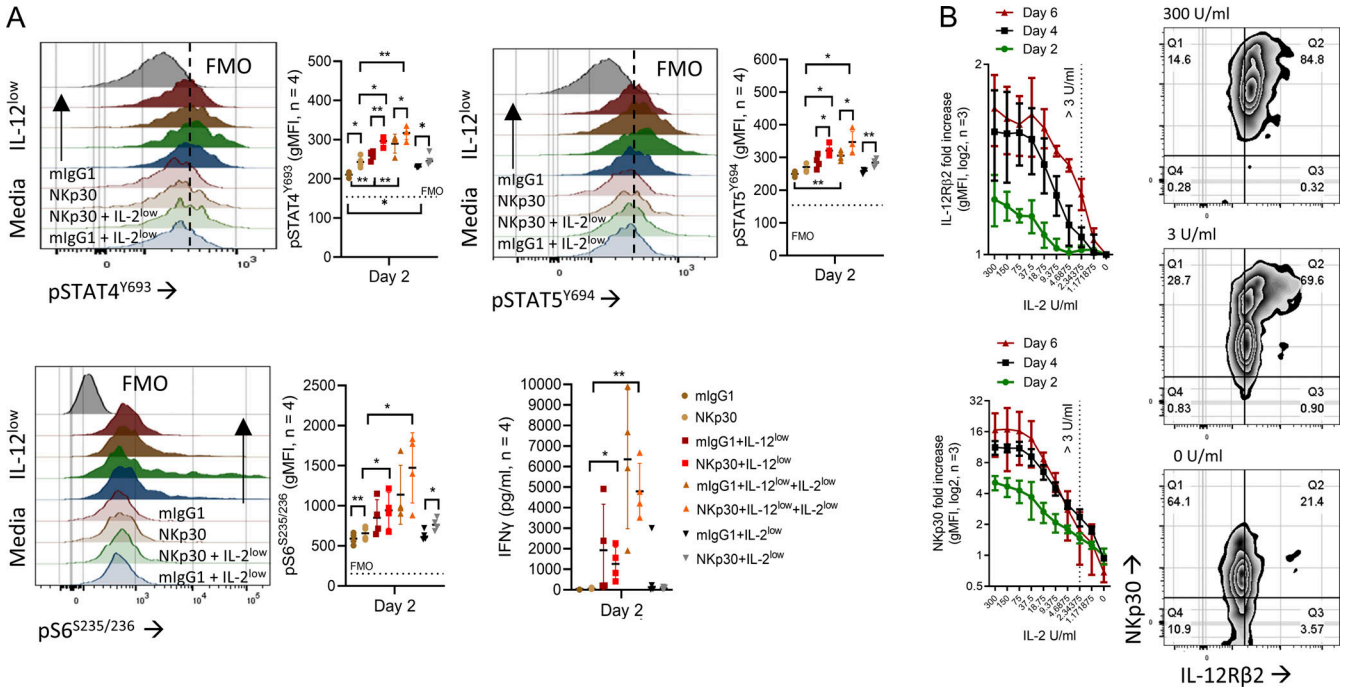

Figure 3. **NKp30 co-stimulation increases STAT signaling in the presence of IL-12.** Purified human peripheral blood NK cells were stimulated as indicated for 48 h. Cytokines were supplemented at the beginning of the cell culture in U-shaped 96-well plates. *n* = number of donors. Mean ± SD. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01. IL-12^{low} = 2.5 ng/ml. IL-2^{low} = 3 U/ml. **(A)** Cell signaling (pS6$^{S235/236}$, pSTAT4^{Y693}, pSTAT5^{Y694}) of 48-h-stimulated NK cells. IFNγ secretion was used as a positive control for IL-12 stimulation (two independent experiments). **(B)** IL-12Rβ2 or NKp30 protein expression levels following IL-2–mediated priming. Data are normalized to minimize variations between donors. *n* = 3 (three independent experiments).

IL-2–primed NK cells respond to IL-12^{low} and activate NK receptor co-stimulation (Fig. 6 A). In accordance with our early observations, IL-12 did not mediate cell proliferation relative to IL-2^{high}, even with IL-18 co-stimulation (Fig. 6 B; and Fig. 1, C and D). Low IL-12 concentrations sustained NK cell viability similar to IL-2^{low} and relative to media without cytokines (Fig. 6, B and C). Activating NK receptor co-stimulation promoted IL-12–mediated proliferation associated with activating NK receptor upregulation, as was observed for NKp30 and NKp44 (Fig. 6 D and Fig. S2 B). We confirmed that activating receptor co-stimulation leading to cell proliferation could be induced by the natural ligand of the activating NK receptors, in addition to agonist antibodies (Fig. S5). Accordingly, low IL-12 concentrations promoted NK cell expansion by NK receptor ligand–recognition during co-culture with mouse Ba/F3 target cells transfected with the NKp30 ligand, B7-H6, or the NKG2D ligand, MHC class I polypeptide–related sequence A (MICA; Fig. 6 E and Fig. S5). These results demonstrate that low IL-12 concentrations are sufficient to promote the expansion of prolonged IL-2–cultured primary NK cells during activating NK receptor co-stimulation (Tait Wojno et al., 2019; Sheppard and Sun, 2021; Tugues et al., 2015).

Discussion

IL-12 is a potent mediator of tumor rejection with pleiotropic effects on the immune system (Berraondo et al., 2018; Tugues et al., 2015). IL-12 signaling is essential for memory formation and ligand-specific expansion of mouse NK cells during MCMV infection (Sun et al., 2012). Our initial objective was to characterize the capacity of IL-12 to promote human NK cell proliferation, analogous to its role in the generation of memory mouse NK cells, and to explore the potential role of IL-12 on NK cells in cancer immunotherapy in humans.

We discovered that activating NK receptor stimulation, mediated by agonist antibodies or cognate activating receptor-ligand recognition, can enhance human NK cell proliferation, due to mTOR activation, in the presence of low concentrations of IL-12 without providing concomitant IL-2 or IL-15 co-stimulation. This mechanism was associated with the upregulation of activating NK receptors by IL-2 priming and the duration of effector–target cell interactions, as seen with different effector to target ratios. Therefore, upregulation and stimulation of an activating NK receptor can orchestrate IL-12–mediated responses to enable ligand-specific expansion of human NK cells. STAT signaling can induce the upregulation of CISH and SOCS3 proteins to limit mTOR activation (Wiedemann et al., 2021; Daher et al., 2021), which might explain the limited mTOR activation by IL-12 after IL-2 priming (Daher et al., 2021). However, we showed that low IL-12 concentrations are sufficient to promote NK cell proliferation during innate-activating NK receptor stimulation. Our results support the prior observation that during MCMV infection high cell surface density of the Ly49H receptor on mouse NK cells facilitates the NK cell memory response (Grassmann et al., 2019). Other studies have reported that NKp30, NKp46, or/and NKG2D mediate human NK cell–myeloid cell crosstalk and NK cell–tumor interactions to

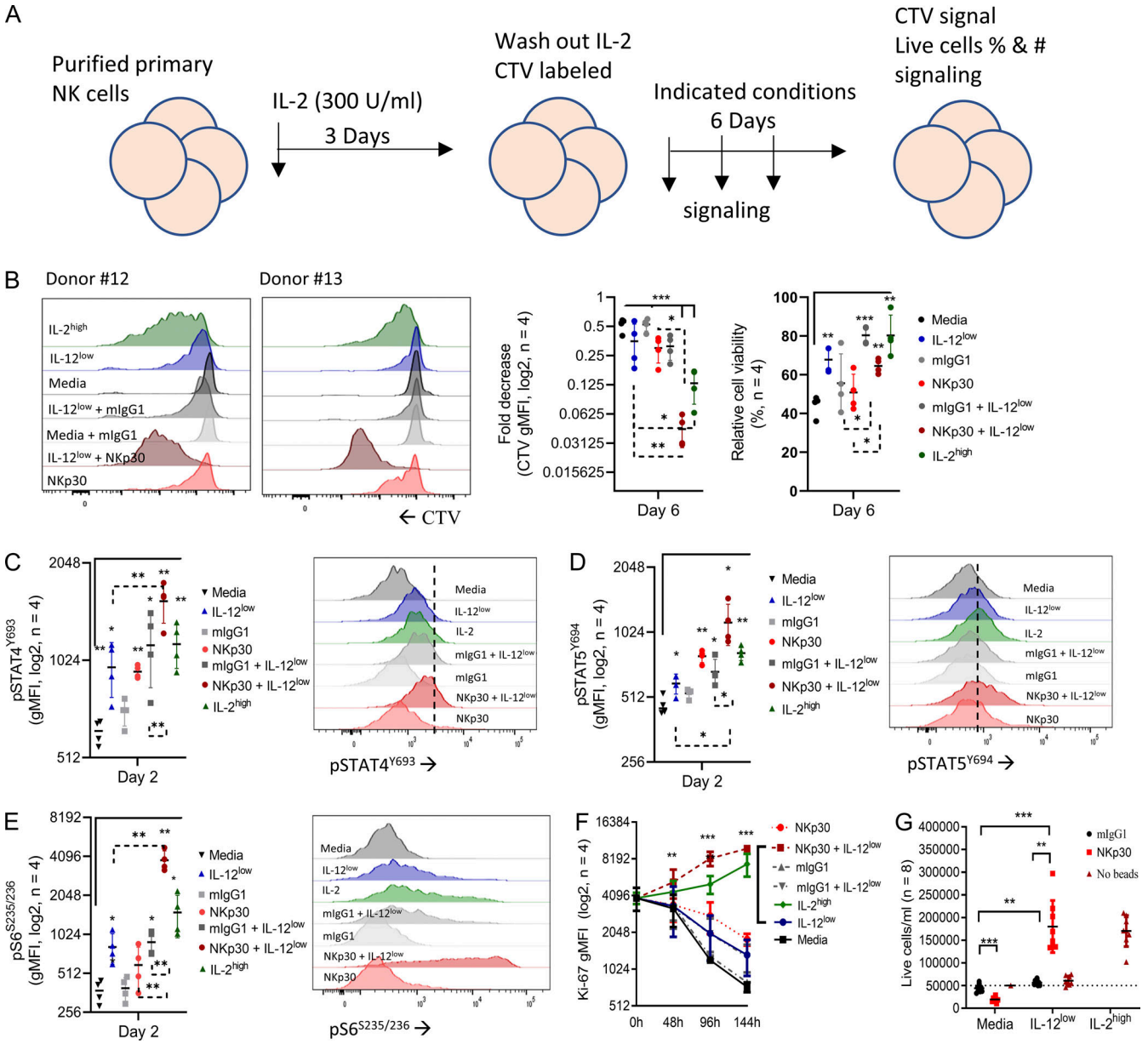

Figure 4. **Activating NK receptor stimulation promotes differential IL-12 signaling leading to IL-2–primed NK cell expansion. (A)** Assays' design: Purified human peripheral blood NK cells were primed with IL-2^{high} (300 U/ml) for 3 d before IL-12^{low} (2.5 ng/ml) stimulation. Cytokines were added only at the beginning of the culture, and IL-2 was washed out before the subsequent stimulation. Data are displayed as *n* = number of donors. Mean ± SD. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01; ***, P < 0.001. mIgG1-coated beads were used as a negative control (≥2 independent experiments). **(B)** Proliferation (CTV) of IL-2–primed NK cells induced by IL-12^{low} with or without anti-NKp30 beads. Left, CTV histograms from two representative donors; middle, CTV gMFI; right, relative cell viability. **(C)** pSTAT4^{Y693} gMFI at 48 h (right: representative histograms, color-coded). **(D)** pSTAT^{Y694} gMFI at 48 h (right: representative histograms, color-coded). **(E)** pS6$^{S235/236}$ gMFI at 48 h (right: representative histograms, color-coded). **(F)** Ki-67 gMFI at days 0, 2, 4, and 6. **(G)** Live NK cell numbers following the indicated stimulation. Fold-change in live cells was evaluated by cell counting and trypan blue stain exclusion. Integrated data from two independent experiments, *n* = 4/experiment (total of *n* = 8).

facilitate innate lymphoid cell expansion (Matta et al., 2013; Trabanelli et al., 2017; Hart et al., 2019; Walk and Sauerwein, 2019). This suggests that the in vivo persistence observed in acute myeloid leukemia patients undergoing cytokine-induced memory-like NK cell therapy might be partly due to higher activating NK receptor levels, as was reported for NKp30, NKp44, NKp46, and NKG2D (Romee et al., 2016; Foltz et al., 2019). Our results also suggest the memory-like features of NK cells attributed to NKG2C stimulation during HCMV infection likely involve the nature of the inflammatory response, the level of NKG2C expression on the NK cells, and the amount of the NKG2C ligand on the virus-infected cell (Rölle et al., 2014; Rölle et al., 2018a). In the case of NKG2C, designed NK cell engagers can promote tumor rejection in preclinical models (Chiu et al., 2021). Thus, applying these technologies to target NKp30 or other activating NK receptors with nontoxic

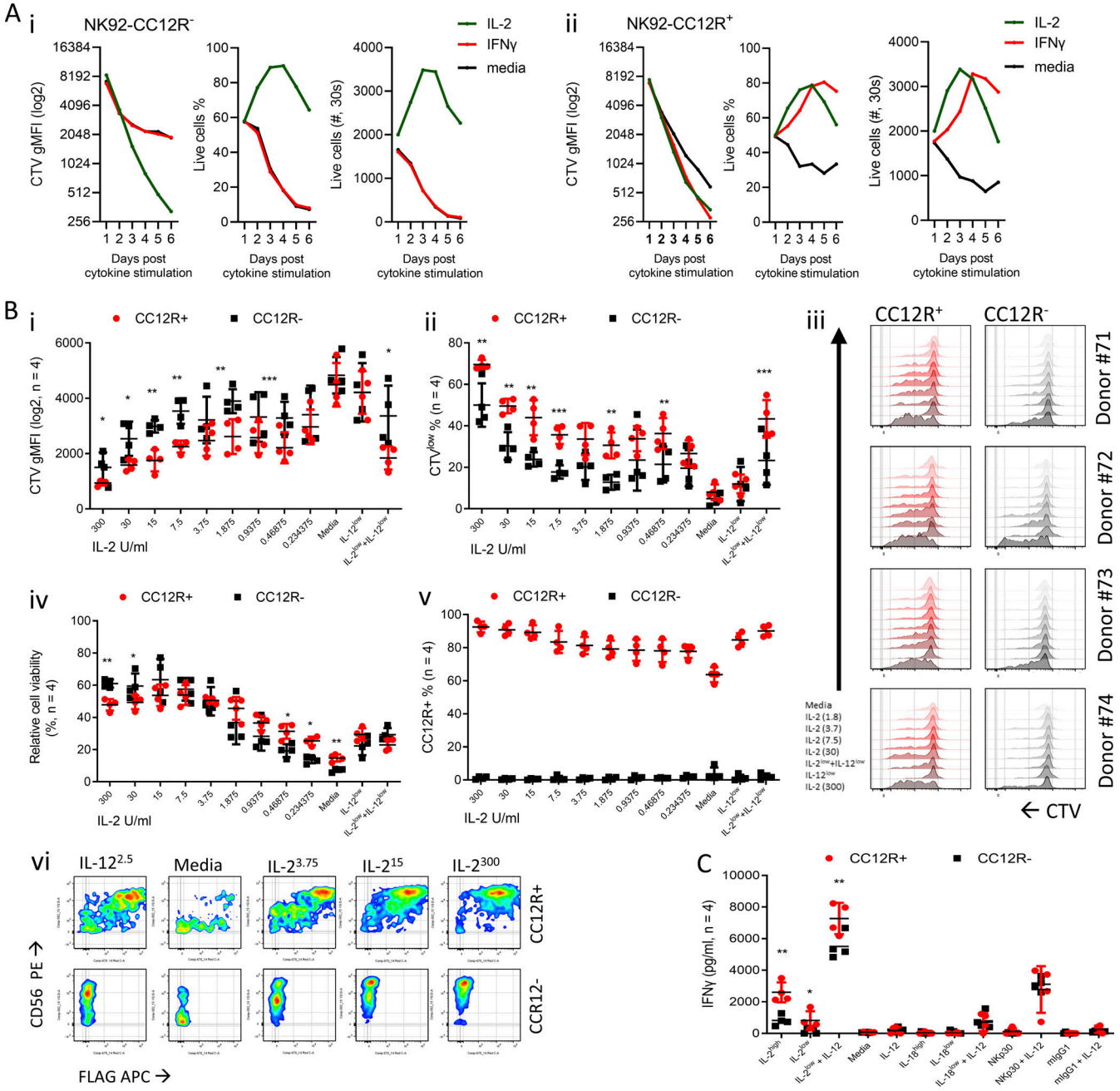

Figure 5. **Chimeric IFNγR-hIL-12R receptor (CC12R) enhances human primary NK cell proliferation. (A)** NK92 cells were cultured in the presence of IL-2 (200 U/ml), IFNγ (100 ng/ml), or without cytokines (media) and measured for (left to right) cell proliferation (CTV gMFI dilution), percentage of live-cell (fixable near-infrared viability dye negative cells), and relative cell numbers (30-s sample acquisition). **(A.i)** CC12R-negative NK92. **(A.ii)** CC12R-positive NK92 (≥2 independent experiments). **(B)** CC12R-transduced human primary NK cells were cultured for 6 d with the indicated stimulation. Cell proliferation, viability, and CC12R expression were assessed relative to donor-matched CC12R-negative cells. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01; ***, P < 0.001. Mean ± SD. *n* = number of donors (≥2 independent experiments). **(B.i)** Cell proliferation (CTV gMFI). **(B.ii)** Percentage of CTV^{low} cells. **(B.iii)** Representative histograms showing CTV dilution. **(B.iv)** Relative cell viability (live fixable near-infrared viability dye negative cells). **(B.v)** Percentage of $CC12R^+$ cells evaluated by FLAG expression. **(B.vi)** Expression of CD56 (y axis) vs. FLAG (CC12R; x axis) relative to the indicated stimulation. **(C)** IFNγ amounts (pg/ml) after 6 d of culture in IL-2^{high} (300 U/ml), IL-2^{low} (15 U/ml) IL-18^{high} (25 ng/ml) IL-18^{low} (2.5 ng/ml), IL-12 (2.5 ng/ml) in CC12R-positive samples relative to donor-matched negative samples. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01; ***, P < 0.001. Mean ± SD. *n* = number of donors.

amounts of IL-12 or IL-2 will likely improve their clinical efficacy.

We demonstrated that after short- or long-term IL-2 priming, IL-12 or IL-2 restimulation is necessary for a "recall expansion" during activating NK receptor stimulation. This observation indicates that cytokine sensitivity, i.e., the ability of NK cells to sense cytokines, plays a vital role in the regulation of NK cell expansion and might explain the difference between the responses in different NK cell donors or subsets. Reduced sensitivity to cytokine-only stimulation and an increasing

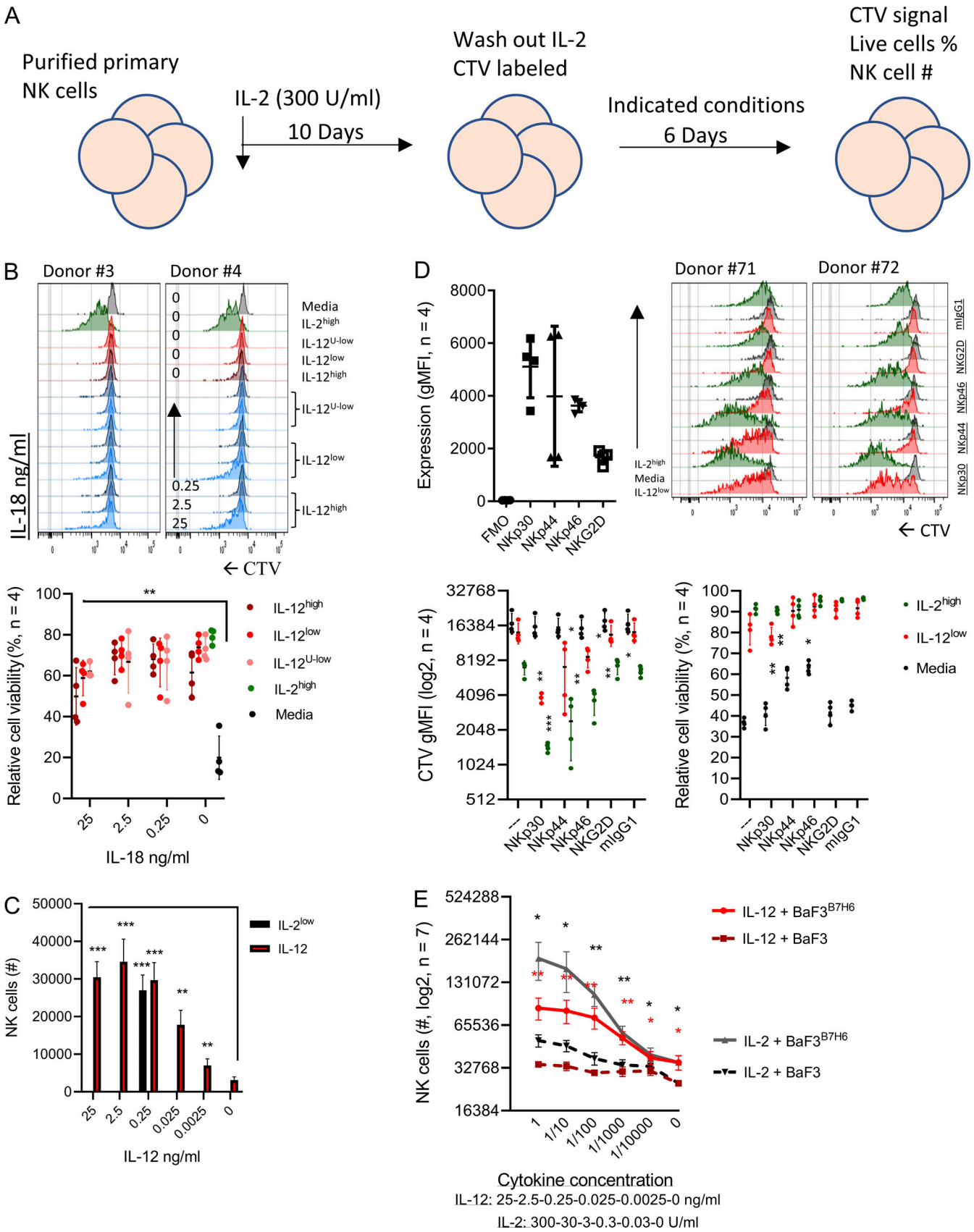

Figure 6. **Low IL-12 concentrations promote NK receptor ligand-specific expansion of long-term IL-2 culture primary NK cells. (A)** Assays' design: Purified human peripheral blood NK cells were primed with IL-2^{high} (300 U/ml) for 10 d before IL-12^{low} (2.5 ng/ml) stimulation. Cytokines were added only at the beginning of the culture, and IL-2 was washed out before the restimulation. Data are displayed as *n* = number of donors. Mean ± SD. Paired *t* test,

parametric; *, P < 0.05; **, < P < 0.01; ***, P < 0.001. mIgG1-coated beads were used as a negative control (≥2 independent experiments). **(B)** Proliferation (CTV) assay. Long-term primed NK cells were co-cultured with IL-12$^{high, low,}$ or $^{ultra\text{-}low}$ (25, 2.5, or 0.25 ng/ml, respectively) in the presence of increasing IL-18 concentrations (25, 2.5, or 0.25–0 ng/ml, respectively). IL-2^{high} (300 U/ml) or media without cytokines were used as positive or negative controls. Lower panel: relative cell viability. **(C)** Live NK cell numbers during IL-12 stimulation relative to IL-2^{low} (3 U/ml). **(D)** Proliferation (CTV) assay. Primed NK cells were stimulated using beads coated with antibodies against the indicated activating receptors with IL-2^{high}, IL-12^{low}, or media without cytokines. Upper left: activating NK receptor expression levels; upper right: CTV histograms; lower left: CTV gMFI; lower right: relative cell viability. **(E)** Live NK cell numbers of IL-2–primed NK cells after co-cultured for 6 d with Ba/F3 cells expressing NKp30 ligand (B7-H6), relative to no ligand in the presence of variable IL-2 or IL-12 concentrations.

dependency on activating NK receptor stimulation are hallmarks of human and mouse memory NK cells (Min-Oo and Lanier, 2014; Lee et al., 2015). On the other hand, differential cytokine signaling can influence several downstream pathways, as was shown in the case of IL-15 (Marçais et al., 2014; Mukherjee et al., 2017), and can be regulated by homotypic or heterotypic cellular interactions (Sun et al., 2012; Kim et al., 2014), and by combinatorial cytokine stimulation (Wiedemann et al., 2021). As demonstrated here, IL-12 signaling in human NK cells is strongly regulated by activating NK receptor stimulation, which leads to differential signaling to promote NK cell proliferation even in "naive" ex vivo NK cells.

We showed that IL-12 sensitivity was essential to sustain NK cell viability and could suppress activation-induced cell death by activating NK receptor stimulation and could mediate STAT5 phosphorylation necessary for NK cell proliferation (Eckelhart et al., 2011; Wang and Zhao, 2021). Similar to IL-2 or IL-15 stimulation, IL-12–mediated NK cell proliferation was sensitive to TGFβ, indicating shared signaling pathways and the need to antagonize TGFβ during IL-12 treatment. Distinct from the response of human NK cells, IL-12 stimulation alone or with other stimuli of mouse NK cells ex vivo did not mediate NK cell viability or cell proliferation (unpublished data; Marçais et al., 2014; Wiedemann et al., 2021). This observation suggests a functional difference between mouse and human NK cells. We conclude that STAT5 phosphorylation and maintenance of cell viability by the human IL-12 receptors support NK receptor–dependent ligand-specific expansion of in vivo– or ex vivo–primed human NK cells during activation of NK receptor stimulation, similar to the observation in memory or memory-like NK cells. Further studies are warranted to deconvolute the contribution of combined signaling molecules, such STAT4, STAT5, immunoreceptor tyrosine-based activation motifs, and mTOR activation, on downstream effector molecules and their effects on human NK cell proliferation mediated by IL-12 during activating receptor stimulation.

Collectively, we have shown that low IL-12 concentrations can efficiently mediate human NK cell expansion during innate-activating NK receptor stimulation and promote human NK cell persistence. As human NK cells are being genetically engineered for optimal activating receptor stimulation, our findings provide insights into the contribution of IL-12 signaling in the establishment of human NK cell ligand-specific memory-like features and could be helpful for the implementation of IL-12 and NK cells for cancer immunotherapy.

Materials and methods

Patient samples and data collection

Plasma IL-12 levels were measured using 38-plex Luminex multi-bead arrays from Millipore. Raw mean fluorescence intensity (MFI) values were batch-corrected using the ComBat algorithm, followed by $\log_2$ transformation to fit a normal distribution. Kidney transplant recipients were enrolled after transplantation at Ronald Reagan Medical Center, and all patients gave informed consent. The University of California, Los Angeles Institutional Review Board approved this observational study (#11-001387; Pickering et al., 2021).

Primary NK cells isolation and culture

Human primary NK cells were obtained from healthy donors' peripheral blood after donors gave informed consent in accordance with approval by the University of California, San Francisco (UCSF) Institutional Review Board (#10-00265) or from plateletpheresis leukoreduction filters (Vitalant, https://vitalant.org/Home.aspx). NK cells were isolated by using the negative selection "RosetteSep human NK Cell Enrichment Cocktail" kit (STEMCELL Technologies) according to the company's protocol. Purified NK cells ($CD56^+CD3^-$) were used on the same day (day 0, ex vivo) or after priming with IL-2, as indicated. NK cell culture media: GMP SCGM (CellGenix) supplemented with 1% L16 glutamine, 1% penicillin and streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 10 mM Hepes, and 10% human serum (heat-inactivated, sterile-filtered, male AB plasma; Sigma-Aldrich). Purified NK cells were used fresh or frozen ex vivo; freezing media: culture media 40% + FCS 50% + DMSO 10%. NK cells were primed at a cell density of 2–3 × 10^6 cells/well in 24-well plates, in 2 ml culture media supplemented with 300 U/ml of human IL-2 (TECIN; teceleukin; Roche, generously provided by NCI Biological Resources Branch).

Antibody-conjugated beads

Antibody-conjugated beads were prepared according to the company's protocol (Invitrogen Dynabeads Antibody Coupling Kit) at 10 µg antibody per 1 mg beads. Following conjugation, beads were resuspended in sterile PBS at an antibody concentration of 0.1 µg/µl. Antibody conjugation was evaluated by flow cytometry with APC-conjugated anti-mouse or rat IgG. BioLegend: anti-CD16 (cat. 302002, IgG1k), anti-NKp30 (cat. 325204, IgG1k), anti-NKp44 (cat. 325102, IgG1k), anti-NKp46 (cat. 331904, IgG1k), and anti-NKG2D (cat. 320802, IgG1k). R&D Systems: anti-NKG2C (clone; 134572.111, IgG1; generously provided by R&D Systems). UCSF Monoclonal Antibody Core: mouse IgG1 isotype-matched control (clone; MOPC-21). Antibody-conjugated beads were kept at 4°C.

Cytokines

All cytokines were resuspended in sterile PBS: human IL-2, 1,000 U/µl (TECIN); human IL-15, 250 µg/ml (247-IL/CF; R&D Systems); human IL-12, 50 µg/ml (219-IL; R&D Systems); human

IL-18, 50 µg/ml (9124-IL/CF; R&D Systems); human IFNα, 100 µg/ml (cat. 78076; STEMCELL Technologies); human IFNγ, 100 ng/ml (cat. 285-IF; R&D Systems); and human TGFβ1, 50 µg/ml (cat. 580706; BioLegend). Cytokines were kept at −20°C.

B7-H6 and CCR cloning and lentivirus preparation

B7-H6 cDNA (generously provided by Dr. A. Cerwenka, University of Heidelberg, Heidelberg, Germany) or human IFNγR-hIL-12R CCR constructs were cloned into the lentivirus vectors pHR containing the EF1a or SFFV promotor using an In-Fusion HD Cloning Kit (TAKARA). The chimeric cytokine receptor contained amino acids 1–245 of IFNγR1 and amino acids 1–247 of IFNγR2 extracellular domains, and amino acids 546–662 of IL-12Rβ1 and amino acids 663–862 of IL-12Rβ2 containing the transmembrane and intracellular domains of the human IL-12R (Integrated DNA Technologies). Myc-tag was integrated into the N-terminus of the first CC12R chain, while FLAG-tag was integrated into the N-terminus of the second CC12R chain to allow surface detection. CC12R chains were separated by a T2A sequence. Lentivirus preparation was done by using the pMD2.G and pCMV dr8.91 packaging vectors and transfection of the Lenti-X 293T cell line (TAKARA) cultured in complete DMEM plus 10% FCS. Lentivirus was concentrated using a Lenti-X concentrator (TAKARA) and resuspended in 1 ml RPMI-1640 + 10% FCS with protamine sulfate (1 µg/ml). Aliquots were kept at −20°C.

Lentiviral transduction

Lentivirus particles containing pHR-EF1a-B7-H6 were used to transduce the mouse pro-B cell line Ba/F3 pre-engineered to express mouse IL-3. Lentiviral-transduced Ba/F3 cells were cultured at 37°C and sorted for B7-H6 expression after staining with APC-conjugated anti-B7-H6 (FAB7144A; R&D Systems). Ba/F3-human MICA cells were used as previously described (Rosen et al., 2004). Ba/F3 cells culture media: complete RPMI-1640 + 10% heat-inactivated FCS. Lentivirus particles containing pHR-SFFV-hIFNγR-hIL-12R CCR were used to transduce NK92 cells (ATCC CRL-2407) or primary NK cells. Briefly, 5×10^5 NK cells were resuspended in 200 µl of the equivalent NK92 media (100 U/ml IL-2) or primary NK cell culture media (3 U/ml IL-2). After adding the virus, cells were centrifuged at 1,000 *g* relative centrifugal force (rcf) for 1 h at room temperature and incubated with the virus for 3 d at 37°C. CC12R expression was analyzed by staining with anti-myc-tag (3739S; Cell Signaling) and anti-FLAG (367308; BioLegend) antibodies.

STAT and mTORC1 inhibitors

All inhibitors were resuspended at 100% DMSO and stored as recommended by the company's protocol. Reagents used included rapamycin mTORC1 inhibitor (cat. 553210; Calbiochem, IC_{50} = 0.1 µM) WP1066 STAT3#1 inhibitor (cat. 573097; Millipore Sigma, IC_{50} = 5.6 µM), CAS-1041438-68-9 STAT3#2 inhibitor (cat. 573103; Millipore Sigma, IC_{50} = 0.17 µM), CAS-285986-31-4 STAT5#1 inhibitor (cat. 573108-M; Millipore Sigma, IC_{50} = 47 µM), and Pimozide STAT5#2 inhibitor (cat. 573110; Millipore Sigma, IC_{50} = 5 µM).

Antibody-coated bead stimulation

Ex vivo NK cells or IL-2–primed NK cells were labeled with CTV according to the company's protocol (cat. C34557; Invitrogen). As indicated, antibody-coated beads were diluted at 1:1,000 in cytokine-free NK cell culture media and used by adding 50 µl/well to a final concentration of 25 ng/ml antibody. Cytokines or/and inhibitors were added at 100 or 150 µl/well to a final concentration as indicated in each experiment. CTV-labeled NK cells (5×10^4 cells/well) were added at 50 µl/well in cytokine-free NK cell culture media. The final culture volume during the assays was 200 µl/well. The assays were performed in 96-well round-bottom plates. NK cells were incubated at 37°C with 5% CO_2 for the duration of the assay. Cells were analyzed by flow cytometry (LSR-II; Becton Dickinson Immunocytometry Systems).

B7-H6 and MICA Ba/F3 stimulation

IL-2–primed NK cells were labeled with CTV according to the company's protocol (cat. C34557; Invitrogen). Ba/F3 were labeled with CFSE (cat. C34554; Invitrogen) at the beginning of the assay. As indicated, Ba/F3 cells were added to the effectors (5×10^4 NK cell/well) to the desired target ratio in cytokine-free NK cell culture media. Cytokines were added at 100 or 150 µl/well to the final concentration as indicated in each experiment. CTV-labeled NK cells (5×10^4 cells/well) were added in 50 µl/well in cytokine-free NK cell culture media. The final culture volume during the assay was 200 µl/well. The assays were performed in 96-well round-bottom plates. NK cells were incubated at 37°C with 5% CO_2 for the assay duration. At the end of the assay, PE-conjugated anti-mouse CD45.2 (1:100, cat. 109808; BioLegend) was used to detect Ba/F3 cells. Both CFSE and CD45.2 were used as gating markers to distinguish Ba/F3 cells from CTV-labeled NK cells. Samples were analyzed by flow cytometry (LSR-II; Becton Dickinson Immunocytometry Systems).

NK cell CC12R stimulation

For assessing CC12R function, frozen purified primary NK cells were thawed and immediately labeled with CTV to minimize NK cell handling after transduction and then transduced with lentiviral particles in NK cell culture media with 3 U/ml IL-2. After 3 d, the virus was washed out, and cells were resuspended in fresh cytokine-free NK cell culture media and split equally between the assay conditions at 50 µl/well. Cytokines were added at 100 or 150 µl/well to a final concentration as indicated in each experiment. The final culture volume during the assay was 200 µl/well. The assays were performed in 96-well round-bottom plates. NK cells were incubated at 37°C with 5% CO_2 for the duration of the assay. Cells were analyzed by flow cytometry (LSR-II; Becton Dickinson Immunocytometry Systems).

Flow cytometry and antibodies

For membrane staining, cells were antibody-labeled for 30 min at 4°C at 50 µl/well. Flow cytometry buffer was PBS + 2% FCS. Dead cells were labeled by using propidium iodide (1 mg/ml, 1:500) or near-infrared fixable dye (1:1,000, cat. L34976; Invitrogen). For the wash step, 150 µl/well of buffer was added, the plate was centrifuged at 600 *g* rcf for 5 min at 4°C, and culture media was discarded. For intracellular antigen detection, cells were incubated for 20 min at 4°C with 100 µl/well Cytofix/Cytoperm buffer (51-2090KZ; Becton Dickinson). Following

incubation, cells were washed twice using 150 µl/well Perm Wash buffer (cat. 421002; BioLegend) diluted 1:10 in PBS, and then antibodies against intracellular markers were added and incubated with the cells for 60 min at 4°C. Following incubation, cells were washed twice with 150 µl/well cytoplasm buffer. Before analyzing the samples for membrane or/and intracellular markers, cells were resuspended in a 300 µl flow cytometry buffer. Samples were kept at 4°C until analyzed using an LSR-II flow cytometer. Surface and intracellular markers: BioLegend: PerCep-Cy5.5–conjugated anti-CD56 (cat. 318322), APC-Cy7–conjugated anti-CD3 (cat. 300318), APC-conjugated anti-NKp30 (cat. 325210), AF647-conjugated anti-NKp44 (cat. 325112), APC-conjugated anti-NKp46 (cat. 331918), APC-conjugated anti-NKG2D (cat. FAB139A), and APC-conjugated anti-CD16 (cat. 302012), BV605-conjugated streptavidin (cat. 405229). Becton Dickinson: AF-647–conjugated anti-pSTAT4^{Y693} (cat. 562074), AF-647–conjugated anti-pSTAT5^{Y694} (cat. 612599), and AF-647–conjugated anti–Ki-67 (cat. 558615). Cell Signaling: AF-647–conjugated anti-pS6$^{S235/236}$ (cat. 4851S), PE-conjugated pBad^{S112} (cat. 11865S), Biotin-conjugated Bcl-xL (cat. 87979S), AF594-conjugated Mcl-1 (cat. 88169S), AF647-conjugated Bcl2 (cat. 82655S). R&D Systems: PE-conjugated anti–IL-18Rα (cat. FAB840P) and PE-conjugated anti–IL-18Rβ (cat. FAB118P).

IFNγ ELISA

For the detection of IFNγ, NK cell-containing cell-culture plates were centrifuged at 600 *g* rcf for 5 min at 4°C. 75 µl/well media was collected and stored at −20°C until analysis. ELISA MAX Deluxe Set Human IFN-γ (cat. 430115; BioLegend) was used according to the company's protocol. An IFNγ standard curve was performed by serial twofold dilutions, starting from 10,000–4.88 pg/ml, while 0 pg/ml was used to calculate the background signal. 50 µl of the sample was used to detect IFNγ levels following the indicated stimulation.

Graphics and statistical analysis

Graphs were generated using GraphPad Prism 9 or FlowJo_V10. Statistical analysis is indicated in figure legends and was calculated using Graphpad Prism 9 or Excel (Microsoft 365). *, $P < 0.05$; **, $< P < 0.01$; ***, $P < 0.001$.

Online supplemental material

Fig. S1 shows IL-2, IL-15, IL-18, IFNα, and NKp30 contribution to ex vivo human NK cell expansion, proliferation, and viability by IL-12. Fig. S2 shows contribution of innate-activating receptors to IL-12–mediated NK cell proliferation. Fig. S3 shows activating NK receptor stimulation promotes mTOR activation by IL-12. Fig. S4 shows chimeric hIFNγR-hIL-12R receptor (CC12R) design and expression in human primary NK cells. Fig. S5 shows IL-12–mediated NK cell proliferation during target cell co-culture.

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Supplemental material

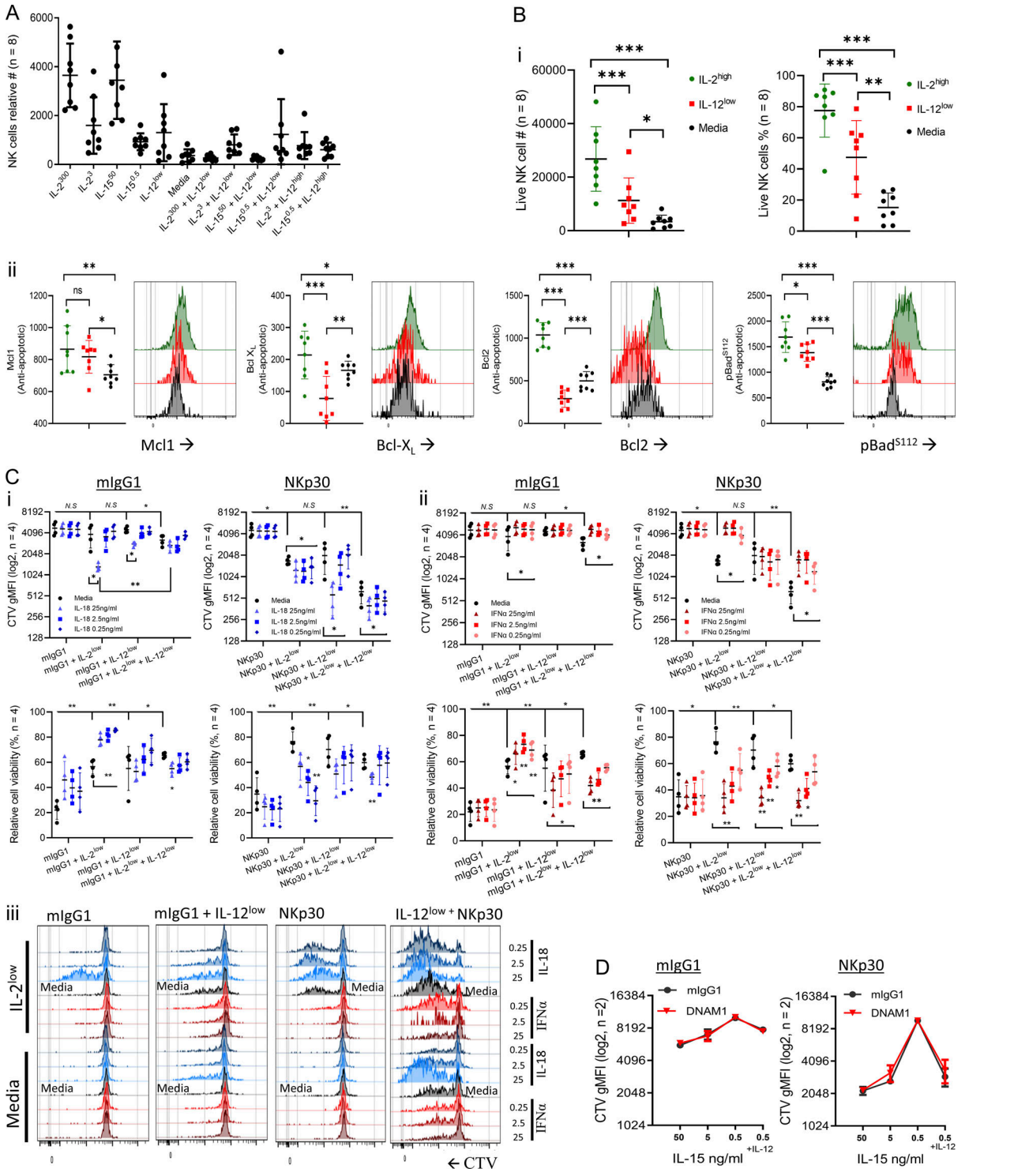

Figure S1. **IL-2, IL-15, IL-18, or IFNα, and NKp30 contribution to ex vivo human NK cell expansion, proliferation, and viability by IL-12. (A)** Primary NK cell relative numbers. Samples were measured by flow cytometry on day 6 of the indicated stimulation. *n* = 4 (number of donors). **(B)** Purified human peripheral blood NK cells (*n* = 8, number of donors) were cultured for 6 d with IL-2^{high} (300 U/ml), IL-12^{low} (2.5 ng/ml), or media without cytokines. Expression levels of the indicated Bcl2 family protein were assessed by intracellular staining and flow cytometry. Mean ± SD. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01; ***, P < 0.001. **(B.i)** Live NK cell number per reading (left) and matched live NK cells percentage (right). **(B.ii)** Expression of anti-apoptotic proteins (left to right): Mcl1, Bcl-X_L, Bcl2, pBad^{S112}, and representative histograms. **(C)** Proliferation (CTV) assay: ex vivo NK cells were stimulated with IL-12^{low} (2.5 ng/ml) and/or IL-2^{low} (3 U/ml) with or without IL-18 (C.i) or IFNα (C.ii). Upper panels: cell proliferation measured by CTV; lower panels: cell viability measured by fixable near-infrared viability dye. **(C.iii)** Representative CTV histograms. Stimulation was done in the presence of mIgG1- or anti-NKp30–coated beads. Mean ± SD. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01. *n* = 4 (≥2 independent experiments). **(D)** Cell proliferation (CTV) assays: ex vivo NK cells were stimulated with anti-NKp30 beads with or without anti-DNAM1 beads in the presence of the indicated IL-15 concentrations and IL-12 (2.5 ng/ml) as indicated. *n* = number of donors, mean ± SD. *n* = 4, mean ± SD.

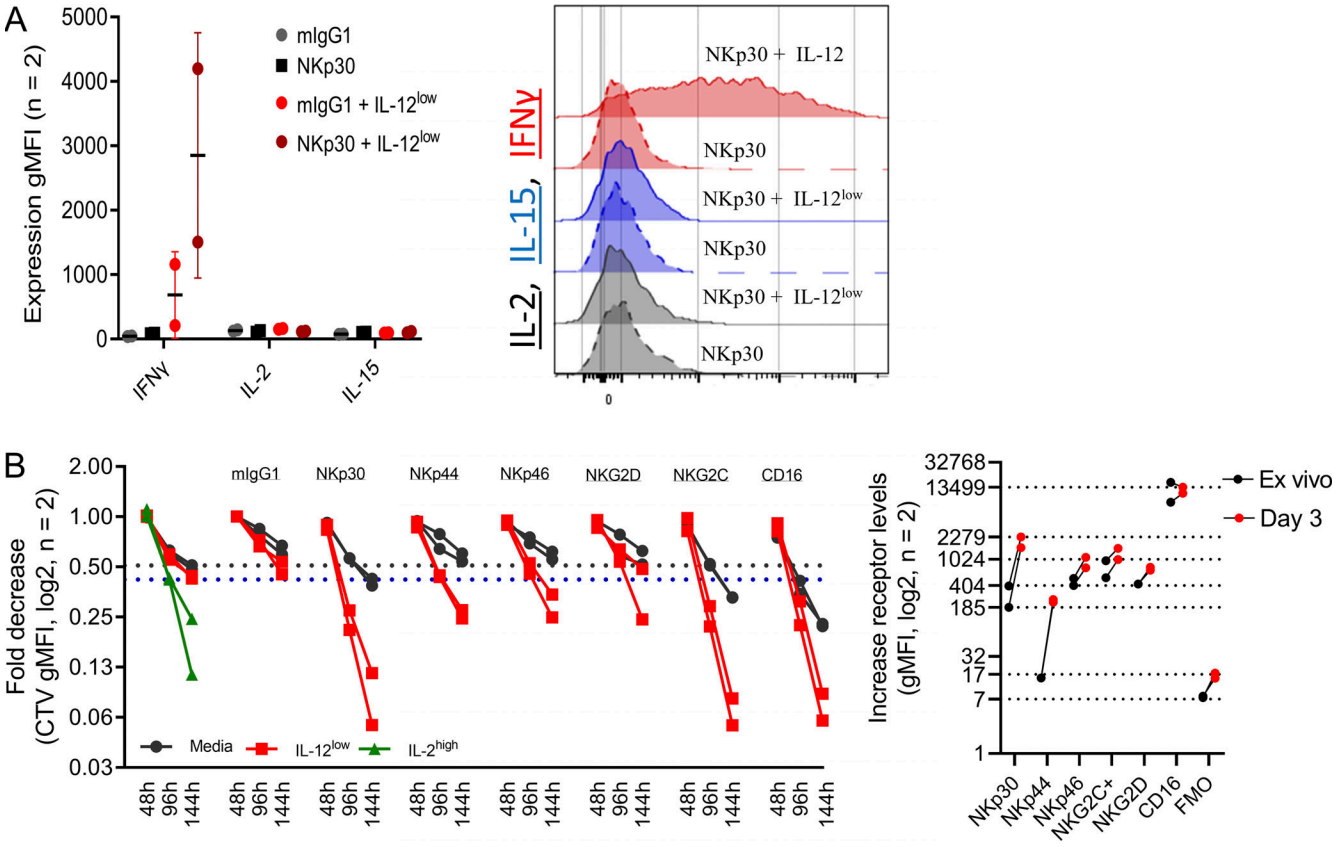

Figure S2. **Contribution of innate-activating receptors to IL-12 mediated NK cell proliferation.** Purified human peripheral blood NK cells were primed with IL-2^{high} (300 U/ml) for 3 d before IL-12^{low} (2.5 ng/ml) stimulation. Cytokines were added only at the beginning of the culture. IL-2 was washed out before the subsequent stimulation. *n* = number of donors. Mean ± SD. mIgG1-coated beads were used as a negative control (≥2 independent experiments). **(A)** Intracellular IFNγ, IL-2, and IL-15 levels in IL-2–primed NK cells after 24 h of the indicated stimulation. **(B)** Proliferation (CTV) assay: CTV gMFI levels of IL-2–primed NK cells from 2 donors with a NKG2C^{high} adaptive NK cell subset (>30%) following the indicated innate-activating receptor stimulation with or without IL-12^{low}. IL-2^{high} (300 U/ml) was used as a positive control. Media without cytokines was used as a negative control. Right: NK activating receptor expression levels ex vivo (black) or after 3 d of IL-2 priming (red).

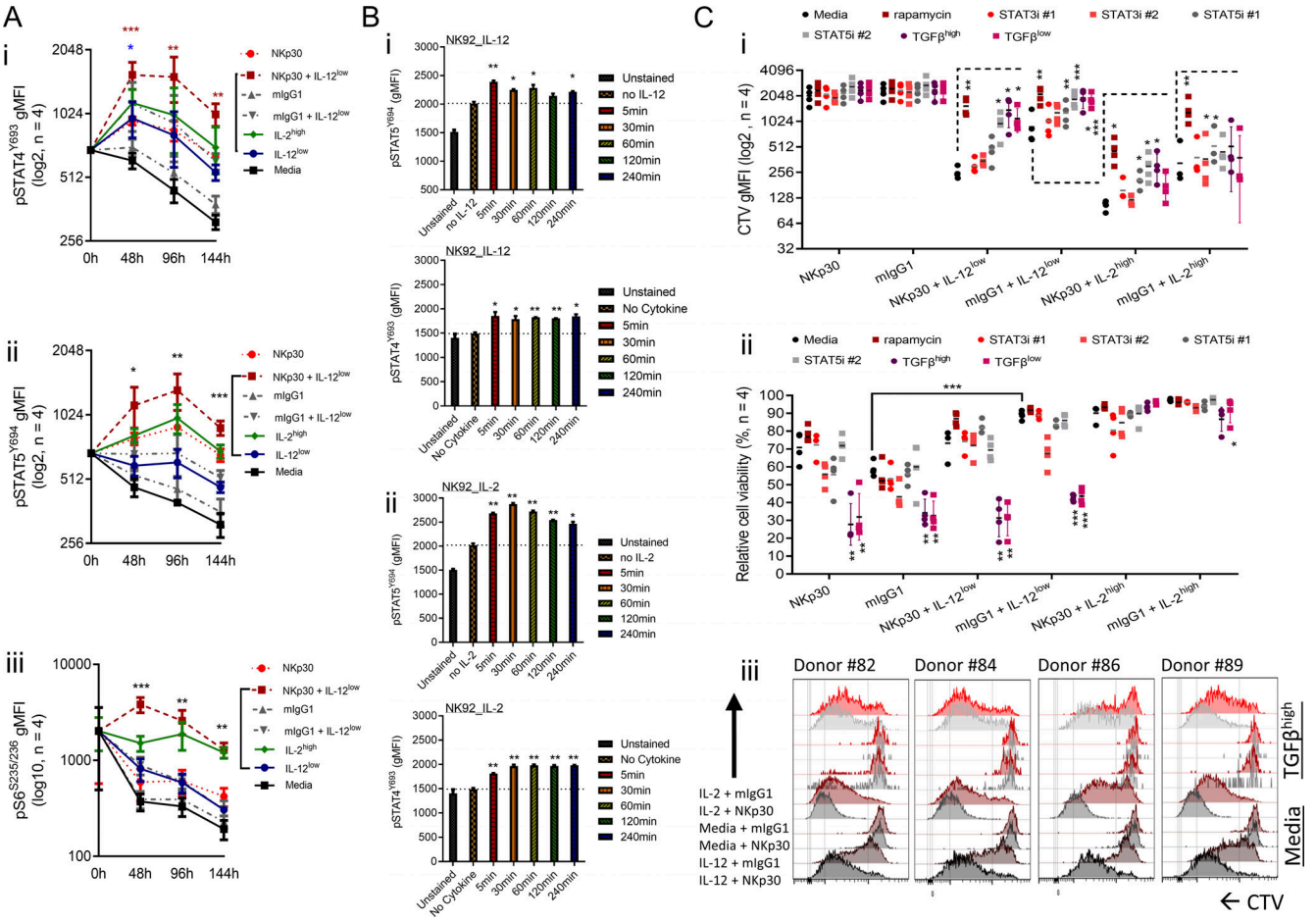

Figure S3. **Activating NK receptor stimulation promotes mTOR activation by IL-12. (A)** Purified human peripheral blood NK cells were primed with IL-2^{high} (300 U/ml) for 3 d before IL-12^{low} (2.5 ng/ml) and NKp30 stimulation. IL-2 was washed out before the subsequent stimulation, and cytokines were added only at the beginning of the culture. *n* = number of donors. Mean ± SD. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01; ***, P < 0.0001. mIgG1-coated beads were used as a negative control. **(A.i)** $pSTAT4^{Y693}$. **(A.ii)** $pSTAT5^{Y694}$. **(A.iii)** $pS6^{S235/236}$ (≥2 independent experiments). **(B)** NK92 cells were stimulated with IL-12 (2.5 ng/ml; B.i) or IL-2 (200 U/ml; B.ii) for the indicated amount of time and tested for $pSTAT4^{Y693}$ or $pSTAT5^{Y694}$. Cytokines were added first at 240 min and then as indicated. Mean ± SEM; unpaired *t* test, parametric; *, P < 0.05; **, < P < 0.01 (≥2 independent experiments). **(C)** 3-d IL-2^{high} primed NK cells were stimulated by anti-NKp30 beads or control mIgG1 beads and/or IL-12 (2.5 ng/ml), or IL-2 (300 U/ml) or media without cytokines in the presence of different inhibitors against mTOR, STAT3, or STAT5, or with $TGFβ^{high\ or\ low}$ (12.5–1.25 ng/ml) for 6 d. **(C.i)** Proliferation (CTV gMFI) assay. **(C.ii)** Relative cell viability. **(C.iii)** CTV histograms during TGFβ co-stimulation. Statistical analysis was calculated relative to media. *n* = number of donors, mean ± SD. Paired *t* test, parametric; *, P < 0.05; **, < P < 0.01; ***, P < 0.0001 (≥2 independent experiments). STAT3 inhibitors were used as a control for STAT5 inhibitors.

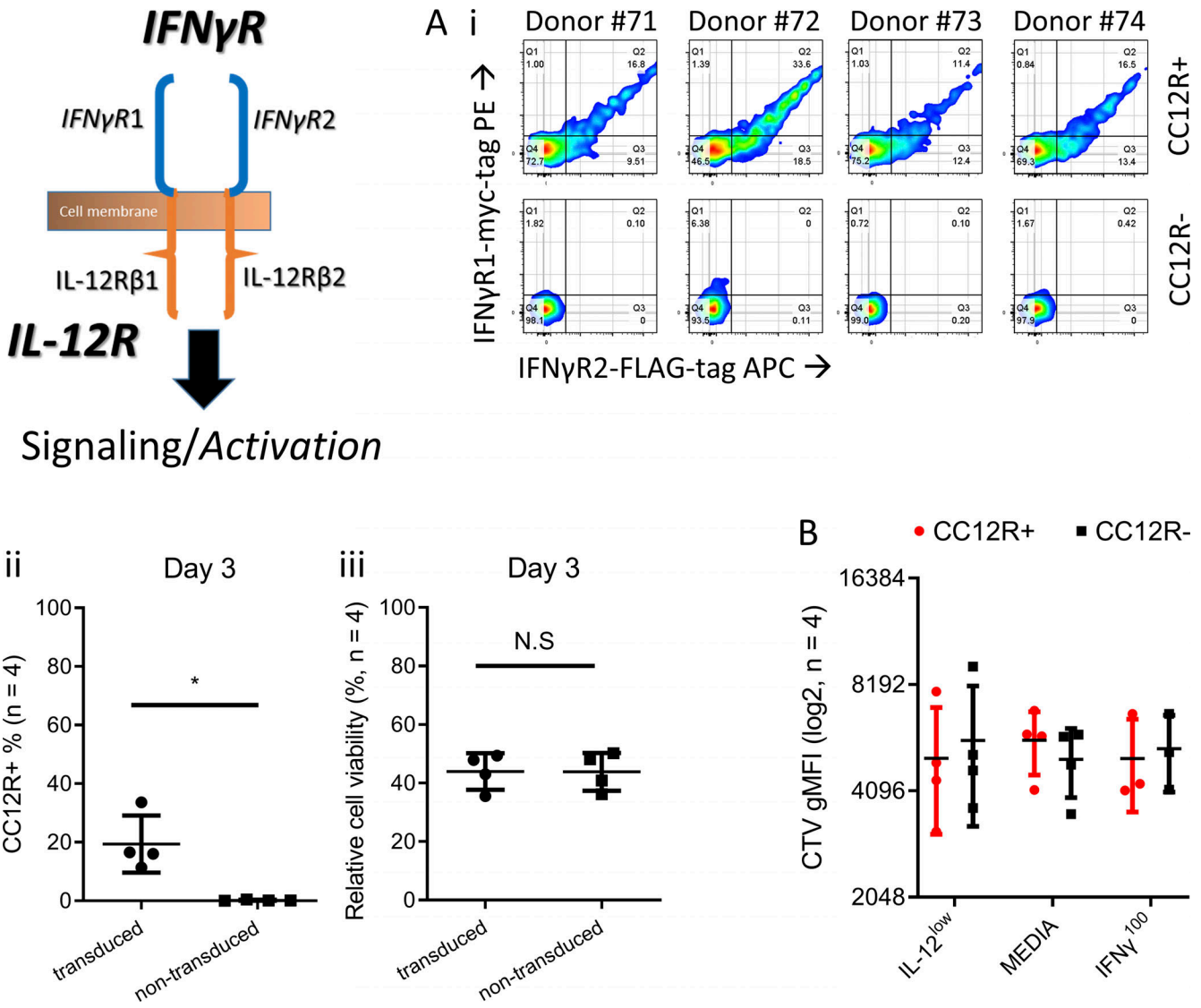

Figure S4. **Chimeric hIFNγR-hIL-12R receptor (CC12R) design and expression in human primary NK cells.** Purified frozen human primary NK cells were cultured for 3 d with or without lentiviral particles containing the CC12R construct and IL-2 (3 U/ml). CC12R expression was evaluated by anti-myc-tag (first CC12R chain) and anti-FLAG (second CC12R chain). Expression was evaluated relative to donor-matched control samples (CC12R–). Cell viability was assessed using a near-infrared live/dead marker. Four donors (n = 4). Mean ± SD. Paired t test, parametric; *, $P < 0.05$. **(A) (A.i)** Expression of CC12R after 3 d of transduction. **(A.ii)** Percentage of CC12R+ cells evaluated by FLAG expression. **(A.iii)** Relative cell viability. **(B)** Influence of IFNγ stimulation on cell proliferation of CC12R$^+$ cells.

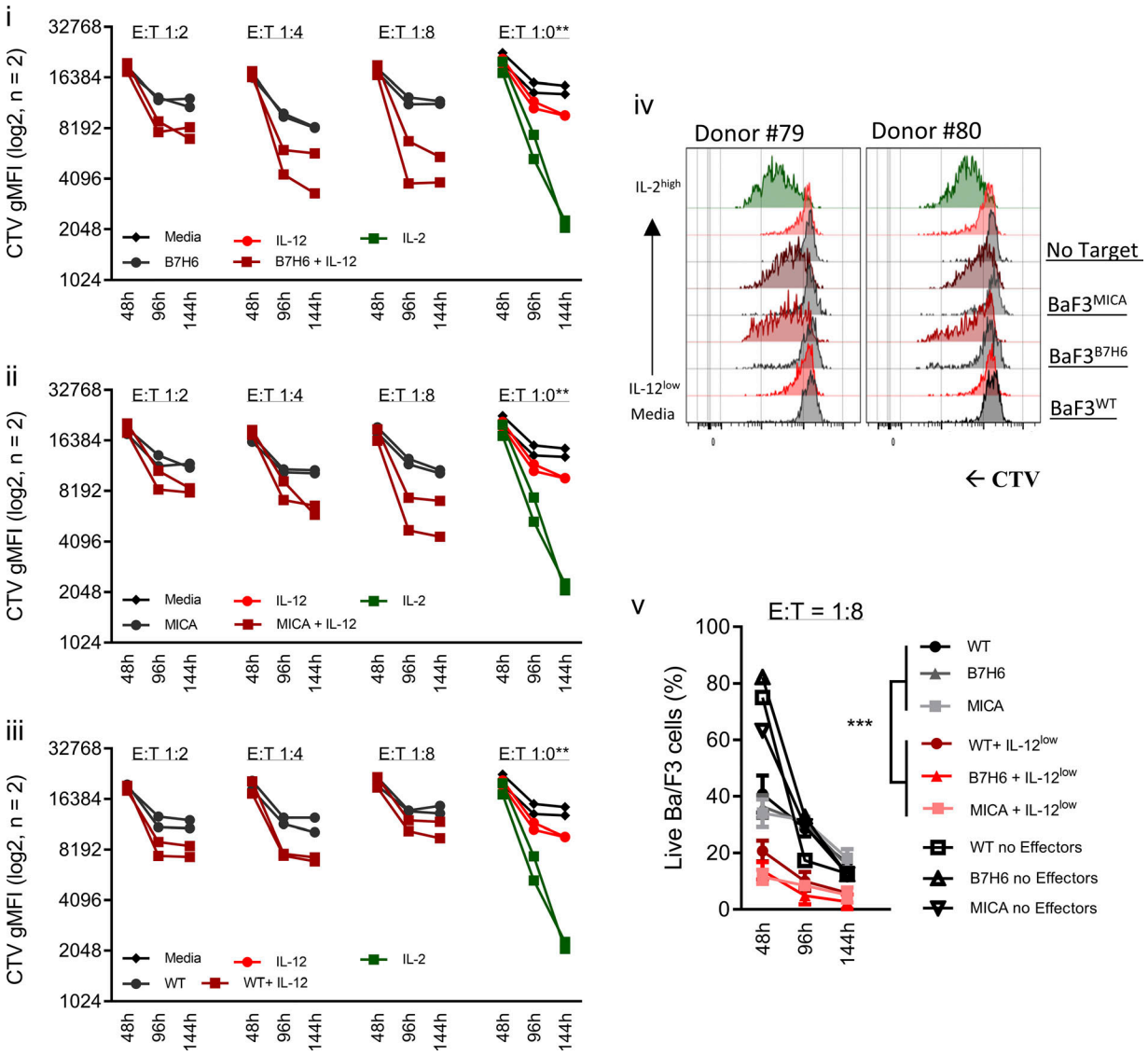

Figure S5. **IL-12–mediated NK cell proliferation during target cell co-culture.** Purified human peripheral blood NK cells were primed with IL-2^{high} (300 U/ml) for 10 d (long-term) prior to the indicated assays. Cytokines were added at the beginning of the cell culture. IL-2 was washed out prior to target cell co-culture. *n* = number of donors, each data line represents one donor measurement. **(i–iii)** Primed NK cells were co-cultured with Ba/F3 cells expressing NKp30 ligand (B7-H6; i), NKG2D ligand (MICA; ii), or without ligands (WT; iii), supplemented with IL-12^{low} (2.5 ng/ml) or media without cytokine. **(iv)** Representative histograms for CTV levels during NK cell and Ba/F3 cells co-culture. **(v)** Percentage of live target cells during the assay. Unpaired *t* test; ***, P < 0.001. **, NK cells cultured without Ba/F3 cells (no target, repeated data for comparison) were supplemented with IL-2 (300 U/ml), IL-12^{low}, or media without cytokines. Effector target ratio = 1:2, 1:4, 1:8. Ba/F3 cell viability without effectors decreased over time due to culture conditions (200 µl media, U-shaped 96-well plate).