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Celia C Cubitt

Ethan McClain

Michelle Becker-Hapak

Jennifer A Foltz

Pamela Wong

See next page for additional authors

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A novel fusion protein scaffold 18/12/TxM activates the IL-12, IL-15, and IL-18 receptors to induce human memory-like natural killer cells

Celia C. Cubitt,¹ Ethan McClain,¹ Michelle Becker-Hapak,¹ Jennifer A. Foltz,¹ Pamela Wong,¹ Julia A. Wagner,¹ Carly C. Neal,¹ Nancy D. Marin,¹ Lynne Marsala,¹ Mark Foster,¹ Timothy Schappe,¹ Patrick Soon-Shiong,² John Lee,² Melissa M. Berrien-Elliott,¹ and Todd A. Fehniger¹

¹Washington University School of Medicine, 425 South Euclid Avenue, Campus Box 8007, St. Louis, MO 63110, USA; ²ImmunityBio, Culver City, CA 90232, USA

Natural killer (NK) cells are cytotoxic innate lymphoid cells that are emerging as a cellular immunotherapy for various malignancies. NK cells are particularly dependent on interleukin (IL)-15 for their survival, proliferation, and cytotoxic function. NK cells differentiate into memory-like cells with enhanced effector function after a brief activation with IL-12, IL-15, and IL-18. N-803 is an IL-15 superagonist composed of an IL-15 mutant (IL-15N72D) bound to the sushi domain of IL-15R α fused to the Fc region of IgG1, which results in physiological trans-presentation of IL-15. Here, we describe the creation of a novel triple-cytokine fusion molecule, 18/12/TxM, using the N-803 scaffold fused to IL-18 via the IL-15N72D domain and linked to a heteromeric single-chain IL-12 p70 by the sushi domain of the IL-15R α . This molecule displays trispesific cytokine activity through its binding and signaling through the individual cytokine receptors. Compared with activation with the individual cytokines, 18/12/TxM induces similar short-term activation and memory-like differentiation of NK cells on both the transcriptional and protein level and identical *in vitro* and *in vivo* anti-tumor activity. Thus, N-803 can be modified as a functional scaffold for the creation of cytokine immunotherapies with multiple receptor specificities to activate NK cells for adoptive cellular therapy.

INTRODUCTION

Natural killer (NK) cells are cytotoxic innate lymphoid cells that make up approximately 5%–20% of circulating blood lymphocytes and are important in the elimination of virally infected and malignantly transformed cells.^{1,2} NK cell function is tightly regulated by a balance of germline encoded activating, co-stimulatory, and inhibitory receptors expressed at the cell surface.^{3,4} Through these receptors, NK cells are able to recognize and spontaneously kill cells through the loss of self-identifying molecules, such as major histocompatibility complex (MHC) class I, that bind to inhibitory receptors on NK cells (detection of missing self) or by upregulating ligands recognized by activating receptors on NK cells that can overcome inhibitory signals.⁵ Human NK cells are identified by surface expression of CD56 and the absence of CD3 and can be categorized based on relative CD56

expression into the distinct CD56^{bright} and CD56^{dim} subsets, where CD56^{dim} NK cells typically express the Fc γ RIII (CD16), while CD56^{bright} NK cells have low or no expression.^{1,6}

NK cells constitutively express a number of cytokine receptors and are particularly dependent on IL-15 for development, homeostasis, and function.^{7–9} IL-15 signaling has been shown to promote the survival, proliferation, and priming (at higher doses) of CD56^{bright} NK cells and to enhance the cytotoxicity of the CD56^{dim} subset.^{10,11} There are three receptor subunits for IL-15 receptor forms: IL-15R α (CD25), IL-15R β (CD122), and IL-15R γ (CD132). The signaling components of the IL-15 receptor are not private, with its β subunit shared with IL-2, and its γ subunit (common γ chain) with IL-2, IL-4, IL-7, IL-9, and IL-21. Physiologically, IL-15 mediates its effects through trans-presentation, whereby the high-affinity IL-15R α is expressed on the surface of accessory cells (such as dendritic cells and monocytes/macrophages) that present IL-15 to NK cells bearing the IL-15R $\beta\gamma$.^{12–14} In addition to the effects mediated by IL-15, the cytokines IL-12 and IL-18 are also important for NK cell survival and function. The primary effect of IL-12 on NK cells occurs via STAT4-mediated signaling and includes interferon- γ (IFN- γ) and tumor necrosis factor (TNF) production.^{15,16} IL-18 transduces signals that lead to mitogen-activated protein kinase (MAPK) and nuclear factor κ B (NF- κ B) activation and has been described to function synergistically with IL-12 and IL-15, while also priming NK cells for IFN- γ production.^{17–20} Indeed, paradigm-shifting studies have demonstrated that combined activation with IL-12, -15, and -18 induces memory-like (ML) NK cells defined by enhanced proliferation, expression of the high-affinity IL-2 receptor $\alpha\beta\gamma$ (IL-2R $\alpha\beta\gamma$), and increased IFN- γ production after re-stimulation with cytokines, tumors, or via activating receptors.^{21–23} These cytokine-induced ML NK cells represent a promising NK cell therapy and have shown encouraging results in

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Correspondence: Todd A. Fehniger, MD/PhD, Washington University School of Medicine, 425 South Euclid Avenue, Campus Box 8007, St. Louis, MO 63110, USA.
E-mail: tfehnige@wustl.edu



first-in-human clinical trials for relapsed/refractory acute myeloid leukemia (AML) patients.^{21,24}

N-803 is an IL-15 superagonist comprised of an IL-15 mutant (IL-15N72D) bound to the N-terminal structural (sushi) domain of IL-15R α fused to the Fc region of immunoglobulin G1 (IgG1).²⁵ This results in accessory-cell-independent trans-presentation of IL-15, prolonged *in vivo* pharmacokinetics, increased *in vivo* biological activity, and increased effector functions compared with IL-15.^{25–30} Given the potent functional effects induced by the combined stimulation of IL-12, -15, and -18, we hypothesized that the construction of a single molecule that could signal through all three cytokine pathways would be beneficial for generating ML NK cells for both research and clinical applications. As such, we constructed a fusion protein, 18/12/TxM, employing N-803 as a scaffold, linking IL-18 to the IL-15N72D domain and a heteromeric single-chain IL-12 p70 to the sushi domain of IL-15R α , which is already linked to the Fc domain of human IgG1. This non-covalently associated, heterodimeric homodimer, triple-cytokine fusion protein retained specific and unique IL-18, IL-12, and IL-15 activities *in vitro* and *in vivo*.

The stability of the N-803 structure provides a biochemical strategy to decorate the backbone with additional components, while maintaining IL-15-based signals. Here we investigated the ability of the novel 18/12/TxM fusion protein to generate ML NK cells, compared with the combination of individual recombinant human (rh) IL-12, -15, and -18. This included *in vitro* examination of individual activity of each cytokine receptor signaling, short-term effector function, and ability to generate ML NK cells.

RESULTS

The fusion 18/12/TxM superkine induces signaling via all targeted cytokine receptors

We first sought to construct a fusion protein consisting of human IL-18, IL-12, and IL-15 to replace individual use of the recombinant cytokines. To this end, N-803 (an IL-15 superagonist formerly known as ALT-803) was linked to IL-18 via the IL-15N72D domain and linked to heteromeric single-chain IL-12 p70 by the sushi domain of the IL-15R α connected to the Fc domain of human IgG1 (Figure 1A). The ability of this triple-cytokine fusion protein (henceforth referred to as 18/12/TxM) to induce signaling via all targeted cytokine receptors was assessed. Freshly isolated and purified NK cells were stimulated with 18/12/TxM (38.8 nM) or the optimal combination of rhIL-12 (10 ng/mL), IL-18 (50 ng/mL), and IL-15 (50 ng/mL) (IL-12/15/18) and assessed for their ability to induce phosphorylation of key signaling intermediates at various time points; 18/12/TxM induced IL-15 signaling through phosphorylation of STAT5, AKT, and ERK with similar efficiency to IL-12/15/18 stimulation in both CD56^{bright} and CD56^{dim} NK cell subsets (Figure 1B). At higher concentrations, 18/12/TxM (77.6 nM) induced slightly higher phosphorylated (p) ERK in CD56^{dim} cells and slightly lower pAKT in CD56^{bright} NK cells (Figure S1A). Phosphorylation of STAT4 by IL-12 signaling displayed modest but statistically significant difference at the lower TxM dose (38.8 nM) in CD56^{bright} NK cells ($p = 0.005$), with no difference

observed at the higher TxM concentration (77.6 nM) or in CD56^{dim} NK cells (Figures 1C and S1B). Phosphorylation of p65 via IL-18 signaling was similarly induced by 18/12/TxM and IL-12/15/18 (Figures 1D and S1C). Next, the ability of 18/12/TxM to activate individual cytokine bioassays was assessed. To elucidate the IL-15 activity, proliferation of a mouse hematopoietic cell line, the IL-2/15-dependent 32D-IL-2/15R β (32D β) cells, was assessed. The ability of 18/12/TxM to promote cell proliferation was reduced compared with that of N-803 (half-maximal effective concentration [EC₅₀] of 1.7 nM versus 0.03 nM for N-803), possibly due to the linkage of IL-18 to the IL-15N72D domain (Figure 1E). To determine the IL-12 activity of 18/12/TxM, activation of the IL-12 reporter HEK-Blue (HEK12) cells, which express a STAT4-inducible secreted embryonic alkaline phosphatase (SEAP) gene, was assessed. The EC₅₀ of 18/12/TxM was 99.1 pM and 86.1 pM for rhIL-12, which demonstrates similar bioactivity to recombinant IL-12 (Figure 1F). Finally for IL-18, IL-18 reporter HEK-Blue (HEK18) cells, which express an NF- κ B/activator protein (AP)-1-inducible SEAP gene, were plated with increasing concentrations of 18/12/TxM. The EC₅₀ of 18/12/TxM was 7.1 pM, which was about 13-fold reduced compared with recombinant IL-18 (EC₅₀ of 0.54 pM), potentially due to the linkage of IL-18 to IL-15N72D (Figure 1G). Collectively, these data support that 18/12/TxM at adequate concentrations stimulates signals via the IL-12, -15, and -18 receptors.

Short-term activation with 18/12/TxM superkine results in NK cell activation

Short-term activation of human NK cells with IL-12, -15, and -18 leads to increased expression of the IL-2 receptor α (IL-2R α , CD25) and enhanced production of IFN- γ .^{21,23,31} To evaluate the optimal concentration for 18/12/TxM activation, purified human NK cells were activated *ex vivo* for 16 h with increasing concentrations of 18/12/TxM or IL-12/15/18.²³ Induction of an activated phenotype was assessed as increased cell surface CD25 expression and intracellular IFN- γ , as compared with control, resting NK cells, as determined by flow cytometry (Figure 2A). The optimal concentration for maximal induction of CD25 was reached at 38.8 nM 18/12/TxM, with an EC₅₀ of 2.095 nM (Figure 2B). Short-term activation of purified human NK cells with 18/12/TxM at 38.8 nM or IL-12/15/18 demonstrated similar induction of CD25 over control NK cells (Figures 2C and 2D). Similarly, near maximal induction of IFN- γ was reached at 38.8 nM, with an EC₅₀ of 2.64 nM (Figure 2E). Short-term activation demonstrated similar induction of intracellular IFN- γ with 18/12/TxM compared with IL-12/15/18, although both were significantly higher than the IL-15 (1 ng/mL) control (Figures 2F and 2G). Collectively, these data show that the 18/12/TxM fusion protein results in nearly identical short-term activation via the IL-12, -15, and -18 receptors, resulting in IFN- γ and CD25 expression, compared with the combination of rhIL-12, -15, and -18.

Activation with 18/12/TxM superkine stimulates NK cell proliferation

Previous studies have demonstrated that activation with IL-12, -15, and -18 leads to an ML phenotype that includes robust proliferation and expansion of NK cells.^{21–23} To address the ability of 18/12/TxM

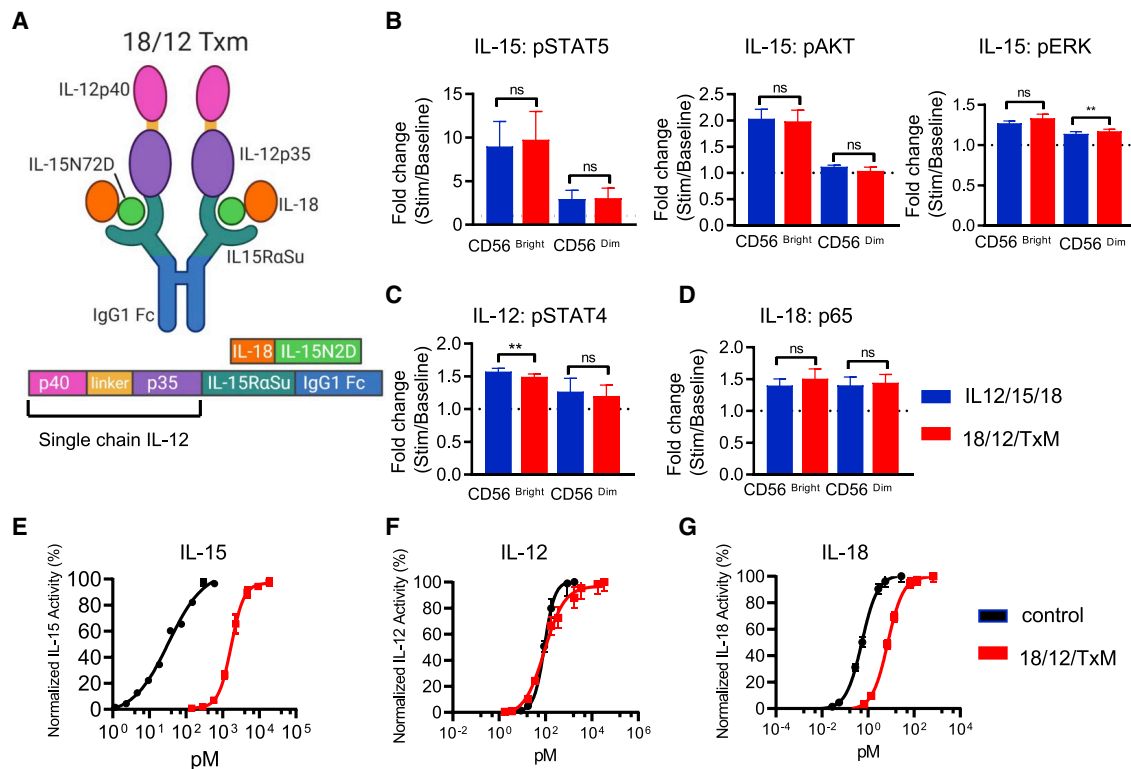


Figure 1. A fusion protein superkine (18/12/TxM) combining IL-15, -12, and -18 induces signaling via all targeted cytokine receptors

(A) Diagram showing structure of the 18/12/TxM molecule. (B–G) Freshly isolated NK cells from 3 to 5 healthy donors were stimulated with IL-12 (10 ng/mL), IL-15 (50 ng/mL), or IL-18 (50 ng/mL) (IL-12/15/18) or 18/12/TxM (38.8 nM) and assessed at various time intervals, gating on CD56^{bright} and CD56^{dim} NK cells. Summary data showing fold change of phosphorylated (B) STAT5, AKT, and ERK, (C) STAT4, or (D) p65 after stimulation. Fold change is calculated as the median fluorescence intensity of cytokine-stimulated (stim) over unstimulated (baseline) NK cells. Dotted line at $y = 1$ represents no change over the unstimulated condition. Data shown are mean \pm SEM and compared using paired t test. $N = 3$ –5 human donors. ns, not significant, * $p < 0.05$, ** $p < 0.01$) (E–G) Assessment of individual cytokine activity was performed using reporter cell lines. (E) Proliferation of the IL-15-dependent 32 β cell line was assessed 3 days after incubation with various concentrations of 18/12/TxM or N-803 (F). Bioactivity was measured following incubation with various concentrations of IL-12 or 18/12/TxM with HEK12 cells (G). Bioactivity was measured following incubation with various concentrations of IL-18 or 18/12/TxM with HEK18 cells.

to induce proliferation, purified human NK cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), activated *ex vivo* for 16 h with 18/12/TxM (38.8 nM), IL-12/15/18, or IL-15 only. After activation, NK cells (including both CD56^{dim} and CD56^{bright} subsets) were washed and rested in IL-15 for 6 days. In agreement with previous data, activation with IL-12/15/18 or 18/12/TxM induced robust proliferation, as compared with those activated with IL-15 only (Figures 3A and 3B).^{21,24} Interestingly, activation with 18/12/TxM in this set of experiments resulted in modestly enhanced proliferation compared with IL-12/15/18, with an increased proportion and number of NK cells expanding beyond 3 generations (Figures 3B and S2A). This increased cell cycling with 18/12/TxM was not attributed to differences in viability between the activating conditions (Figure S2B).

Multidimensional phenotypic changes are similar between IL-12/15/18- and 18/12/TxM-induced memory-like NK cells

ML NK cells undergo dramatic changes in a large number of cell surface and intracellular markers both immediately after activation with

IL-12/15/18 and 6 days after differentiation.²⁴ A custom mass cytometry panel was previously developed, including markers for NK cells lineage, maturation, and functional capacity (Table S1) and identified an ML NK cell multidimensional phenotype.^{21,24} To compare the multidimensional phenotypes, human NK cells were profiled using mass cytometry before activation (baseline), after 16 h of incubation with IL-12/15/18 or 18/12/TxM (D1) and 6 days post-activation to allow time for ML differentiation (D6). Using the mean expression of markers, t-distributed stochastic neighbor embedding (tSNE) analysis revealed distinct NK cell populations for baseline, day 1 (D1), and day 6 (D6) after activation. Notably, the same particular clustering NK cell subsets was identified when activated with either IL-12/15/18 or 18/12/TxM (Figure 4A). Furthermore, comparison of the changes in mean expression after overnight activation of well-defined markers of acute NK cell activation, such as increased CD25, CD69, and CD137 and decreased CD56 and CD16, were identical between IL-12/15/18- and 18/12/TxM-activated NK cells (Figures 4B and S3A). In accordance with previous studies of differentiated ML NK cells, both IL-12/15/18- and 18/12/TxM-activated NK cells

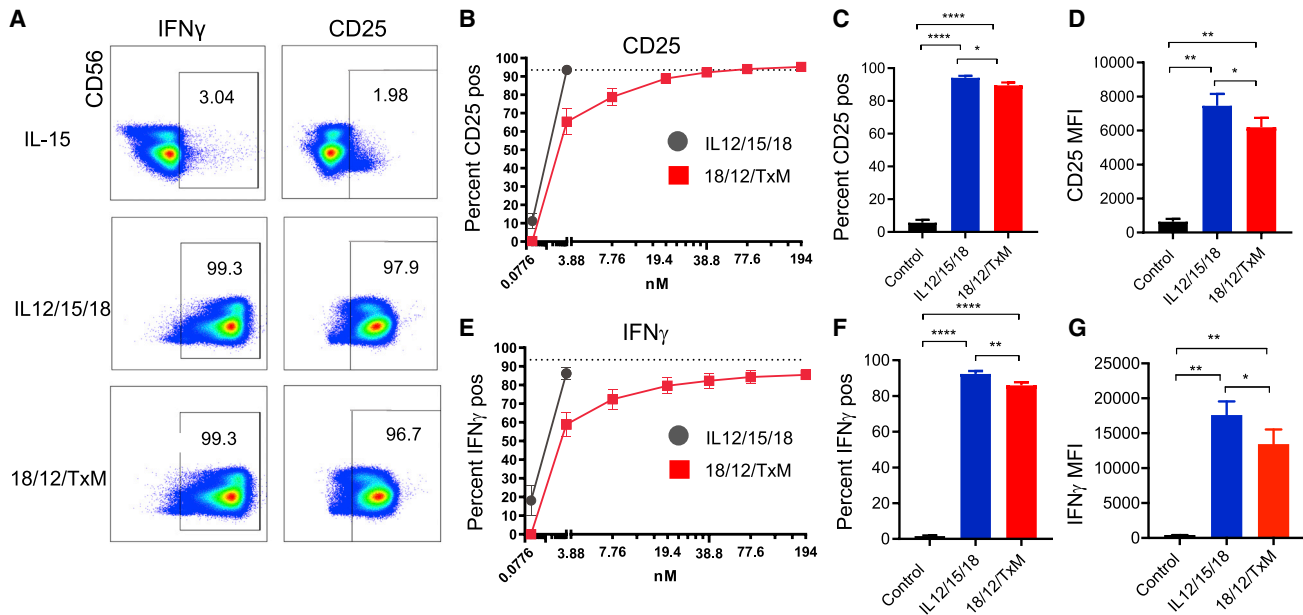


Figure 2. Short-term activation with 18/12/TxM superkine results in NK cell activation, inducing IFN- γ and CD25 expression, and increased cytotoxicity (A–G) Freshly isolated NK cells were activated for 16 h with increasing concentrations of 18/12/TxM or IL-12 (10 ng/mL) + IL-15 (50 ng/mL) + IL-18 (50 ng/mL) and assessed for the expression of indicated markers. (A) Representative flow plots showing IFN- γ and CD25 expression. (B) NK cells were incubated with varying concentrations of 18/12/TxM to identify the optimal concentration for maximal induction of CD25. (C and D) Summary data from NK cells stimulated for 16 h with 38.8 nM 18/12/TxM. CD25 expression shown as (C) percent CD25-positive NK cells and (D) CD25 mean fluorescence intensity (MFI). (E) NK cells were incubated with varying concentrations of 18/12/TxM to identify to optimal concentration for maximal induction of IFN- γ . (F and G) Summary data from NK cells stimulated for 16 h with 38.8 nM 18/12/TxM. IFN- γ expression shown as (F) percent IFN- γ -positive NK cells and (G) IFN- γ MFI. Data shown as mean \pm SEM and were compared using RM one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 6$ donors, 2 independent experiments.

demonstrated similar upregulation of NKG2A, CD69, Ki67, CD25, CD137, granzyme B, perforin, and the activating receptors NKp44, NKG2D, and CD94 at D6. They also demonstrated similar downregulation of CD56, CD16, CD57, NKp30, and NKp80, as has been previously reported (Figure 4C).^{21,24} The RNA expression levels of inhibitory receptors, such as TIGIT, TIM3, and LAG3, were also similar between activating conditions (Figure S3B). These data indicate that 18/12/TxM activation results in short- and long-term changes in NK cells, similar to IL-12, -15, and -18 activation.

18/12/TxM induces functional memory-like NK cells *in vitro*

Previous studies have shown that ML NK cells can be induced *ex vivo* following overnight stimulation of purified NK cells with saturating amounts of IL-12/15/18. These cells exhibit ML properties, such as (1) enhanced proliferation, (2) expression of IL-2R α , (3) increased IFN- γ production, and (4) augmented cytotoxicity mediated by perforin and granzymes.^{21–23} To demonstrate generation of ML NK cells by 18/12/TxM, primary human NK cells were activated with 18/12/TxM (38.8 nM), IL-12/15/18, or IL-15 for 16 h, washed, and supported in IL-15 for 6 days to allow ML differentiation (Figure 5A). The production of IFN- γ as a functional readout for the generation of ML NK cells was assessed following 6 h re-stimulation with cytokines (IL-12 [10 ng/mL] and IL-15 [50 ng/mL]) or leukemia targets (K562 cells) (Figure 5B). Activation with 18/12/TxM induced

IFN- γ expression to a slightly greater extent than IL-12/15/18 after K562 stimulation (Figure 5C). Expression of IFN- γ after IL-12 + IL-15 stimulation was slightly higher in IL-12/15/18-activated NK cells, compared with 18/12/TxM, but was robustly induced over the IL-15 control in both conditions (Figure 5C). Induction of CD107a (a surrogate marker for degranulation) between IL-15, IL-12/15/18, and 18/12/TxM after K562 stimulation was similar, which is consistent with previous reports that degranulation is not affected by ML differentiation (Figures S4A–S4C).²² Interestingly, activation with 18/12/TxM resulted in higher expression of TNF even without stimulation, suggesting that 18/12/TxM is inducing higher baseline expression of this cytokine (Figures S4D–S4F). In addition to cytokine secretion, the ability of 18/12/TxM to promote tumor killing was assessed in a standard 4-h cytotoxicity assay using K562 target cells. Specific killing of target cells was identical between IL-12/15/18- and 18/12/TxM-activated NK cells and greater than IL-15 NK cells at all effector to target (E:T) ratios evaluated (Figure 5D).

18/12/TxM activation induces a molecular program similar to IL-12, IL-15, and IL-18

To complement the phenotypic and functional similarities induced by 18/12/TxM and IL-12/15/18, we performed bulk RNA sequencing (RNA-seq). Purified NK cells from three different donors were isolated, and RNA was isolated before (baseline), after overnight

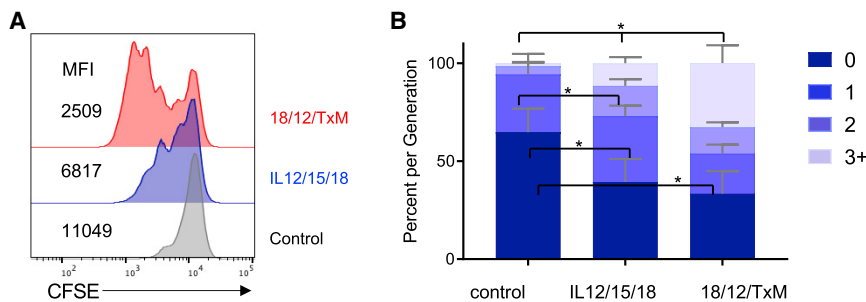


Figure 3. Activation with 18/12/TxM superkine stimulates NK cell proliferation, similar to IL-12/15/18

Purified NK cells were labeled with CFSE to track cell division and activated for 16 h with either IL-15 (1 ng/mL IL-15) or IL-12/15/18 (10 ng/mL IL-12 + 50 ng/mL IL-15 + 50 ng/mL IL-18) or with 38.8 nM 18/12/TxM. After incubation, cells were washed 3 times to remove the pre-activating cytokines and cultured in IL-15. After 7 days, cells were analyzed for CFSE dilution. (A) Representative bivariate plots of both CD56^{bright} and CD56^{dim} NK cells demonstrating cell division (CFSE dilution) with 18/12/TxM and IL-12/15/18. (B) Summary results showing

enhanced proliferation of both CD56^{bright} and CD56^{dim} NK cells 7 days after activation with 18/12/TxM or IL-12/15/18, compared with IL-15 controls. Summary results are shown as the mean \pm SEM of the percentage of cells per generation ($n = 4$ donors, 2 independent experiments). Comparisons made individually between conditions using one-way repeated measures ANOVA ($*p < 0.05$, $**p < 0.01$). Purified NK cells were $\geq 95\%$ CD56⁺CD3⁻ with $< 0.5\%$ CD3⁺ T cells.

activation (D1), and 6 days supported by IL-15 (D6) after activation with 18/12/TxM, IL-12/15/18, or IL-15. Analysis of transcript counts revealed a similar gene expression profile on D1 after activation between IL-12/15/18- and 18/12/TxM-activated NK cells, when contrasted to IL-15 only. Analysis of the genes that were significantly differentially expressed ($p < 0.05$) on D1 following IL-12/15/18 or 18/12/TxM stimulation demonstrated that the vast majority (5,812 genes) of changes were shared after either treatment, with 808 unique genes expressed in the TxM condition and 332 unique genes following IL-12/15/18 treatment (Figure 6A). Indeed, when directly comparing genes expressed in NK cells activated with 18/12/TxM or IL-12/15/18, their expression profiles were nearly identical ($r^2 = 0.9679$) 1 day after activation (Figure 6B). Consistent with phenotypic observations on D1, brief activation with both 18/12/TxM and IL-12/15/18 led to a dramatic increase in expression of *IL2RA* (CD25), *IFNG* (IFN- γ), *GZMB* (granzyme B), *LTA* (lymphotoxin alpha), *TNFSF4* (OX40L), *NIFK* (nucleolar protein interacting with the FHA domain of MKI67), *CCL3* (*C-C motif chemokine ligand 3*), and *CSF2* (granulocyte-macrophage colony-stimulating factor [GM-CSF]) (Figures 6C and 6D; Table S2). On D6 of differentiation after activation supported by IL-15, there were no statistically significant differentially expressed genes between IL-15- and 18/12/TxM- or IL-12/15/18-activated NK cells. While the vast majority of gene expression changes were minimal (log fold change [FC] < -0.5 or > 0.5), a direct comparison of genes induced in 18/12/TxM- and IL-12/15/18-activated NK cells on D6 that trended differently compared with IL-15 only (logFC > 1 or < -1) revealed similar changes between the two treatment groups (Figure 6E). Despite the dramatic molecular activation profile 1 day after activation, by D6 there were no statistically significant genes that were differentially expressed between IL-15- and IL-12/15/18-activated NK cells (Table S2). This is consistent with previous work done in our laboratory and is likely due to the heterogeneity of NK cells at D6 that underwent ML differentiation and the overwhelming IL-15-induced transcriptional profile that may mask a unique transcriptional profile. Some differentially expressed genes trended differently between IL-15- and 18/12/TxM- or IL-12/15/18-treated NK cells at D6, including increased expression of *CXCR6* (C-X-C motif chemokine receptor 6), *CCR1* (C-C motif chemokine receptor 1),

and *GZMK* (granzyme K) (Table S2). A direct comparison of the gene expression changes induced by 18/12/TxM or IL-12/15/18 showed no statistically significant differences, suggesting that they are inducing similar transcriptional profiles by D6 (Figure 6F). Thus, using bulk RNA-seq approaches on enriched NK cells, 18/12/TxM and IL-12/15/18 induce nearly identical transcriptional changes after 24 h and were both distinct from control NK cells. However, this analysis approach does not identify significant differences between D6 ML NK cells induced with either initial activation. Based on the subsets of ML NK cells with enhanced function, we expect that only a proportion of cells at D6 represent functional ML NK cells with unique transcriptional signatures. In this setting, single-cell RNA-seq approaches would be required to identify subset-based transcriptome changes.

18/12/TxM induces memory-like NK cell anti-tumor activity *in vivo*

To confirm that the molecular and phenotypic changes induced *in vitro* by 18/12/TxM would also translate to enhanced *in vivo* functionality, we compared the anti-tumor activity in NOD-SCID-IL-2 R $\gamma^{-/-}$ (NSG) mice engrafted with leukemia.^{21,24} Briefly, NSG mice were engrafted with luciferase-expressing K562 tumor cells (1×10^6 cells/mouse) and injected 4 days later with NK cells ($3\text{--}5 \times 10^6$ cells/mouse) that had been pre-activated with 18/12/TxM, IL-12/15/18, or IL-15 only (Figure 7A). Tumor growth was assessed using whole body bioluminescence imaging (BLI) on days 3, 11, 17, and 24 (Figures 7B and S5). ML NK cells induced with either IL-12/15/18 or 18/12/TxM demonstrated enhanced tumor control at day 17 (Figure 7C). These data demonstrate that activation of NK cells with 18/12/TxM can induce an ML NK cell phenotype, similar to that induced by IL-12/15/18, which exerts enhanced control of tumor targets *in vivo*.

DISCUSSION

Here, the novel fusion protein 18/12/TxM was constructed using N-803 as a scaffold, linking IL-18 to the IL-15N72D domain and linking heteromeric single-chain IL-12 p70 to the sushi domain of IL-15R α , which is already linked to the Fc domain of human IgG1.

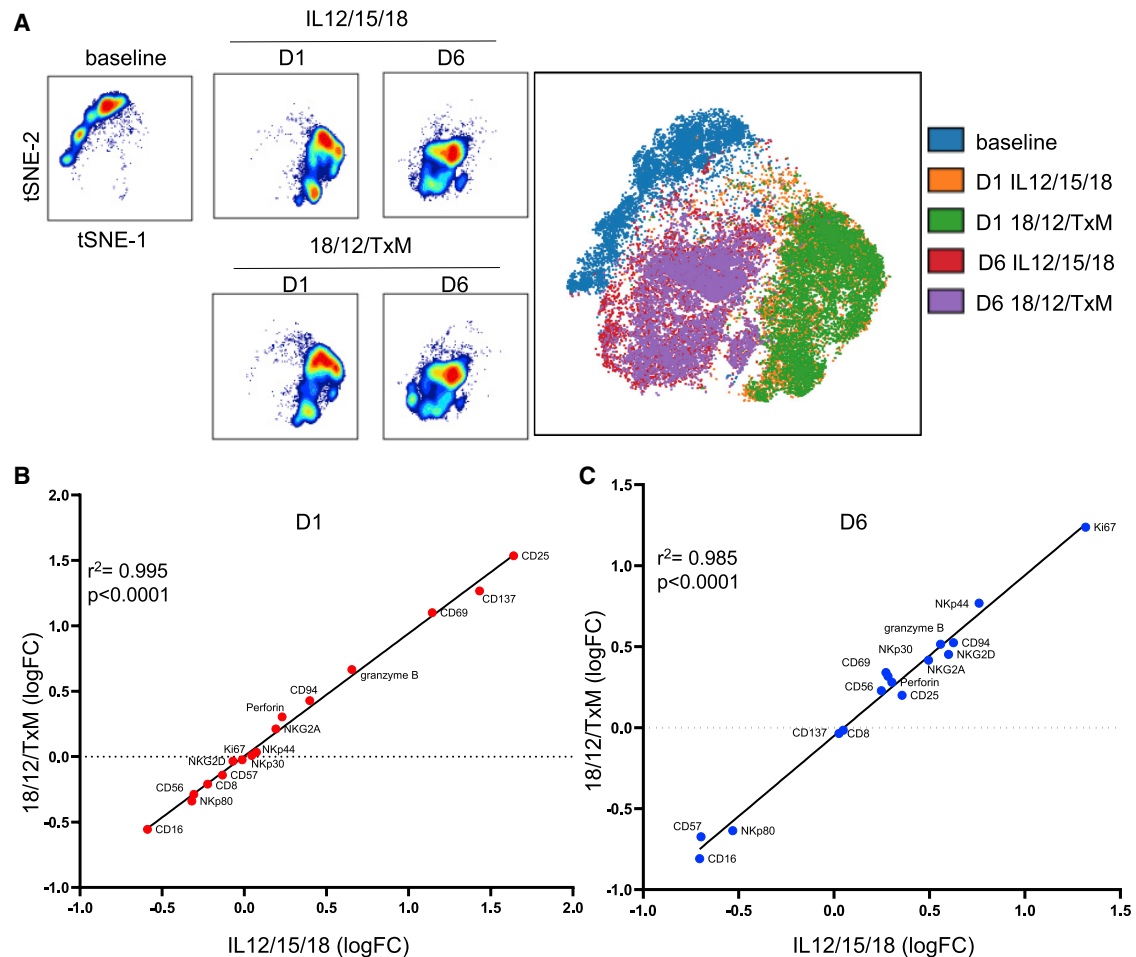


Figure 4. Multidimensional phenotype changes in NK cells are comparable after 18/12/TxM and IL-12/15/18 activation

Mass cytometry analysis reveals similar changes in NK cell phenotype. Freshly isolated human NK cells were activated for 16 h with 18/12/TxM or IL-12/15/18 and assessed for the expression of 36 markers using mass cytometry at baseline or at day 1 or 6 after activation. (A) Representative viSNE maps from 1 donor showing NK cell populations at baseline and at 1 or 6 days after activation with IL-12/15/18 or 18/12/TxM. Overlaying these populations demonstrates similar population-level changes between activating conditions. (B and C) Data reported as the average log fold change over baseline of the mean expression (B) day 1 or (C) day 6 after activation ($n = 4$ donors, 2 independent experiments). r^2 value generated by simple linear regression.

This non-covalently associated, heterodimeric homodimer, triple-cytokine fusion protein retained specific and unique IL-18, -12, and -15 activities *in vitro*, as measured by activation, proliferation, and signaling through cognate receptors. Furthermore, 18/12/TxM exhibited functions equivalent to the combination of the individual cytokines when used at the appropriate concentration on primary NK cells *ex vivo* after overnight stimulation and after 6 days of *in vitro* differentiation into ML NK cells and *in vivo* in NSG mice. The ability of 18/12/TxM to induce this ML phenotype was confirmed at the protein and transcriptional level using high-dimensional methods, including mass cytometry phenotyping and bulk RNA-seq. These phenotypic changes translated to equivalent cytotoxic effector functions *in vitro*, including specific killing and cytokine secretion. Additionally, despite the expected variation in the ability of different human donor NK cells to control K562-engrafted NSG

mice, NK cells activated with IL-12/15/18 or 18/12/TxM demonstrated similar tumor control *in vivo*. Thus, 18/12/TxM is an effective alternative to IL-12, -15, and -18 for the generation of ML NK cells.

Interestingly, activation with 18/12/TxM resulted in slightly greater levels of proliferation in purified NK cells than the individual cytokines combined. This finding suggests that there may be distinct biological outcomes resulting from simultaneous engagement of the IL-12, -15, and -18 receptors on the same cell rather than sequential activation of the receptors on the same or different cells. Another possibility is that the spatial linking of the cytokines on the scaffold that results in enhanced membrane clustering of cytokine receptors and signaling molecules as a result of binding from the trimeric molecule. It may also be possible that the Fc portion of 18/12/TxM is activating NK cells via downstream signaling events following engagement with

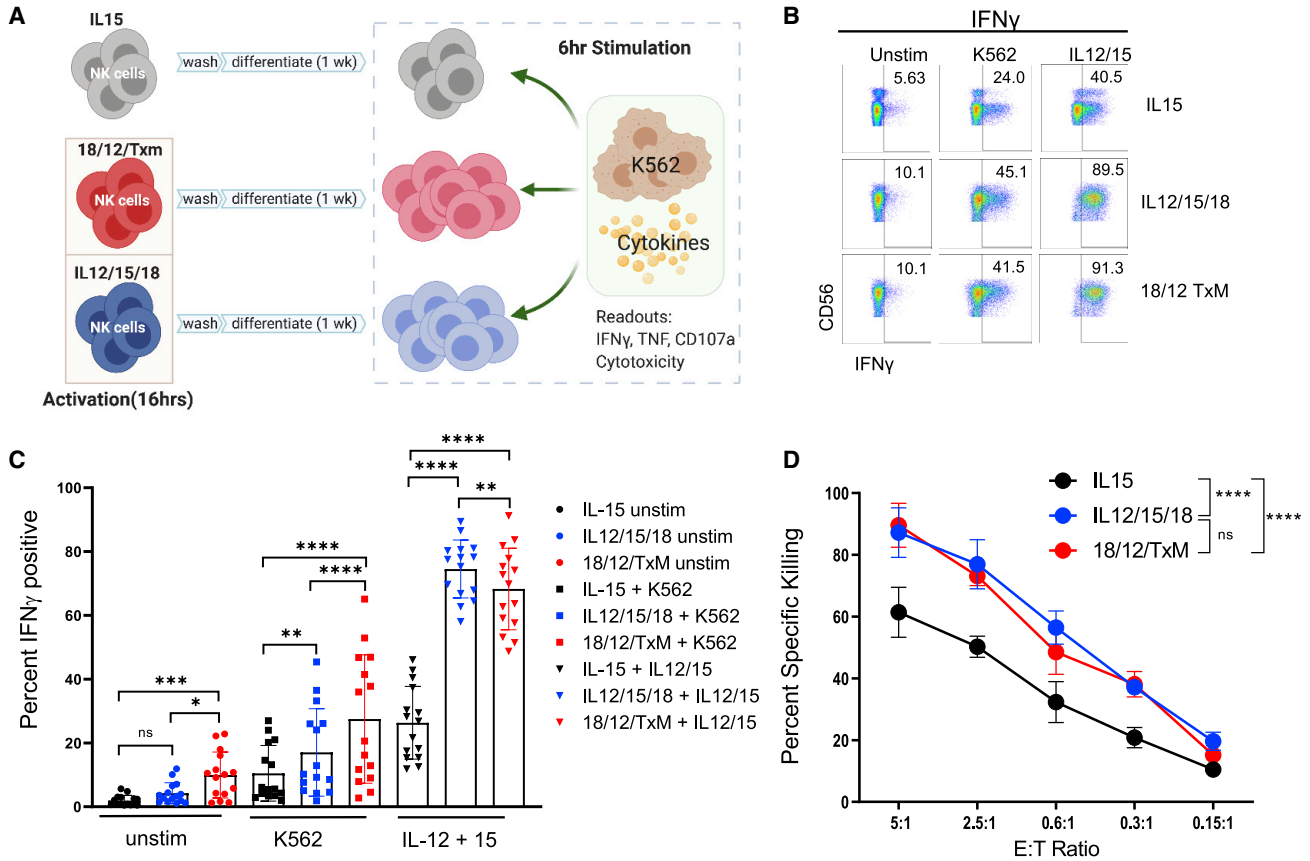


Figure 5. Activation with 18/12/TxM induces functional memory-like NK cells *in vitro*

(A) Functional assay schema. Briefly, NK cells from healthy donors were isolated and activated for 16 h with IL-15, 18/12/TxM, or IL-12/15/18, washed, and incubated in 1 ng/mL IL-15 for 1 week. Functional assessments were performed by stimulating the NK cells with K562 cells (E:T ratio 5:1) or IL-12 and IL-15 and assessed for the indicated markers by flow cytometry (n = 9 donors, 3 independent experiments). (B) Representative flow plot showing IFN- γ induction in K562 and IL-12 + IL-15 stimulated NK cells. (C) Summary data showing percent IFN- γ -positive NK cells stimulated with K562s or IL-12/15 as mean \pm SEM. Analysis performed using two-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). N = 15 unique human donors, 6 independent experiments. (D) Percent specific killing measured by chromium release following incubation with K562 cells at various E:T ratios (n = 4 donors, 2 independent experiments). Analysis performed using two-way ANOVA.

the Fc γ RIII receptor (CD16). However, given the observation that the increased proliferation occurred in both CD56^{dim} (CD16⁺) and CD56^{bright} (CD16⁻) NK cells, it may also be possible that Fc-FcR interactions allow CD16⁺ NK cells to readily trans-present the 18/12/TxM to nearby NK cells. Further investigation using an FcR null variant of the 18/12/TxM would be required to clarify this potential contribution.

Minimal differences in gene expression were observed between IL-15- and IL-12/15/18-activated NK cells at D6. This is consistent with previous observations that only some NK cells are able to fully undergo ML differentiation, therefore making it difficult to identify their gene signatures with bulk RNA-seq methods, following extensive IL-15-supported culture. Further studies using more in-depth sequencing methods, such as single-cell RNA-seq, will be essential for characterizing the unique transcriptional changes in ML differentiated NK cells. It is also possible that ML differentiation is represented primar-

ily by epigenetic rather than transcriptional changes, which can be clarified with methods, such as assay for transposase-accessible chromatin sequencing (ATAC-seq).

IL-15 has been used as an ideal candidate for clinical immunotherapy combinations due to its ability to stimulate NK cell (and CD8⁺ T cell) activation.^{26,32,33} However, physiological activation with IL-15 requires binding to the IL-15R α -chain prior to activating target cells, which limits the research and clinical roles of free IL-15. N-803 consists of human IgG1 Fc fused to two IL-15R α subunits bound to an IL-15 superagonist (N72D mutation that enhances biological activity), resulting in higher biological activity and longer serum half-life compared with free IL-15.^{25,26,28,34} Previous studies have demonstrated that preactivation of NK cells with IL-12/15/18 results in ML NK cell differentiation that represents a promising approach to enhancing adoptive allogeneic NK cell therapy.^{21,24} However, the use of these individual cytokines alone or in combination for research

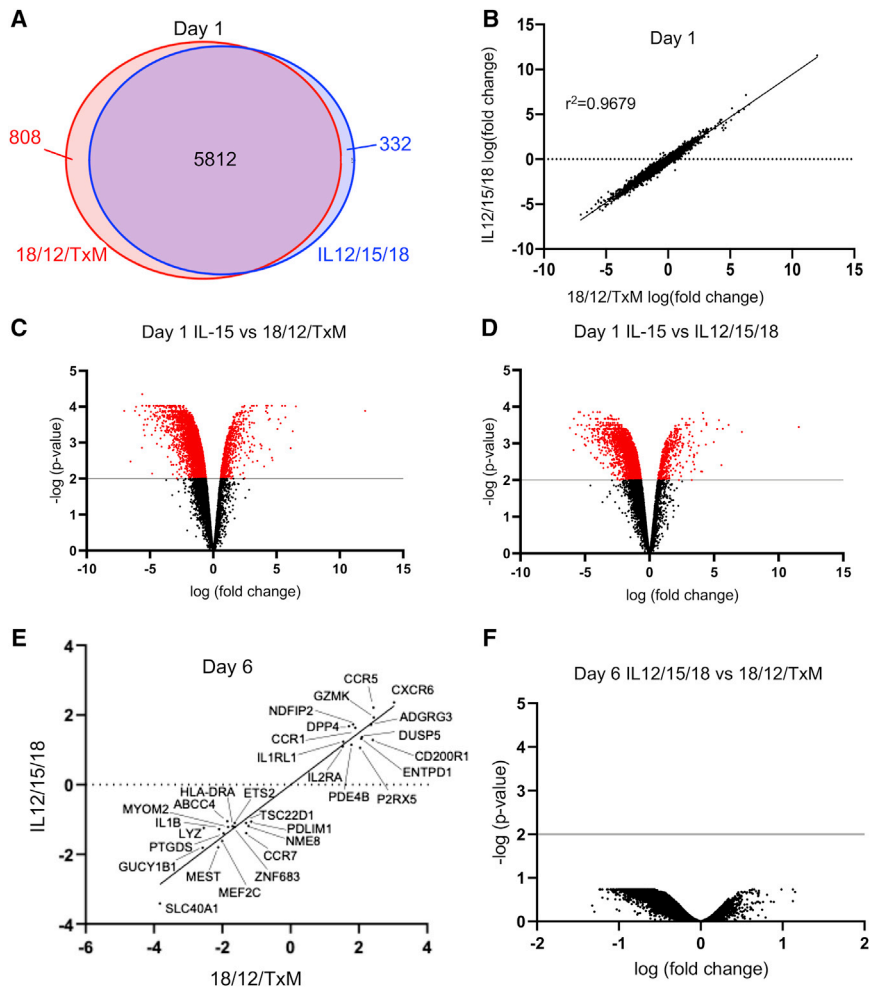


Figure 6. 18/12/TxM activation induces an ML NK cell molecular program

Bulk RNA sequencing data at day 1 (A–D), and (E–F) day 6 after 16 h activation with IL-15, IL-12/15/18, or 18/12/TxM. (A) Venn diagram demonstrating the number of statistically significant differentially expressed genes that were shared (purple) and distinct between IL-15 and 18/12/TxM (red) and IL-12/15/18 (blue) activated NK cells at day 1 ($p < 0.05$). (B) Scatterplot comparing the \log_2 (fold change) of genes induced after IL-12/15/18 or 18/12/TxM activation. (C and D) Volcano plots showing the number of differentially expressed genes between IL15 and (C) 18/12/TxM or (D) IL-12/15/18, 1 day after activation. (E) Scatterplot showing the \log_2 (fold change) of genes induced after IL-12/15/18 or 18/12/TxM activation, filtered to show genes with a \log_2 fold change greater than 1 or less than -1 . (F) Scatterplot showing similar gene induction between conditions at day 6. RNA sequencing analysis performed using Phantasus. Differential gene expression analysis performed using the LIMMA package. $N = 3$ different donors per condition. Data shown are representative from 2 independent experiments using 3 donors each.

beyond the scope of this study. Additionally, the use of the N-803 protein scaffold linked to three distinct cytokine targets represents a novel method to expand and stimulate NK cells *ex vivo* for adoptive cell therapy.

MATERIALS AND METHODS

Recombinant proteins

hIL18/IL12/TxM protein (molecular weight 245 kDa), lot #305-86 and #305-110, and N-803 protein (molecular weight 92.36 kDa), lot #01062016, were manufactured and purified at Altor BioScience (Miramar, FL). Endotoxin-free, rhIL-12 (BioLegend, San Diego, CA), IL-15 (Miltenyi, Bergisch Gladbach, Germany), IL-18 (InvivoGen, San Diego, CA), and IL-2 (R&D Systems, Minneapolis, MN) were used in these studies.

Purification of 18/12/TxM

Purification of 18/12/TxM was from clarified CHO cell culture supernatant by the following steps. Supernatant was loaded onto a MabSelect SuRe (from Cytiva, Marlborough, MA, formerly GE Healthcare) equilibrated with 4 volumes of 50 mM Tris 1M NaCl, pH 7.0, followed by washing the column with 4 volumes of the same buffer. The column was further washed with 4 volumes of 0.1M sodium citrate, pH 5.0 to remove contaminating proteins. The 18/12/TxM protein was eluted off the column with 0.1M glycine-HCl, pH 3.5. The eluted protein was then adjusted to pH 3.6 with 1M citric acid and incubated at room temperature for 1 h to inactivate viruses. A 1M Tris base was then added to this sample to bring the pH to 4.2 and then loaded onto a Capto Q (from Cytiva)

and clinical purposes can be subject to production issues and lot variability. Additionally, this superkine presents a promising platform for exchanging out different NK cell activating cytokines (IL-2 and IL-21) or tumor targeting molecules (e.g., CD20, EGFR, HER2, or CD34) to direct activated NK cells to kill tumor cells. Indeed, N-803 has been used as a functional scaffold fused with CD20 targeting antibody components and demonstrated superior anti-tumor activity than the individual components alone.³⁵ Other studies have demonstrated enhanced anti-tumor activity when N-803 is used in combination with either tumor targeting or checkpoint inhibitory antibodies, which represents a promising avenue for the development of additional fusion proteins.^{30,36,37}

Here, we demonstrate that the fusion of three distinct cytokines via a human IgG1Fc connection induced equivalent activity to the individual cytokines combined, *in vitro* and *in vivo*. While these studies used 18/12/TxM to briefly pre-activate NK cells *in vitro*, before rigorous washing and infusion, the Fc backbone could potentially confer additional *in vivo* half-life, similar to N-803, which could support its use *in vivo* in other contexts that are

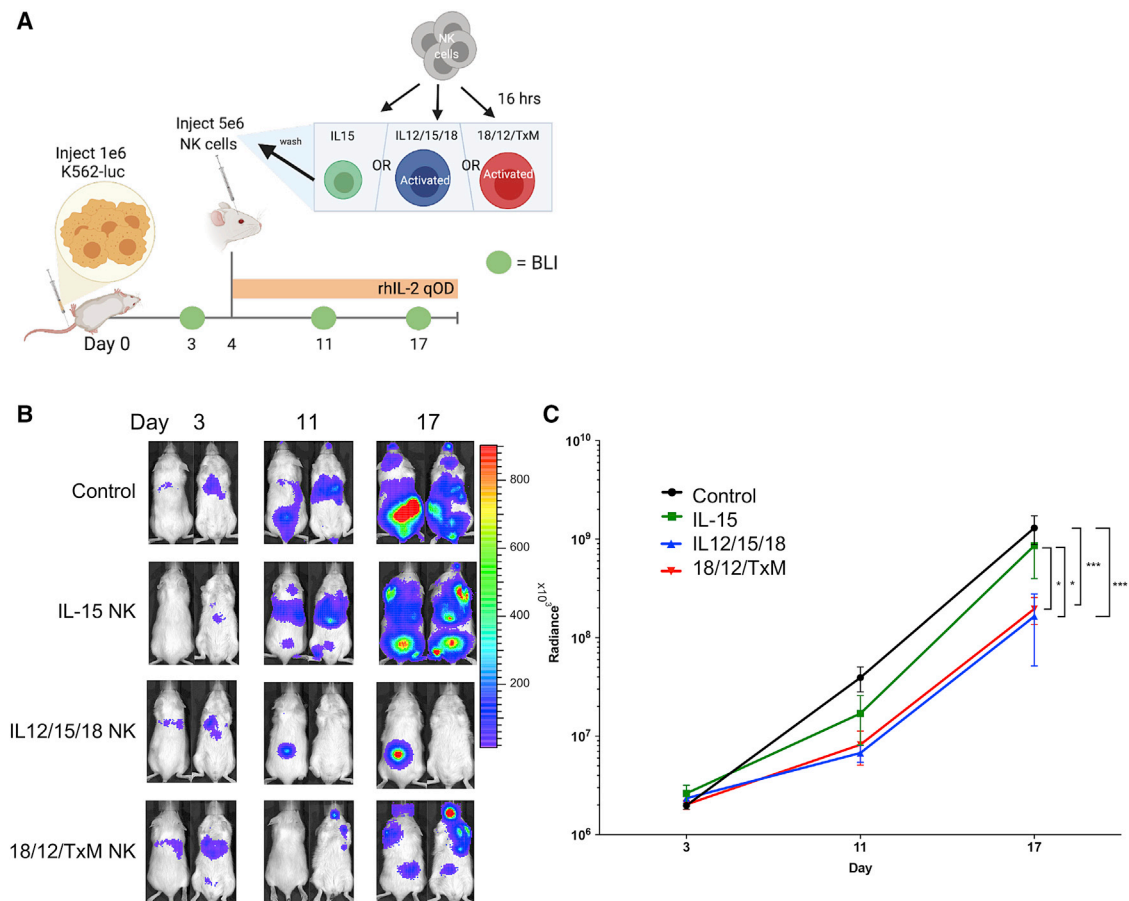


Figure 7. 18/12/TxM induces functional memory-like NK cells *in vivo*, with comparable anti-tumor activity to IL-12/15/18-induced NK cells

(A) Experimental design for (B) and (C). NSG mice were injected intravenously with 1×10^6 K562-luciferase cells. After 3 days, BLI was performed to ensure leukemia engraftment. On day 4, either control (no NK cells) or 5×10^6 NK cells activated with IL-15, IL-12/15/18, or 18/12/TxM were administered retro-orbitally to the mice. The mice were treated with rhIL-2 every other day and monitored for tumor burden (BLI). (B) Representative BLI of recipient mice engrafted with K562-luc on the indicated day after tumor administration. (C) Summary of serial BLI measurements that show tumor burden in mice receiving NK cells activated by IL-12/15/18 or 18/12/TxM. Data presented as mean \pm SEM. Summary data are from two independent experiments with 9–10 mice per group. Differences were determined using two-way analysis of variance (two-way ANOVA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

column equilibrated with 4 volumes of 50 mM sodium acetate, pH 4.2, followed by washing the column with the same buffer until A280 reached to baseline. Protein peak (containing 18/12/TxM) of the Capto Q column flow through during sample loading and a wash step was collected and then formulated in 0.1M glycine and 0.2M sorbitol, pH 4.0. Sodium dodecyl sulfate (SDS) PAGE and size-exclusion chromatography–high-performance liquid chromatography (SEC-HPLC) analysis indicated that 18/12/TxM is at least 90% pure, with approximately 70% of protein existing as a dimer and rest as oligomers.

Flow cytometry antibodies

The following Beckman Coulter antibodies were used: CD3 (clone UCHT1), CD45 (clone A96416), CD56 (clone N901), NKG2A (clone Z199.1), and NKp46 (clone BAB281). The following BD Biosciences antibodies were used: CD16 (clone 3G8), IFN- γ (clone B27), CD107a

(clone H4A3), CD57 (NK-1) CD69 (FN50), CD137 (clone 4-1BB), perforin (clone dG9), Ki67 (clone B56), ERK1/2 (pT202, pY204), AKT (pS473), STAT4 (38/p-Stat4), STAT5 (47/Stat5, pY694), p38 (pT290/pY182), and p65 (pS529). The following BioLegend antibodies were used: NKG2D (clone 1D11), NKp30 (clone P30-15), NKp44 (clone P44-8), and IgG1 control (clone MG1-45). The following eBioscience antibodies were used: granzyme B (GB12) and TNF (clone Mab11).

Cell lines

K562 cells (ATCC, CCL-243) were obtained from ATCC in 2008, viably cryopreserved, thawed for use in these studies, and maintained for no more than 2 months at a time in continuous culture as described.²⁴ Prior to our studies, the K562 cells were authenticated by confirming cell growth morphology (lymphoblast), growth characteristics, and functionally as NK-cell-sensitive targets in 2014 and

2015. Cells were cultured in RPMI 1640 supplemented with L-glutamine, HEPES, non-essential amino acid (NEAA), sodium pyruvate, and penicillin/streptomycin containing 10% fetal bovine serum (FBS) (Hyclone/GE Healthcare, Logan, UT).

HEK-Blue IL-18 cells (IL-18 sensor cells) and HEK-Blue IL-12 cells (IL-12 sensor cells) from InvivoGen were cultured in complete HEK-Blue media (I10 media), consisting of IMDM, 10% FBS (Hyclone/GE Healthcare), 1X penicillin-streptomycin-glutamine (Thermo Fisher Scientific, Dallas, TX); 100 µg/mL normacin, and 1X HEK-Blue selection (InvivoGen). The 32D-IL-2/15Rβ (32Dβ) cells were constructed at Altor BioScience, cultured in complete 32Dβ media containing IMDM-10 media plus 25 ng/mL rhIL-2, and maintained at a cell density between 1.5×10^4 - 2×10^6 cells/mL at 37°C and 5% CO₂.

NK cell purification and cell culture

Human platelet apheresis donor peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll centrifugation. NK cells were purified using RosetteSep (StemCell Technologies, Vancouver, Canada) ($\geq 95\%$ CD56⁺CD3⁻) and used for selected experiments. Cells were plated at $3\text{--}5 \times 10^6$ cells/mL and preactivated for 16 h using 38.8 nM 18/12/TxM (9.5 µg/mL), rhIL-12 (10 ng/mL) + rhIL-18 (50 ng/mL) + rhIL-15 (50 ng/mL), or control low-dose rhIL-15 conditions (1 ng/mL). Cells were washed 3 times to remove cytokines and cultured for 6 days in HAB10 complete media containing RPMI 1640 medium + 10% human AB serum (Sigma-Aldrich, St. Louis, MO) supplemented with rhIL-15 (1 ng/mL) to support survival, with 50% of the medium being replaced every 2–3 days with fresh rhIL-15.

Assessment of IL-18 and IL12 activity

HEK-Blue IL-18 and HEK-Blue IL-12 cells were maintained in complete HEK-Blue Media at 37°C and 5% CO₂. HEK-Blue Selection was added to the growth media after two passages, as per the manufacturers' cell handling recommendations. Growth media was renewed twice a week and cells were passaged when 70%–80% confluency was reached. To measure activity of IL-18 or IL-12, the respective sensor cells were detached in PBS and resuspended in Complete HEK-Blue assay media at 280,000 cells/mL. A total of 20 µL of half-log serially diluted cytokine control and hIL18/IL12/TxM (in the concentration range described below) was added to a 96-well, flat-bottom plate, followed by addition of 180 µL of cells, for a final cell count of $\sim 50,000$ cells in 200 µL. The plates were incubated for ~ 20 h at 37°C and 5% CO₂. To assess the activity of IL-18 or IL-12, the resulting secreted alkaline phosphatase was quantified using QUANTI-Blue detection reagent (InvivoGen). QUANTI-Blue reagent was prepared as per the manufacturers' instructions. After warming QUANTI-Blue to room temperature, 180 µL was added to 20 µL of culture supernatant in a 96-well, flat-bottom plate and incubated for 18 h at 37°C and 5% CO₂. Absorbance was then measured at 650 nm to determine cell activation based on reduction of QUANTI-Blue by secreted alkaline phosphatase. The EC₅₀ of IL-18 or IL-12 bioactivity of 18/12/TxM was determined based on the relationship between absorbance and protein concentration from the

dose-response curve generated using non-linear regression variable slope curve fitting with GraphPad Prism 7.

Concentration range

For detection of IL-18 activity, half-log serial dilution ranging from 10 ng/mL (556 pM) to 0.05 pg/mL (0.0028 pM) for IL-18 and 3,350 ng/mL (13,673 pM) to 0.0167 ng/mL (0.0683 pM) for 18/12/TxM was performed. This corresponds to final pM concentrations of 56 to 0.00028 pM for the IL-18 cytokine and 1,367 to 0.00683 pM for 18/12/TxM. For detection of IL-12 activity, half-log serial dilutions, ranging from 1,000 ng/mL (17,483 pM) to 5 pg/mL (0.0875 pM) for IL-12 and 85.7 µg/mL (349,796 pM) to 0.428 ng/mL (1.748 pM) for 18/12/TxM, was performed. This corresponds to final pM concentrations of 1,748.3 to 0.00875 pM for IL-12 cytokine and 34,979.6 to 0.1748 pM for 18/12/TxM molecule.

Assessment of IL-15 activity

To measure IL-15 activity, the assay plate was prepared as follows: 100 µL of IMDM-10 media was added to each well in a 96-well, flat-bottom plate. Next, 100 µL of 4× concentration of N-803 (225 ng/mL; $\sim 2,400$ pM) or IL-18/IL12/TxM (18,000 ng/mL; $\sim 73,468$ pM) was added to column 1. The drugs were 2-fold serially diluted to column 10, leaving 100 µL in each well. The cells were washed 3 times with IMDM-10 media and resuspended at a density 1×10^5 cell/mL in IMDM-10 media, and 100 µL of the cells was added to the assay plate from column 1 to 11, for a total assay volume of 200 µL; 100 µL of IMDM-10 was added to column 12. The assay plate was placed at 37°C and 5% CO₂ for ~ 72 h. To assess the activity of IL-15, 20 µL of 10× PrestoBlue Cell Viability Reagent was added directly to the assay plate after ~ 72 h, and the plate placed at 37°C and 5% CO₂ for an additional ~ 4 h. Absorbance was measured at 570 nm and at 600 nM for normalization. Using column 11 (cells with no drug) as a negative control, the EC₅₀ of IL-15 bioactivity of hIL18/IL12/TxM was determined from the dose-response curve generated using non-linear regression variable slope curve fitting with GraphPad Prism 7.

Phosphorylation assays

Freshly isolated human NK cells were incubated in HAB10 media without cytokines at 37°C for 30 min. Individual cytokines (IL-12, -15, or -18) were added to wells at the indicated concentration for varying time intervals (2 h stimulation for STAT4, 1 h stimulation for Akt and ERK, and 15 min stimulation for NF-κB-P65, STAT5, and P38 detection). After incubation, cells were fixed with 4% paraformaldehyde (PFA) and incubated at room temperature for 10 min. The cells were then pelleted and resuspended in cold 100% methanol and incubated and 4°C for 30 min. Cells were washed 3 times with fluorescence-activated cell sorting (FACS) buffer (PBS, 0.5% BSA, and 2 mM EDTA). After washing, cells were suspended in surface antibody master mix (CD3, CD16, CD56, and CD45) as well as the appropriate phosphoflow antibodies and stained overnight at 4°C. The next morning, cells were washed twice and samples were acquired on a Beckman Coulter (Indianapolis, IN) Gallios flow cytometer and analyzed using FlowJo version 9.3.2 (Tree Star) software.³⁸

Functional assays to assess cytokine production

Control and ML NK cells were harvested after a rest period of 6 days to allow ML NK cell differentiation to occur. Cells were then re-stimulated in a standard functional assay.^{10,21,24} Briefly, cells were incubated for 6 h with K562 leukemia targets at an E:T ratio of 5:1 unless otherwise noted, in the presence of CD107a. After 1 h of stimulation, Brefeldin A and Monensin (GolgiStop/GolgiPlug, BD Biosciences, San Jose, CA) were added, and 5 h later the cells were stained for CD45, CD3, CD56, and CD25. Cells were fixed (Cytofix/Cytoperm, BD Biosciences) and permeabilized (Perm/Wash, BD Biosciences) before the staining of intracellular IFN- γ and TNF. Cells were acquired on a Gallios 3 flow cytometer and analyzed using FlowJo version 9.3.2 (Tree Star) software.

Assessment of specific killing

On D6 or D7 post-activation, control or ML NK cells were re-suspended in 1 ng/mL IL-15 and challenged with K562 targets at various E:T ratios, in a standard 4 h ⁵¹Cr release assay.³⁸ ⁵¹Cr release was detected on a Wallac MicroBeta Trilux Scintillation Counter. The percent specific lysis was calculated using counts per minute (cpm) as follows: $[(\text{cpm}_{\text{exp}} - \text{cpm}_{\text{spontaneous}})/(\text{cpm}_{\text{max}} - \text{cpm}_{\text{spontaneous}})] \times 100$.

Flow cytometric analysis

Cell staining was performed as described previously,^{10,21,24,38} and data were acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed using Kaluza version 1.2 (Beckman Coulter) or FlowJo version 9.3.2 (Tree Star) software. Statistical analysis was done using GraphPad version 7.0 software.

RNA-sequencing

One million purified NK cells were frozen in Trizol at -80°C until RNA isolation using the Direct-zol RNA MicroPrep kit (Zymo Research, Irvine, CA). NextGen RNA-seq was performed using an Illumina HiSeq 2500 sequencer. RNA-seq reads were aligned to the Ensembl release 76 top-level assembly with STAR version 2.0.4b. Gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:featureCount version 1.4.5. Analysis of sequencing data was performed using Phantasus, a browser-based gene expression analysis software.³⁹ Differential expression analysis was performed using the LIMMA package to analyze for differences between conditions, and the results were filtered for only those genes with false-discovery rate adjusted $p \leq 0.05$. The Gene Expression Omnibus (GEO) database accession number is GSE166268.

Mass cytometry

All mass cytometry data were collected on a CyTOF2 mass cytometer (Fluidigm, San Francisco, CA) and analyzed using Cytobank. Mass cytometry data were analyzed using previously described methods, and sample staining and data collection were performed as previously described.^{10,21,24}

NSG xenograft model and BLI imaging

K562-expressing luciferase tumor cells (1×10^6) were injected intravenously (i.v.) via tail vein into 8–12 week old male and female NSG

mice (The Jackson Laboratory, Bar Harbor, ME) on day 0. All mice were irradiated with 2.5 cGy 2 days before tumor injection. At day 3, BLI was performed to confirm leukemia cells engraftment. On day 4, 5×10^6 control (NK cells in 1 ng/mL IL-15) or NK cells activated with IL-12/15/18 (10 ng/mL IL-12, 50 ng/mL IL-15, and 50 ng/mL IL-18) or 18/12/TxM (38 nM) were administered retro-orbitally to the mice (total 9–10 mice per group from 2 independent experiments). The mice were treated with rhIL-2 (50,000 IU per mouse) every other day and monitored weekly for tumor burden (BLI). All mice were maintained and used in accordance with our animal protocol approved by the Washington University Animal Studies Committee.

In vivo BLI imaging was performed on an IVIS 50 (1–60 s exposure, bin8, field of view [FOV] 12 cm, open filter) (Xenogen, Alameda, CA). For this, mice were injected intraperitoneally with D-luciferin (150 mg/kg in PBS, Gold Biotechnology, St. Louis, MO) and imaged under anesthesia with isoflurane (2% vaporized in O₂). Total photon flux (photons/s) was measured from fixed regions of interest over the entire mouse using the Living Image 2.6 software program.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omto.2022.02.009>.

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AUTHOR CONTRIBUTIONS

C.C.C. and T.A.F. wrote the manuscript. T.A.F., M.B.-H., and M.M.B.-E. conceived and designed the study. C.C.C., M.B.-H., C.C.N., E.M., J.A.F., P.W., N.D.M., L.M., M.F., T.S., and J.A.W. collected, analyzed, and assembled the data. P.S.-S. and J.L. provided critical reagents. All authors reviewed the manuscript, edited, and provided approval of the final version of the manuscript.

DECLARATION OF INTERESTS

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Supplemental information

**A novel fusion protein scaffold 18/12/TxM
activates the IL-12, IL-15, and IL-18 receptors
to induce human memory-like natural killer cells**

Celia C. Cubitt, Ethan McClain, Michelle Becker-Hapak, Jennifer A. Foltz, Pamela Wong, Julia A. Wagner, Carly C. Neal, Nancy D. Marin, Lynne Marsala, Mark Foster, Timothy Schappe, Patrick Soon-Shiong, John Lee, Melissa M. Berrien-Elliott, and Todd A. Fehniger

Figure S1

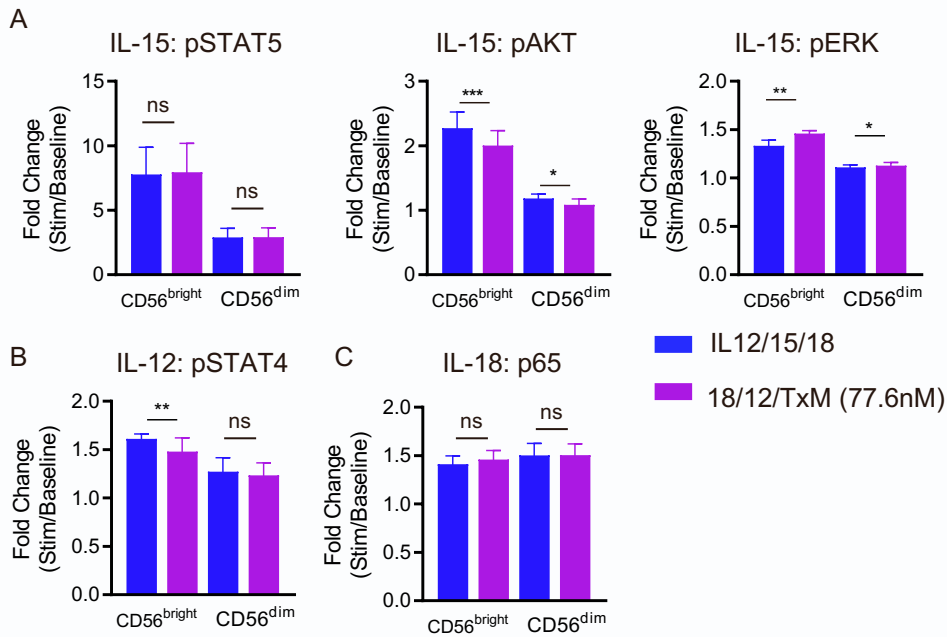


Figure S1. 18/12/TxM induces signaling via all targeted receptors at 77.6 nM. (A-C) Freshly isolated NK cells from 3-5 healthy donors were stimulated with IL-12 (10ng/mL), IL-15 (50ng/mL) and IL-18 (50 ng/mL) or 18/12/TxM (77.6nM) and assessed at various time intervals for CD56^{bright} and CD56^{dim} NK cells. (A) Phosphorylation of the signaling mediators downstream of IL-15 signaling, pSTAT5, pAKT, and pERK. (B). Phosphorylation of pSTAT4, downstream of IL-12 signaling. (C) Phosphorylation of p65, downstream of IL-18 signaling. Data shown is mean +/- SEM and compared using a paired *t*-test. N=3-5 human donors. (**p*<0.05, ***p*<0.01, ****p*<0.001)

Figure S2

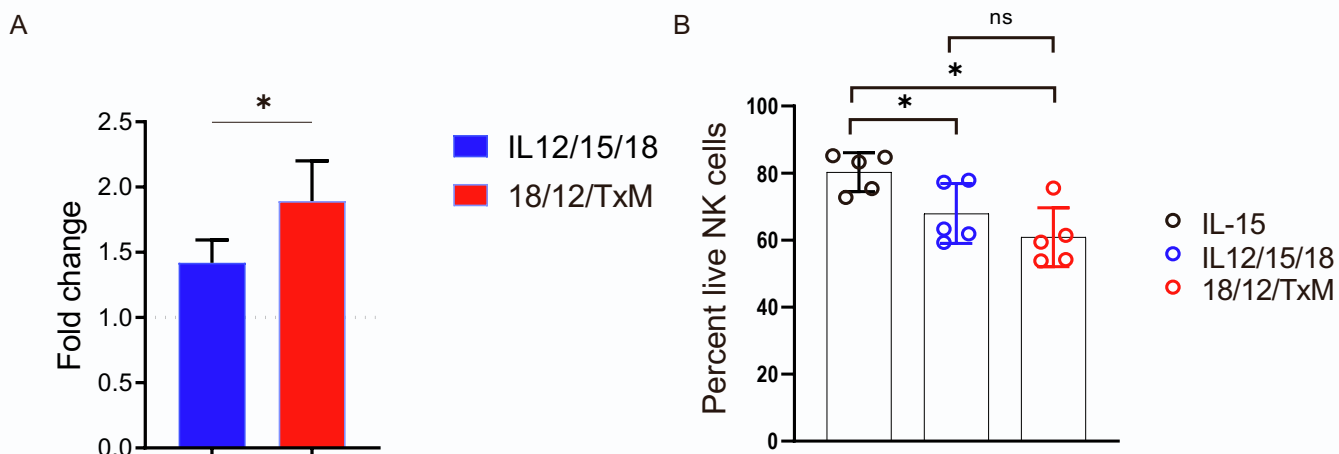
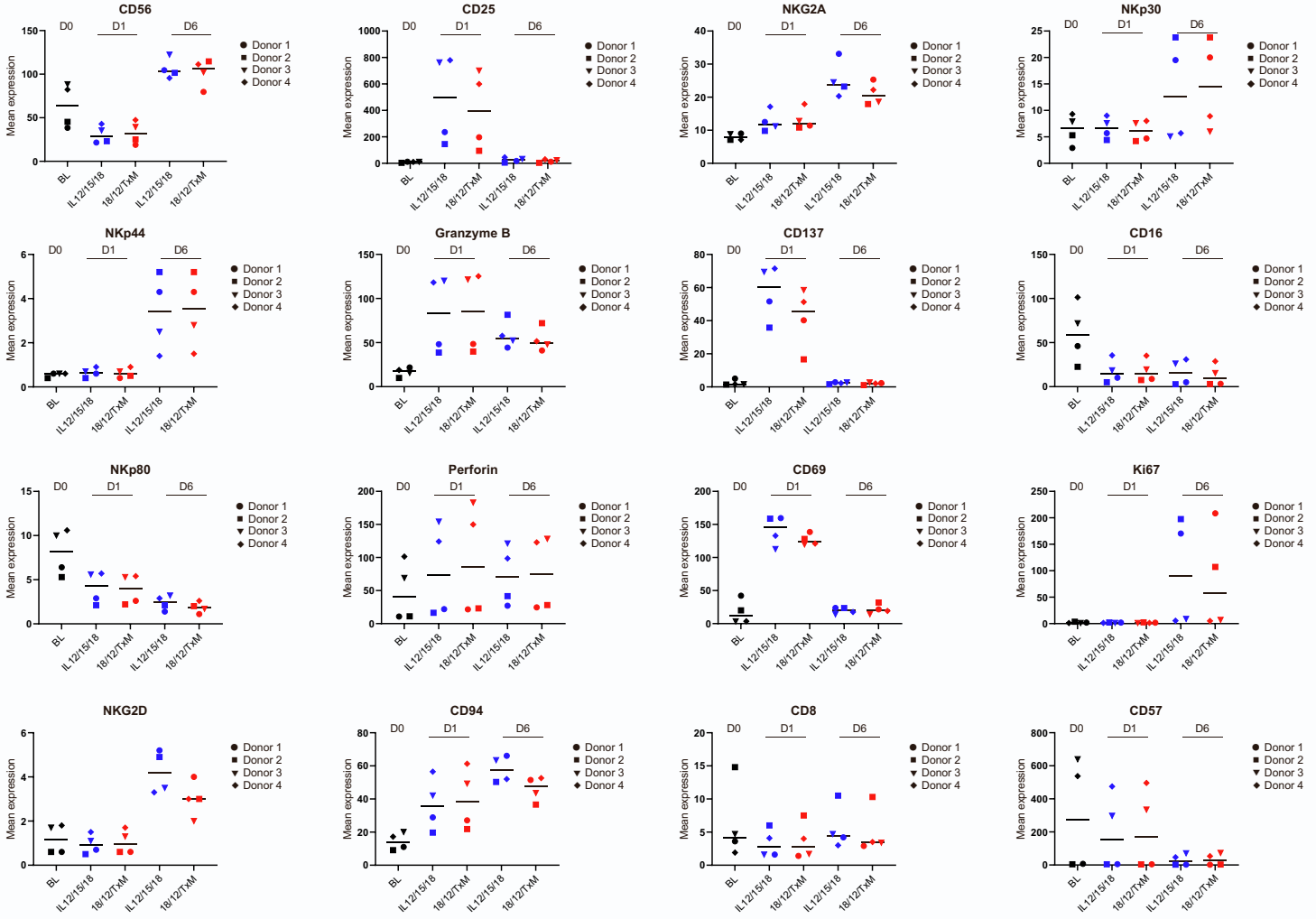


Figure S2. Expansion and viability of NK cells between activating conditions. (A-B) Freshly isolated human NK cells were activated with either IL-15 (1 ng/mL), IL12/15/18, or 18/12/TxM for 16hrs and cultured in IL-15 for 7 days. (A) Fold change calculated as absolute # of NK cells activated by IL12/15/18 or 18/12/TxM / absolute # of NK cells activated with IL-15 only (1 ng/mL) by Day 7. N=4 human donors, 2 independent experiments. Statistical analyses performed using two-tailed paired *t*-test (* $P < 0.05$). (B) Viability was assessed at Day 7 post-activation by flow cytometry by measuring the percent of Zombie-green negative NK cells. N=5 human donors, 2 independent experiments. Statistical analyses performed using one-way ANOVA (* $P < 0.05$). Summary data shown as mean \pm SEM.

Figure S3

A



B

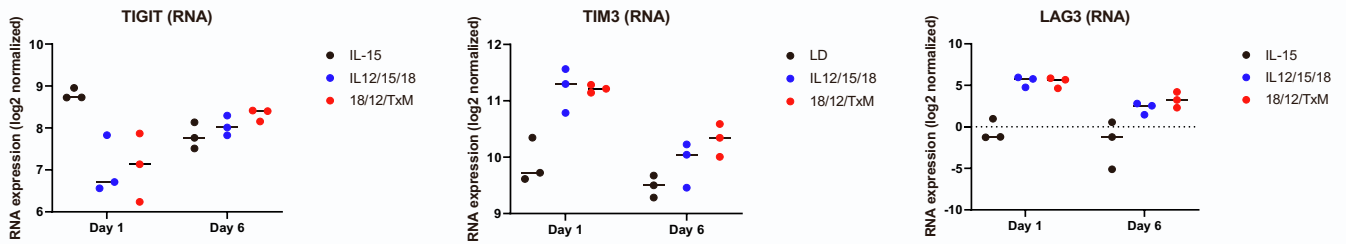


Figure S3. Phenotypic differences in NK cells at baseline, Day 1, and Day 6 after activation with IL-12/15/18 or 18/12/TxM. (A) Summary data demonstrating mean expression of the indicated markers from Figure 4. N=4 human donors, 2 independent experiments. (B) Log₂ normalized RNA expression of inhibitory receptors at day 1 and day 6 after activation with IL12/15/18, 18/12/TxM, or IL-15. N=3 human donors.

Figure S4

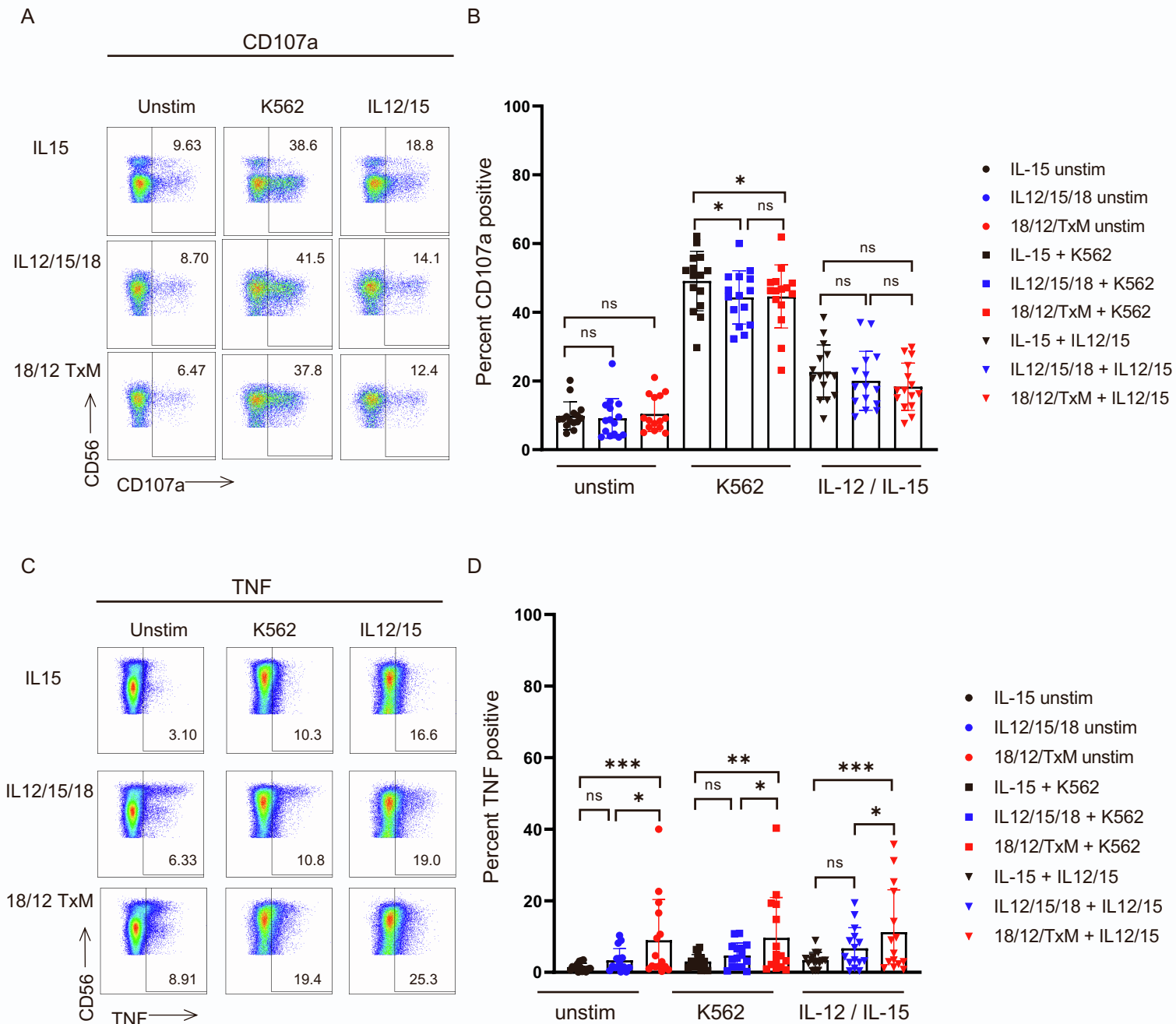
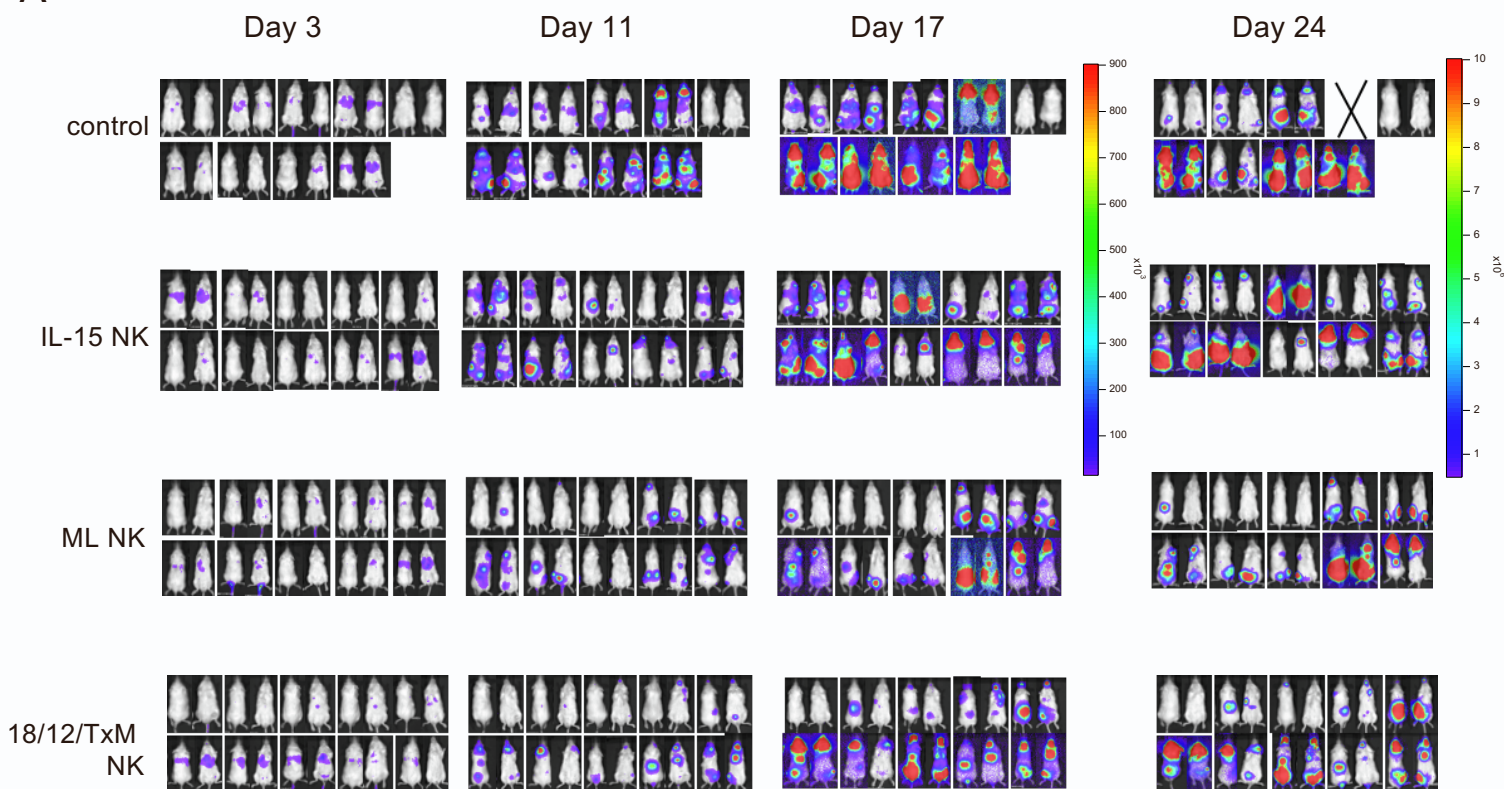


Figure S4: 18/12/TxM induces functional memory-like cells in vitro. Functional assessments were performed as described in Figure 5 and assessed for expression of (A-C) CD107a, and (D-F) TNF. (A) Representative flow plot showing CD107a induction in K562 and IL-12 + IL-15 stimulated NK cells. (B-C) Summary data showing percent CD107a positive NK cells stimulated with (B) K562s or (C) IL-12+IL-15. (D) Representative flow plot showing TNF induction in K562 and IL-12 + IL-15 stimulated NK cells. (D-F) Summary data showing percent TNF positive NK cells stimulated with (E) K562s or (F) IL-12+IL-15. (n=15 donors, 7 independent experiments). Analysis performed using one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Figure S5

A



B

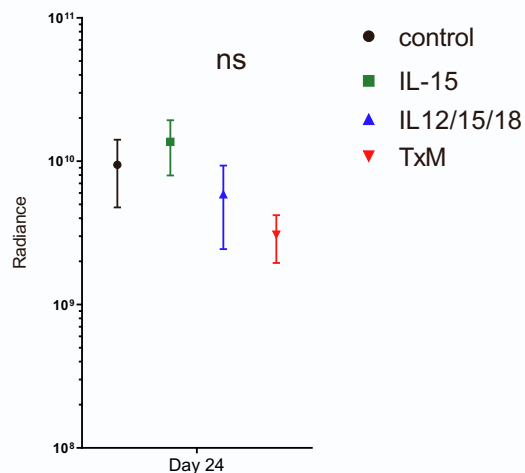


Figure S5. 18/12/TxM induces comparable anti-tumor activity to IL12/15/18 ML NK cells.

(A) BLI images from each K562luc tumor-bearing mouse from experiments described in Figure 7. (B) Summary data of serial BLI measurements on Day 24 in tumor-bearing mice treated with no NK cells (control), or NK cells activated with IL-15, IL12/15/18, or 18/12/TxM. Summary data are from two independent experiments with 9-10 mice per group. Statistical analyses performed using a mixed effects model. * $P < 0.05$

Table S1

Table S1. NK cell phenotypic mass cytometry panels. The metal isotope, marker name, antibody clone, and source are shown for this mass cytometric phenotypic panel. The asterix (*) included after the source indicates antibodies were custom-conjugated using Fluidigm antibody labeling kits, per manufacturers instructions.

Tag	Antibody	Clone	Source
89 Y	CD45	HI30	Fluidigm
141 Pr	CD14	M5E2	BD Pharmingen*
142 Nd	CD19	HIB19	Fluidigm
143 Nd	KIR3DS1/L1 (CD158e1, e2)	DX9	R&D*
145 Nd	KIR2DS4 (CD158i)	FES172	Beckman Coulter*
146 Nd	KIR2DL1/2DS1	EB6B	Beckman Coulter*
147 Sm	NKG2D	1D11	R&D*
148 Nd	KIR2DL2/2DL3 (CD158b)	CH-L	BD Pharmingen*
149 Sm	CD127	AO19D5	Fluidigm
150 Nd	CD4	OKT4	Miltenyi*
151 Eu	TRAIL	RIK-2	Biologend*
152 Sm	CD8	SK1	eBioscience*
153 Eu	CD62L	DREG-56	Fluidigm
154 Sm	KIR2DL5 (CD158f)	UP-R1	Beckman Coulter*
155 Gd	CD27	L128	Fluidigm
156 Gd	PD-L1/PDL2	29E.2A3	Fluidigm
158 Gd	CD137	4B4-1	Fluidigm
159 Tb	NKG2C	134591	R&D*
160 Gd	CD69	FN50	Biologend*
161 Dy	NKp30	P30-15	Biologend*
162 Dy	Ki67	B56	Fluidigm
163 Er	CD94	DX22	Biologend*
164 Dy	TIM-3	F38-2E2	Biologend*
165 Ho	CD16	3G8	Fluidigm
166 Er	NKG2A	Z199	Beckman Coulter*
167 Dy	NKp44	P44-8	Biologend*
168 Er	CD226 (DNAM1)	DX11	Miltenyi*
169 Tm	CD25	2A3	Fluidigm
170 Er	NKp80	239127	R&D*
171 Yb	Granzyme B	GB11	Fluidigm
172 Yb	CD57	HCD57	Fluidigm
173 Yb	CD3	UCHT1	BD Pharmingen*
174 Yb	NKp46	9E2	R&D*
175 Lu	Perforin	B-D48	Fluidigm
176 Yb	CD56 (NCAM)	NCAM16.2	Fluidigm
209 Bi	CD11b	209Bi	Fluidigm