Supplemental Data

Cytokine-activation induces human memory-like NK cells

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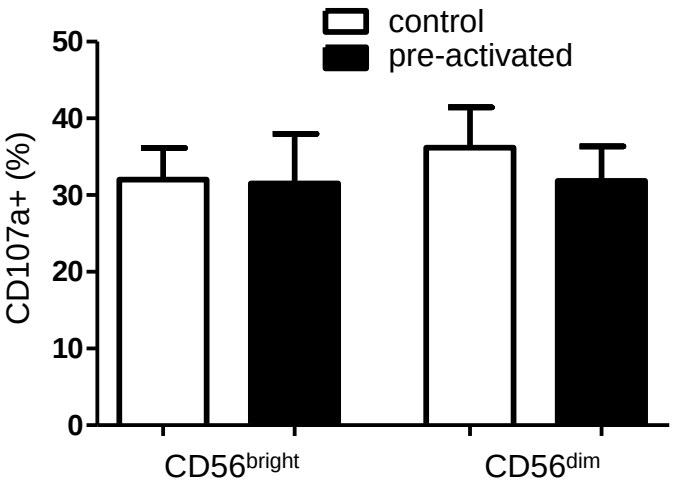
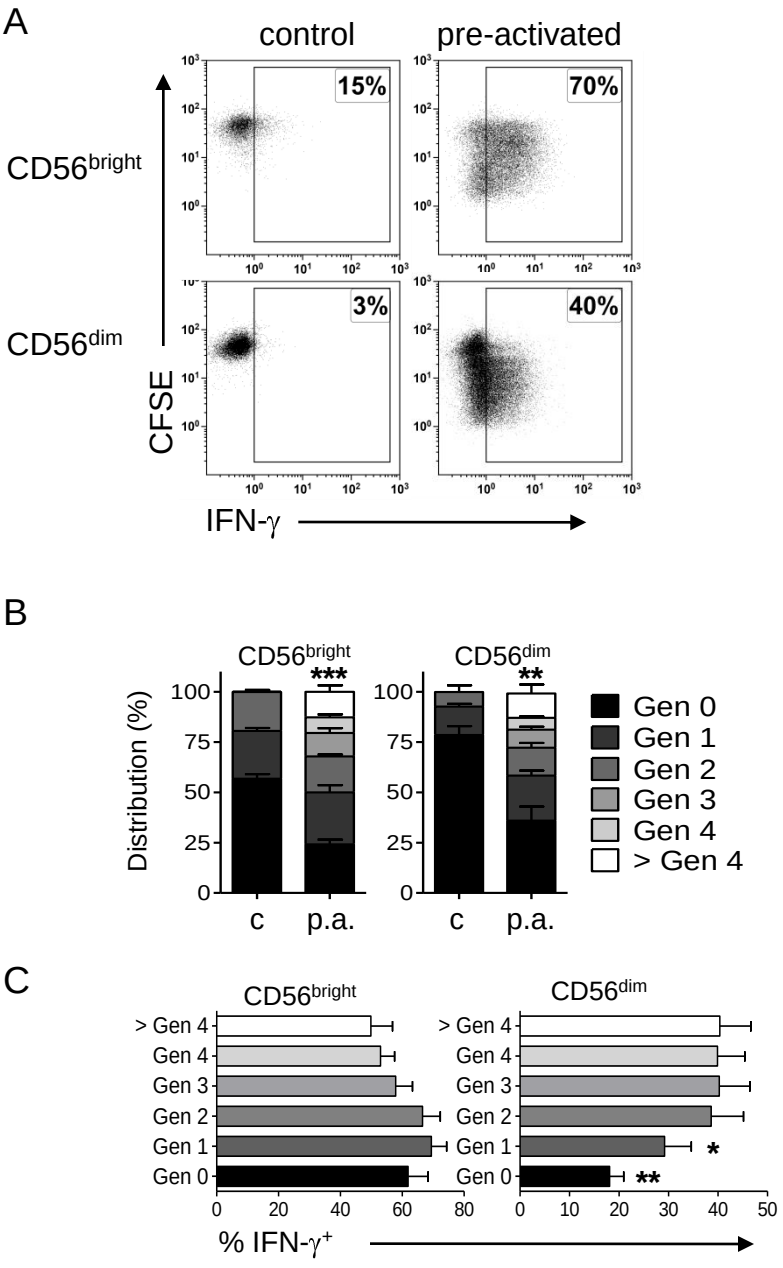


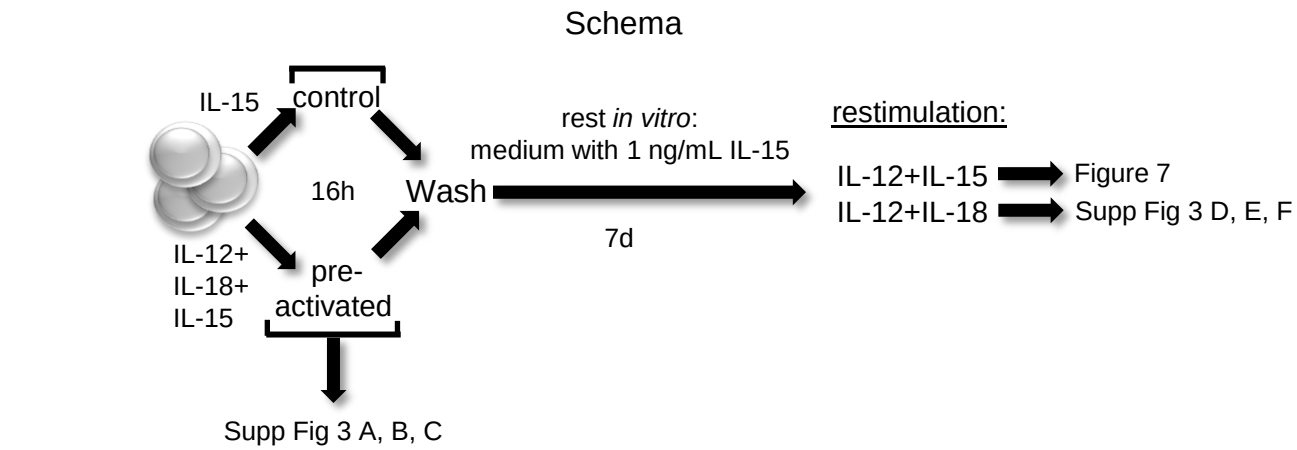
Supplemental Figure 1. Pre-activation of NK cells with IL-12 plus IL-18 does not lead to enhanced CD107a (degranulation) expression. Purified NK cells were pre-activated for 16 hours, washed and rested in media containing low dose IL-15 (1 ng/ml). On day 7 the cells were washed and then restimulated for 6 hours with K562 cells (effector: target of 4:1) to assess CD107a expression. Summary data shown as mean SEM percent CD107a positive $CD56^{bright}$ and $CD56^{dim}$ cells. (N=5, 3 independent experiments) *P<0.05, **P<0.01, ***P<0.001

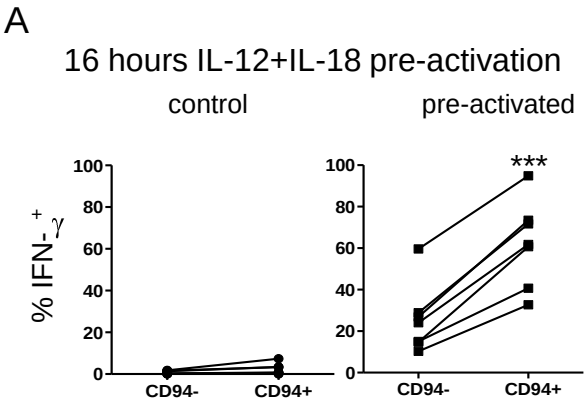
Romee et al., Supplemental Figure 2

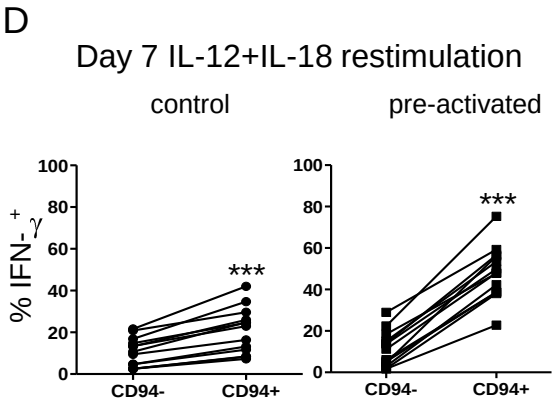


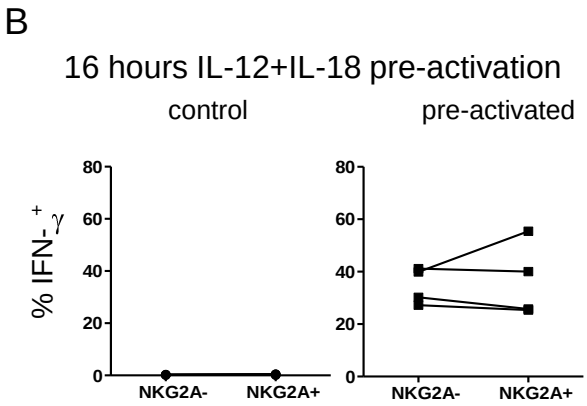
Supplemental Figure 2. Memory-like NK cells proliferate and enhanced IFN-γ production is retained following cell division. PBMCs were labeled with CFSE to track cell division and then pre-activated and rested as per Fig 1A. After 7 days, cells were restimulated with IL-12+IL-15 and analyzed simultaneously for IFN-γ and CFSE to track cell divisions. **(A)** Representative bivariate flow plots of $CD56^{bright}$ and $CD56^{dim}$ NK cells, demonstrating that pre-activation results in both increased cell division (CFSE dilution) and enhanced IFN-γ production. **(B)** Summary results showing the increased proliferation 7 days following pre-activation (p.a.), compared to control (c), in both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. **(C)** Summary results shown as the mean SEM percent IFN-γ^{+} by NK cell generation. Results are from N=4 donors (2 independent experiments). * P<0.05, ** P<0.01, ***P<0.001.

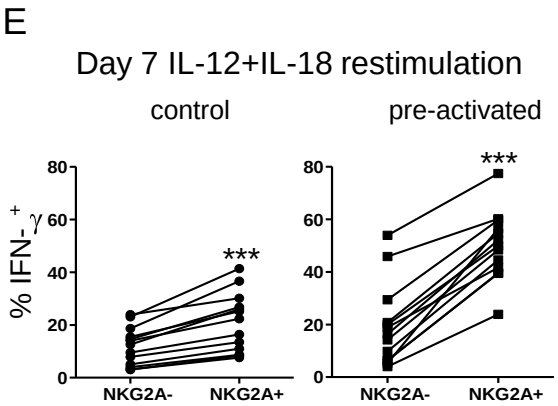
Romee et al., Supplemental Figure 3

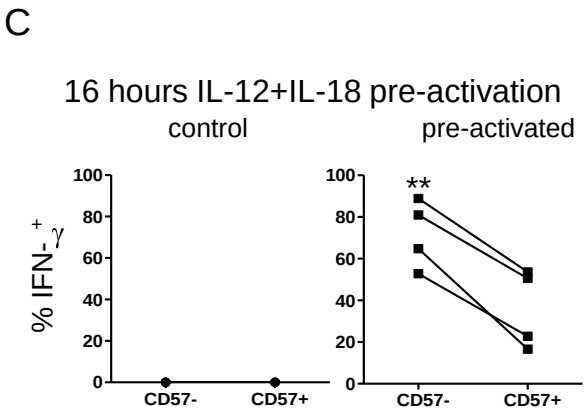


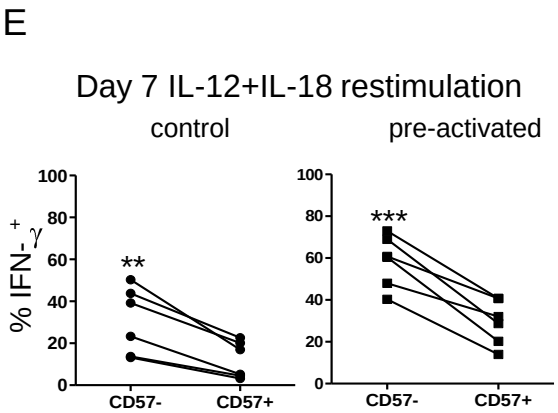






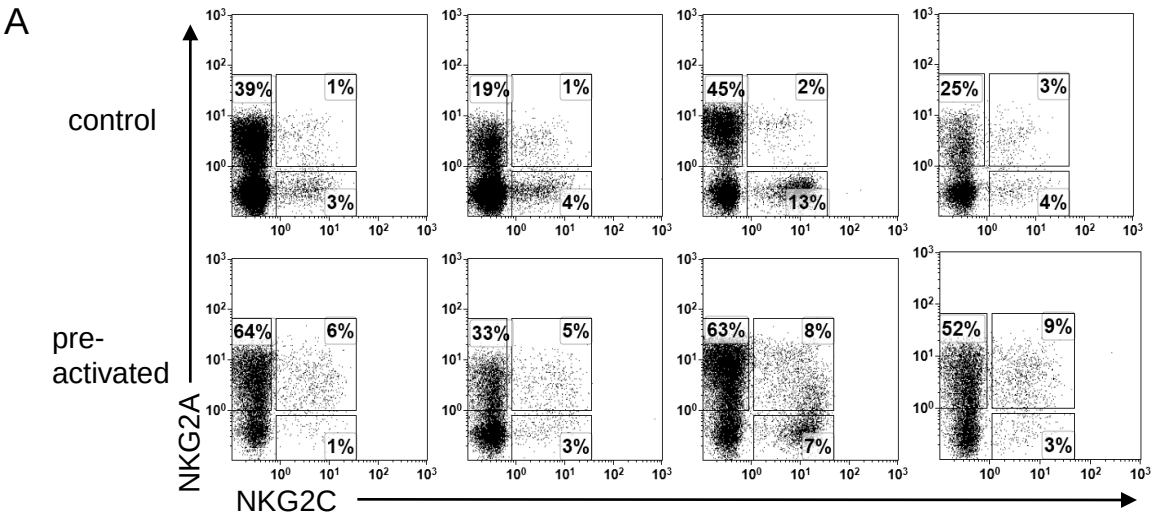


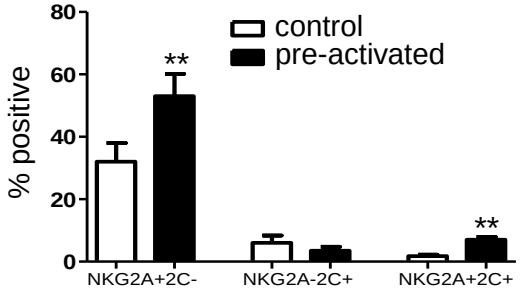


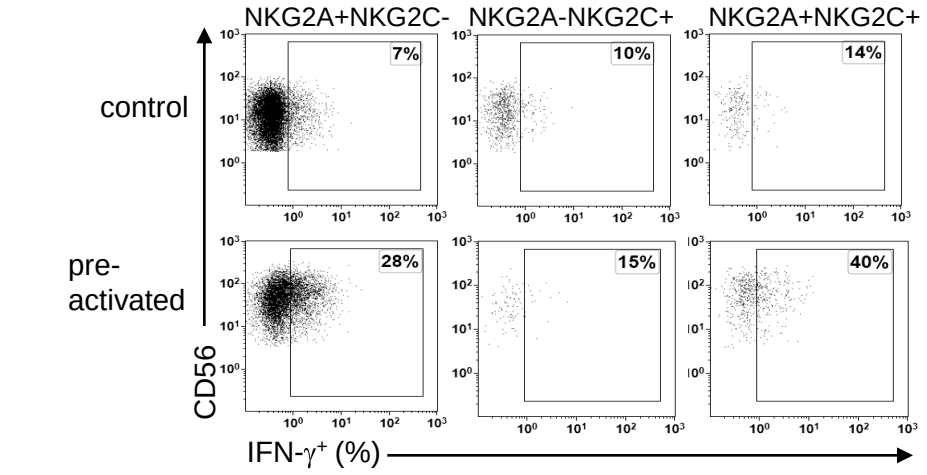


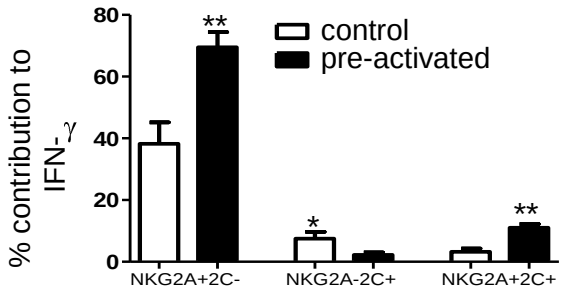
Supplemental Figure 3. Correlation of IFN-γ with CD94, NKG2A or CD57 expression is not altered by pre-activation of NK cells with IL-12 + IL-18 pre-activation. Purified NK cells were pre-activated for 16 hours with control or IL-12 plus IL-18 as in Figure 1A and then initially assessed for IFN-γ production at 16 hours without restimulation. After 7 days of rest in low dose IL-15 (1 ng/ml) to support survival, cells were restimulated with IL-12 (10 ng/ml) plus IL-18 (50 ng/ml) for 6 hours to reassess for IFN-γ production. IFN-γ correlation with (A) CD94, (B) NKG2A and (C) CD69 in the $CD56^{dim}$ NK cells at 16 hours and 7 days (D, E, F) after pre-activation. Results are from N=4-19 donors in ≥2 independent experiments. * P<0.05, ** P<0.01, ***P<0.001.

Romee et al., Supplemental Figure 4





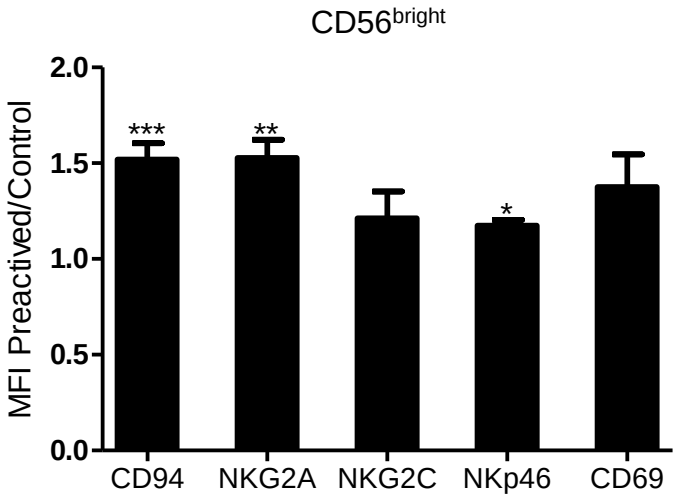




Supplemental figure 4. **Pre-activated CD56^{dim} NK cells co-express NKG2A and NKG2C have enhanced IFN-γ production following restimulation with IL-12 + IL-15.** Purified NK cells (>95%) were pre-activated for 16 hours, washed and rested as in figure 1A and then restimulated for 6 hours with IL-12 (10 ng/ml) plus IL-15 (100 ng/ml) to assess for IFN-γ production. (A) Bivariate flow plots from the four donors showing NKG2A and NKG2C expression in the control and pre-activated CD56^{dim} NK cells at day 7 without restimulation. (B) Summary data shown as mean SEM percent positive NKG2A+NKG2C-, NKG2A-NKG2C+ and NKG2A+NKG2C+ control and pre-activated CD56^{dim} NK cells. (C) Representative bivariate flow plots from control and pre-activated CD56^{dim} NK cells showing IFN-γ production in NKG2A+NKG2C-, NKG2A-NKG2C+ and NKG2A+NKG2C+ subsets. (D) Summary data shown as mean SEM percent contribution to IFN-γ production from NKG2A+NKG2C-, NKG2A-NKG2C+ and NKG2A+NKG2C+ subsets in control and pre-activated CD56^{dim} NK cells. Results are from N= 4 donors in 2 independent experiments. * P<0.05, ** P<0.01, ***P<0.001

Romee et al., Supplemental Figure 5

A



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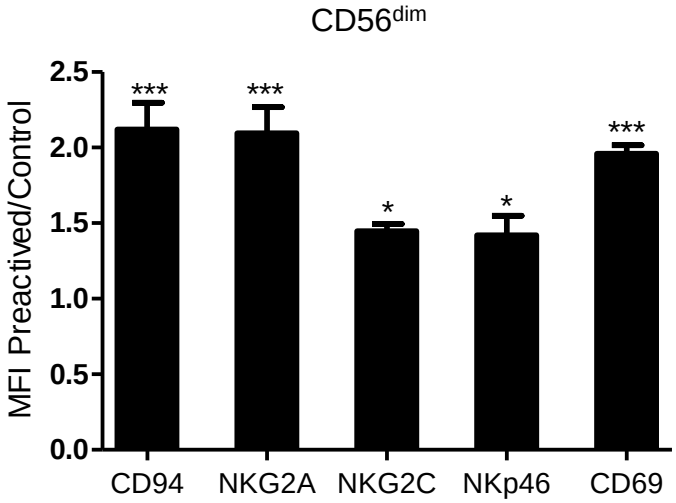
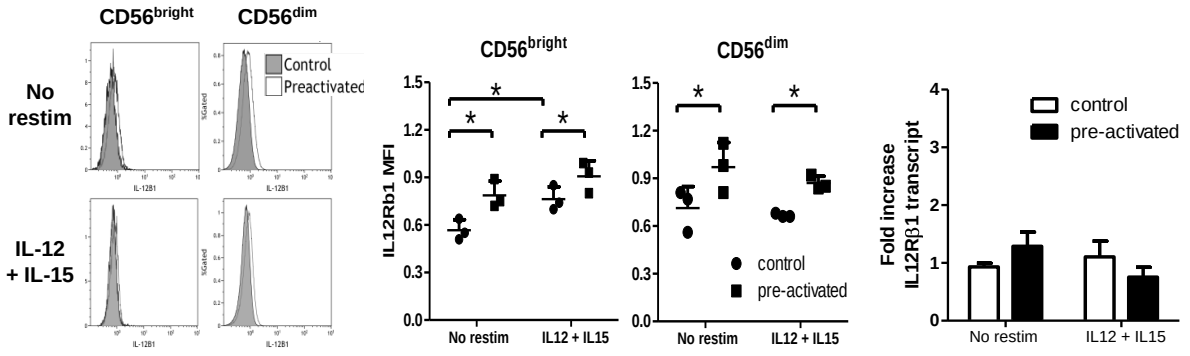


Supplemental Figure 5. IFN-γ production correlates with CD94, NKG2A, NKG2C, NKp46 and CD69 MFI's in the IFN-γ positive NK cells. Purified NK cells (>95% $CD56^{+}CD3^{-}$) were pre-activated, washed, rested for 7 days (as in Figure 1A) and then restimulated with IL-12 + IL-15 for 6 hours to assess IFN-γ production. Summary data shown as mean SEM MFI ratio for the marker of interest in IFN-γ positive pre-activated and control $CD56^{bright}$ (A) and $CD56^{dim}$ (B) NK cells. (N= 4-13, in ≥2 independent experiments) *P<0.05, **P<0.01, ***P<0.001

Romee et al., Supplemental Figure 6

A



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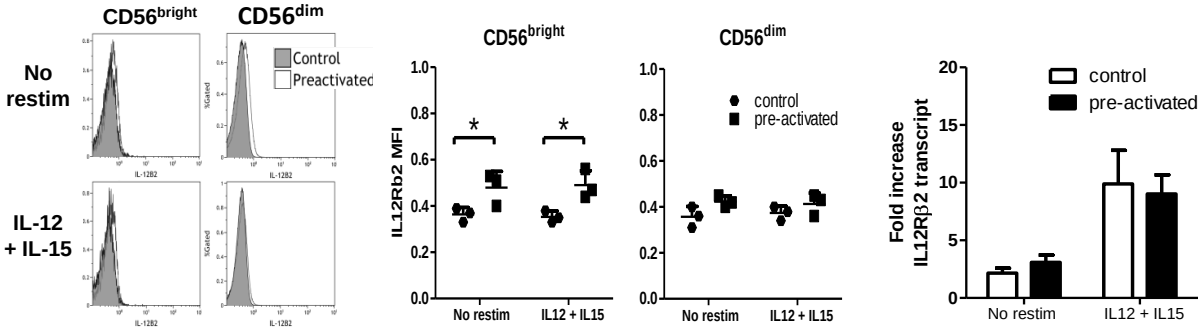

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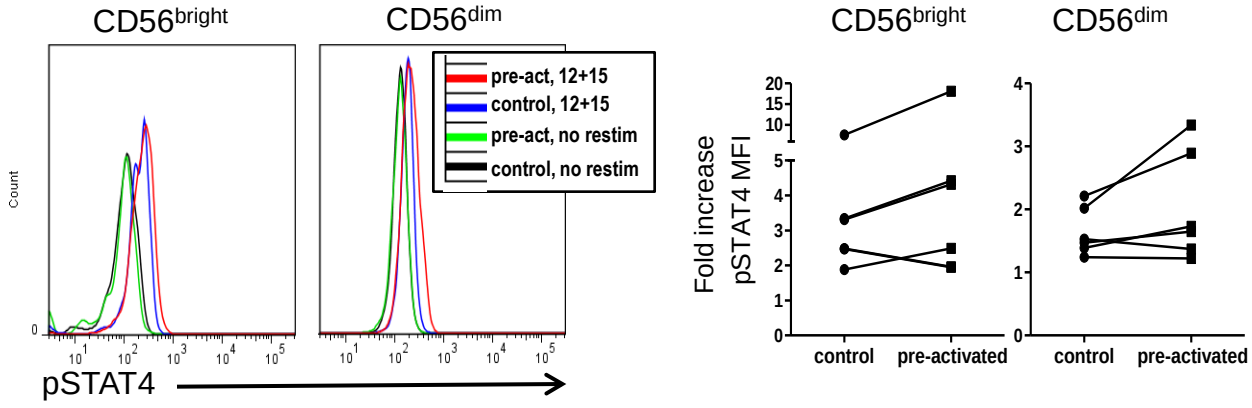


Supplemental Figure 6. Pre-activation leads to a modest increase in IL-12 receptor expression without a significant change in pSTAT4. Purified NK cells were pre-activated, washed, rested (as in Figure 1A). On day 7 of culture, cells were restimulated for 6h with IL-12 (10 ng/mL) plus IL-15 (100 ng/mL). Surface expression of cytokine receptors was assessed via staining with fluorescent antibodies and flow cytometry. Representative histograms, aggregate MFI data and quantitative RT-PCR analysis of cytokine receptor chain transcript IL-12Rb1 **(A)** and IL-12Rb2 **(B)**. Each point represents a single donor. Data are pooled from 3-5 donors, over 3 independent experiments. Additionally, NK cells were cultured in 5 ng/mL of IL-12 for 36h, to induce surface expression both IL-12 receptor chains, as a positive staining control (data not shown). For quantitative RT-PCR analysis of the cytokine receptor chain transcript data are presented as fold change over transcript measured in freshly isolated NK cells from the same donor. Duplicate analyses performed for three independent donors. Representative data are shown. **(C)** Representative flow cytometry histograms and quantitative change in MFI for pSTAT4. Horizontal lines pair data points from a single donor. Data are pooled from 2 independent experiments. * P<0.05, ** P<0.01, ***P<0.001

Methods.

Antibodies and flow cytometry: The following anti-human mAb's were used for detection of IL-12 receptor cell surface expression: BD Biosciences, CD212/IL12Rβ1 (2.4E6), CD212/IL12Rβ2 (2B6/12beta2). For detection of phosphorylated STAT4 molecules, cells were activated with IL-12 (10 ng/mL) and IL-15 (100 ng/mL) for 15 minutes. Cells were then immediately fixed (1% formalin) and methanol permeabilized, followed by intracellular staining for pSTAT4 (38/p-STAT4) (BD Biosciences). Data were collected on a Gallios flow cytometer (Beckman Coulter) and analyzed using Kaluza (Beckman Coulter) or FlowJo (Tree Star).

qPCR: For RNA isolation, NK cells were lysed in Trizol (Invitrogen, Grand Island NY) and prepared according to the manufacturer's protocol. cDNA was generated from RNA using the TaqMan Reverse Transcription Kit (Applied Biosystems) with random hexamer primers. QRT-PCR was performed on a 7300 series ABI instrument, using the standard program and a reaction volume of 20µL. The following specific primer/probe sets were used:

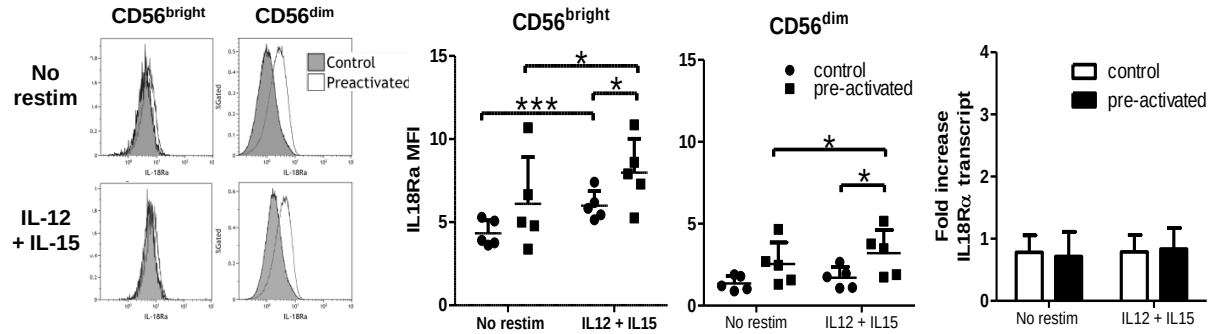
IL-12R1b **(Shi, Y et al, Cancer Gene Therapy, 2008,15:101-107):**
FWD5'-TCTTCCTCTTCCTGCTGTCC-3'
REV 5'-CCTCATACTGCCAGGAGCACTC-3'
Probe 5'-GGACCTGAGATGCTATCGGA-3'.

IL-12Rb2 **(Janefjord, CK et al, Clin Exp Allergy, 2001, 31:1493–1500):**
FWD5'-ACATTCTTGGACATAGTGAGGCC-3'
REV 5'-GTACATCTGCTCACAGAAGCC-3'
Probe 5'-TCCTCCGTGGGACATTAGAATCAAATTTCAAA-3'

All wells also contained a primer/probe set for 18s RNA (TaqMan Ribosomal RNA Control), used to calculate ΔΔCt values.

Romee et al., Supplemental Figure 7

A



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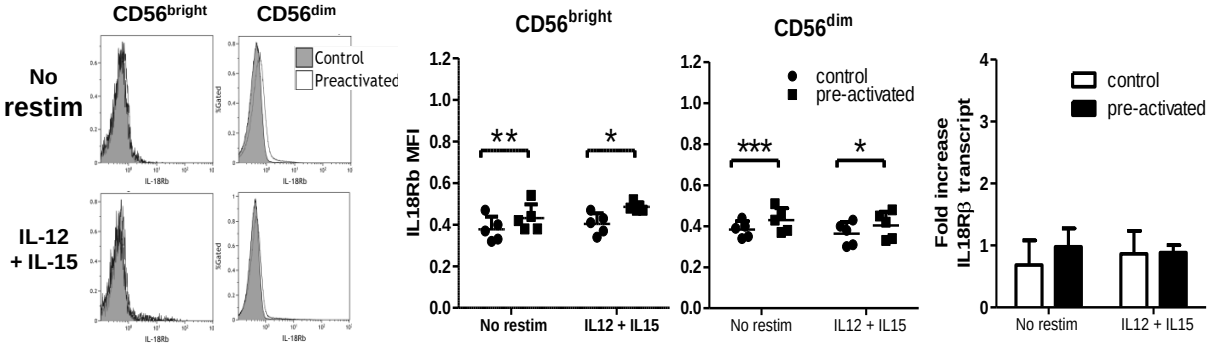


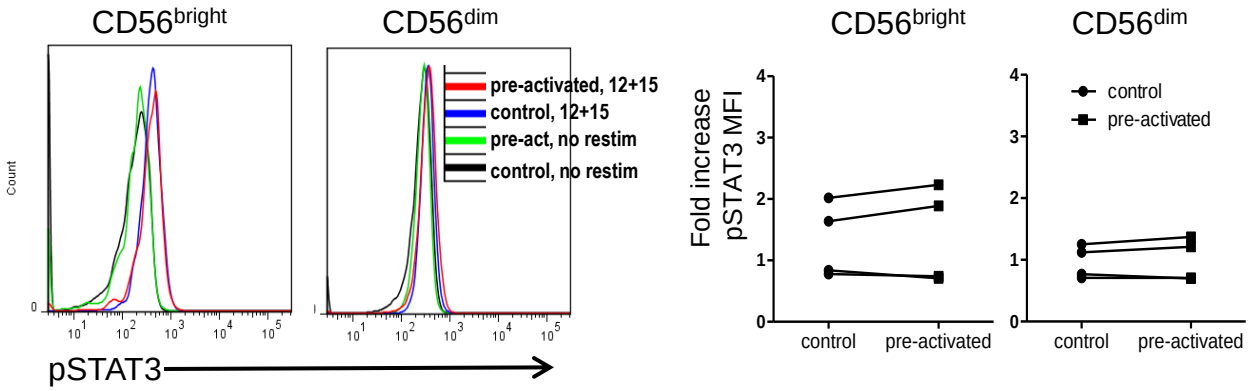
Supplemental Figure 7. Pre-activation leads to a minimal increase in IL-18 receptor expression. Purified NK cells (>95% purity) were pre-activated, washed, rested (as in Fig 1A). After day 7 of culture, cells were restimulated for 6h with IL-12 (10 ng/mL) plus IL-15 (100 ng/mL). Surface expression of cytokine receptors was assessed via staining with fluorescent antibodies and flow cytometry. Representative histograms, aggregate MFI data and quantitative RT-PCR analysis of cytokine receptor chain transcript IL-18Ra **(A)** and IL-18Rb **(B)**. Each point represents a single donor. Data are pooled from 3-5 donors, over 3 independent experiments. For quantitative RT-PCR analysis of the cytokine receptor chain transcript data are presented as fold change over transcript measured in freshly isolated NK cells from the same donor. Duplicate analyses performed for three independent donors. Representative data are shown. * P<0.05, ** P<0.01, ***P<0.001

Methods.

Antibodies and flow cytometry: The following anti-human monoclonal antibodies were used for detection of cytokine receptor cell surface expression: BioLegend: CD218a/IL18Rα (H44); R&D Systems: CD218/IL18Rβ (132029). Data were collected on a Gallios flow cytometer (Beckman Coulter) and analyzed using Kaluza (Beckman Coulter) or FlowJo (Tree Star).

qPCR: NK cells were prepared as in Supplementary Figure 6. The following specific primer/probe sets were used:
IL-18Ra **(Gillett A et al, Multiple Sclerosis, 2010, 16:1056-1065**):
FWD 5'-AGAATGCCGACCTGAAAATG-3'
REV 5'-TTTTCTCTATCAGTGAGTGGATTTCAT-3'
Probe 5'-TAGTGCCTGGAGGAGCTGTT-3'.
IL-18Rb FWD 5'-ATGGTGATGGGGAAATCAGA-3'
REV 5'-CATTGTTCTCCCGTGTTCCT-3'
Probe 5'-CCTGGATATGAATCCCCCTT-3'.
All wells also contained a primer/probe set for 18s RNA (TaqMan Ribosomal RNA Control), used to calculate ΔΔCt values.

Romee et al., Supplemental Figure 8

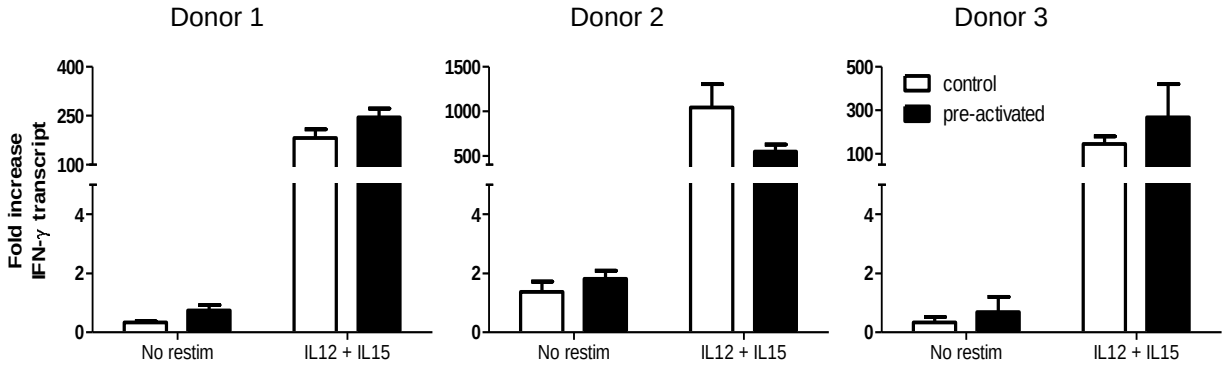


Supplemental Figure 8. Pre-activation does not alter STAT3 signaling in human NK cell subsets. Purified NK cells (>95 purity) were pre-activated, washed and rested (as in Fig 1A). After 7 days of culture, cells were restimulated for 6h with IL-12 (10 ng/mL) plus IL-15 (100 ng/mL) and immediately fixed, permeablized, stained with antibodies against phosphorylated STAT3 molecules, and analyzed via flow cytometry. Representative histograms and aggregate MFI data from pooled from 3-5 donors, over 2 independent experiments. Horizontal lines pair data points from a single donor. * P<0.05, ** P<0.01, ***P<0.001

Methods.

Antibodies and flow cytometry: For detection of phosphorylated STAT3 molecules, cells were activated with IL-12 (10 ng/mL) and IL-18 (50 ng/mL) for 15 minutes. Cells were then immediately fixed (1% formalin) and methanol permeabilized, followed by intracellular staining for pSTAT3 (4/P-STAT3) (BD Biosciences). Data were collected on a Gallios flowcytometer (Beckman Coulter) and analyzed using Kaluza (Beckman Coulter) or FlowJo (Tree Star).

Romee et al., Supplemental Figure 9



Supplemental figure 9. Pre-activation of NK cells is not associated with increased IFN-γ transcripts

Purified NK cells (>95% purity) were pre-activated, washed and rested (as in Figure 1A). After 7 days of culture, cells were restimulated for 6h with IL-12 (10 ng/mL) plus IL-15 (100 ng/ml). Quantitative RT-PCR analysis of the IFN-γ transcripts from individual donors. Data are presented as fold change over transcript measured in freshly isolated NK cells from the same donor. Duplicate analyses performed for three independent donors. Each data set represents a single donor.

Methods: NK cells were lysed in Trizol (Invitrogen, Grand Island NY) and prepared according to the manufacturer's protocol. cDNA was generated from RNA using the TaqMan Reverse Transcription Kit (Applied Biosystems) with random hexamer primers. QRT-PCR was performed on a 7300 series ABI instrument, using the standard program and a reaction volume of 20µL. The following specific primer/probe sets were used to detect IFN-γ transcripts:
FWD 5'- GTTCAGCCATCACTTGGATGAG-3'
REV 5'-GAAAAGCTGACTAATTATTCGGTAACTG-3'
Probe 5'-CTTGAATGTCCAACGCAAAGCAATACATGA-3'.

All wells also contained a primer/probe set for 18s RNA (TaqMan Ribosomal RNA Control), used to calculate ΔΔCt values.