Supplemental Materials

Standard immunological and molecular biology reagents and procedures

Cell lines, Media, and Reagents

HEK293T, K562, HPAC, THP-1, NCIH929, U266, RPMI8226 were purchased from ATCC (Manassas, VA) and maintained in media per the suppliers' recommendation. NK cells were cultured in SCG Medium (CellGenix GmbH, Freiburg, Germany) supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM GlutaMax, and 200 U/ml recombinant human IL-2 (Miltenyi Biotec). Clinical-grade Rim was diluted from a 100 μM stock solution in ethanol for *in vitro* assays. For animal studies, Rim was further diluted into 0.9% saline. Research grade temsirolimus (Sigma, St. Louis, MO) was dissolved in ethanol for *in vitro* assays and in Injection Diluent (10% polyethylene glycol [PEG]-400 + 5% Tween-80, Sigma, St.
Louis, MO) for animal studies.

NK cell isolation

Buffy coats from healthy donors were obtained from the Gulf Coast Regional Blood Center (Houston, TX). Peripheral blood mononuclear cells (PBMCs) were isolated by a density-gradient centrifugation technique (lymphoprep, Accurate Chemical & Scientific Corporation, Westbury, NY). $CD56^+$ NK cells were then enriched from PBMC using an NK isolation magnetic bead kit (Miltenyi Biotech, Inc., San Diego, CA) following manufacture's protocol.

Retroviral and Plasmid Constructs

iMC contains a TIR domain-deleted version of the TLR adaptor protein MyD88, CD40 cytoplasmic region, and two tandem ligand-binding FKBP domains.[1] iRC9 comprises tandem FRB and FKBP domain fused with truncated caspase-9.[2] Synthetic DNA (Integrated DNA Technology, San Diego, CA) encoding human IL-15 was cloned into SFG iRC9.iMC using the enzyme site SacII/PflMI to generate pSFG-iRC9-IL15-ΔCD19-iMC. These SFG retroviral vectors also consist a truncated human CD19 as a marker. P2A and T2A sequences were used to separate encoding genes. A first generation CD123 CAR was generated with the 32716 scFV targeting CD123,[3,4] CD8α stalk transmembrane domain, and the cytoplasmic CD3ζ domain. iMC-BCMA.ζ-IL15 was generated by subcloning ScFV targeting BCMA (C12.A3.2L) and IL15 genes into pSFG-iMCCAR.ζ with XhoI/BamHI and SalI/MluI. All vectors contain CAR also had the QBEnd-10 minimal epitope (CD34 epitope)[5] as the marker. Tumor cells or NKs were transduced with pSFG-eGFPFirefly luciferase (eGFPFfluc) or pSFG-orange Nano-lantern Renilla luciferase (ONLRluc)[6] for labelling.

Flow methods for transduction efficiency

Gene-modified NK cells were stained with anti-CD56-APCcy7 and anti-CD19-BV421 for iMC or iRC9 transgene expression. For CAR transduced cells, anti-CD34 QBEnd-10-PE (Abnova) was used to determine transduction efficiency.

CD107a and intracellular staining

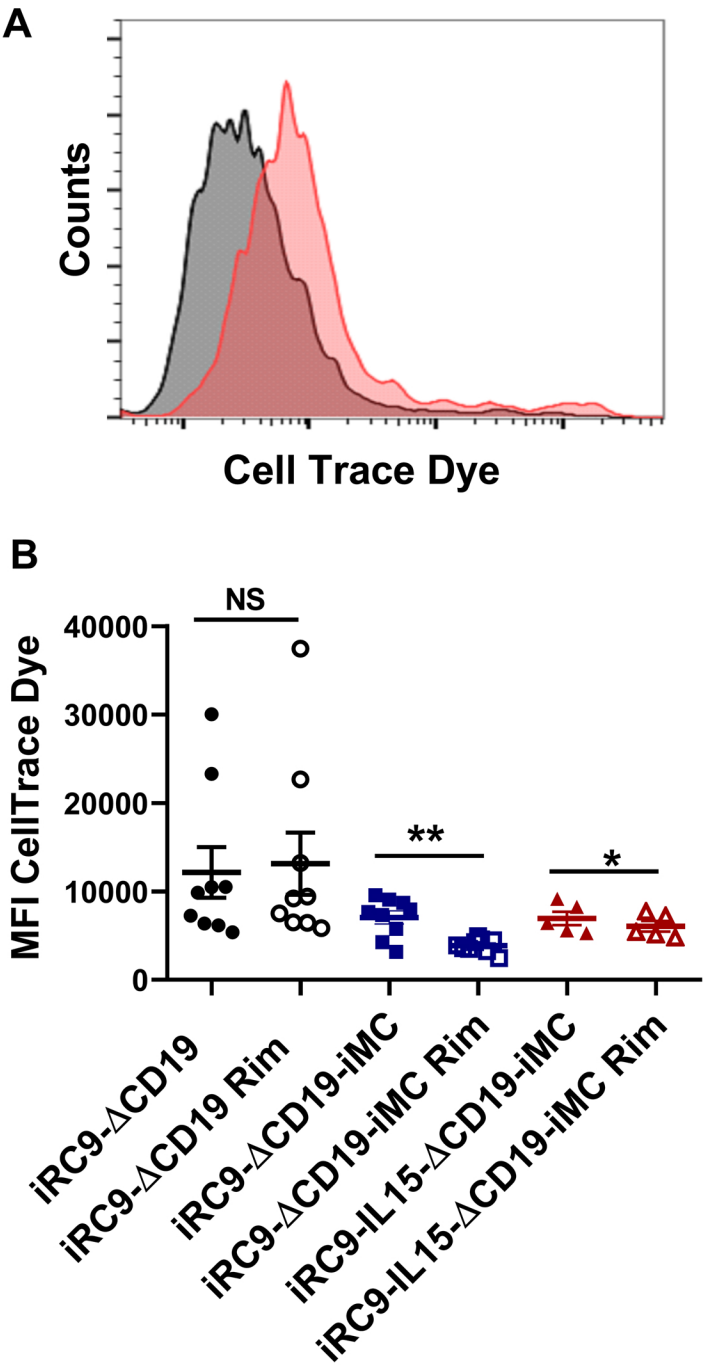
CD107a degranulation and intracellular cytokine IFN-g, TNF-α, perforin, granzyme B productions were measured as reported.[7] Briefly, NK cells modified with iRC9 or iRC9.iMC vectors were cocultured with or without THP-1 tumor targets at 1:1 E/T ratio in the presence or absence of 1 nM Rimiducid for 4-hour incubation. Anti CD107a-PE antibody (eBioscience, San Diego, CA) was added during the first hour, followed by the secretion inhibitor monensin (2 μM, BioLegend) and Brefeldin A (5 μg/ml, BioLegend, San Diego, CA) treatment for the next 3 hrs. Cells were then washed, stained with surface antibodies anti-CD19-BV421, anti-CD56-APCcy7, anti-CD3-FITC (BioLegend). For intracellular staining, gene modified NK cells were co-cultured with or without THP-1 and rimiducid for overnight. Secretion inhibitors were added. Cells were harvested,2 washed, stained with surface antibodies anti-CD56-APC/Cy7 and anti-CD19-PE or anti-CD56 PE and anti-CD19 BV421 (BioLegend), fixed, permeabilized and stained intracellularly with anti-IFN-

γ-APC, anti-TNF-α-APC (BD Biosciences), anti-Perforin-APC/Cy7 (BioLegend), or anti-Granzyme B-AF647 (BD Biosciences). For anti-T-bet PEcy7 and anti-PE Eomes staining, a transcription factor staining kit was used (eBioscience, San Diego, CA) after the NK cells were first stained with viability dye and surface anti-CD56 APC/Cy7 and anti-CD19 BV421.

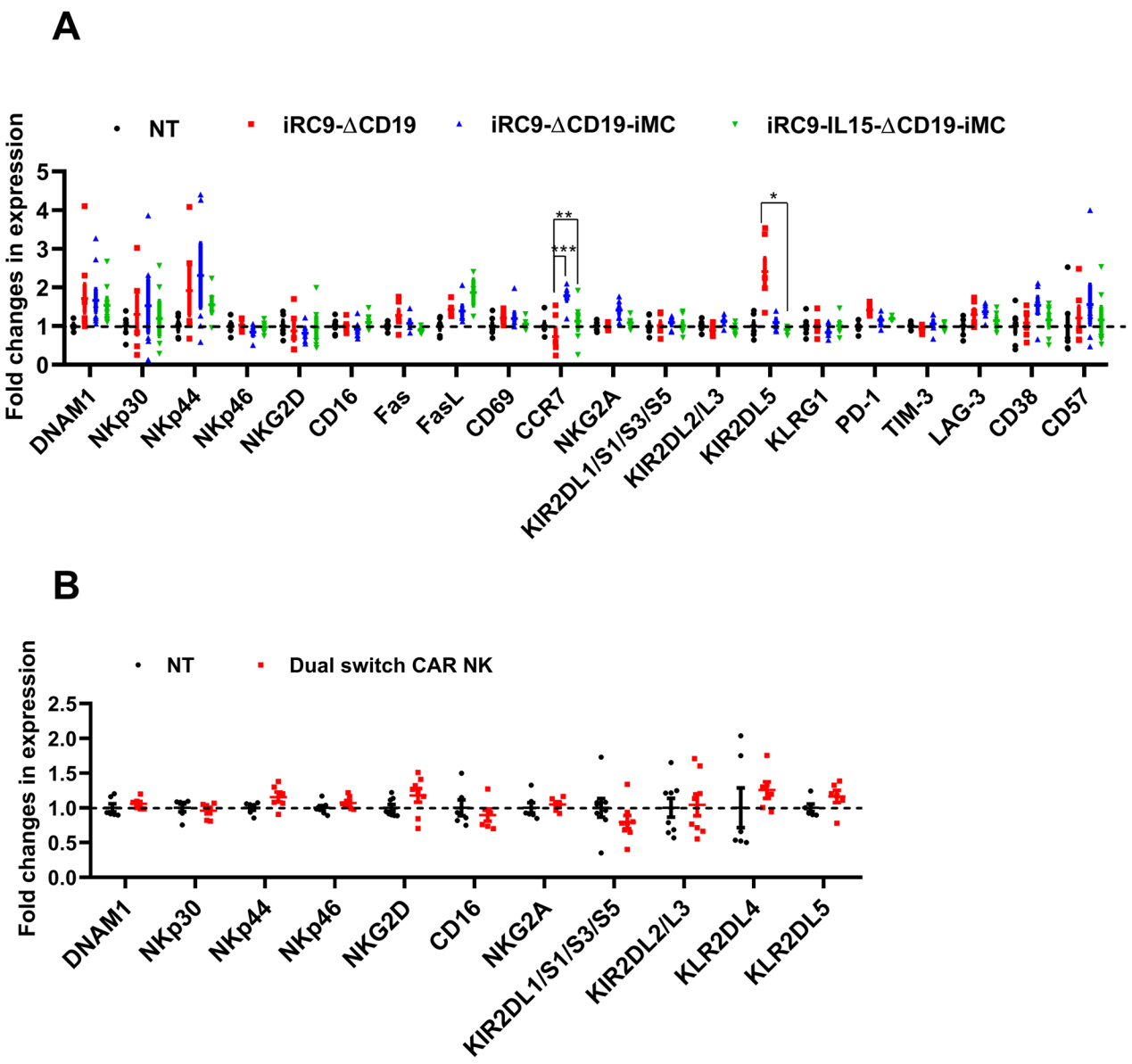
Cytokine production

Production of IFN-γ by NKs modified with iRC9-ΔCD19, iRC9-ΔCD19-iMC, or iRC9-IL15-ΔCD19-iMC in the presence of various concentration Rimiducid was analyzed by ELISA (eBioscience). Ectopic IL-15 production was accessed by ELISA (BioLegend). In some experiments, cytokines production was analyzed using a 29-cytokine/chemokine mutiplex array (Milliplex MAP; Millipore) system with a multiplex reader (Bio-Plex MAGPIX, Bio-rad).

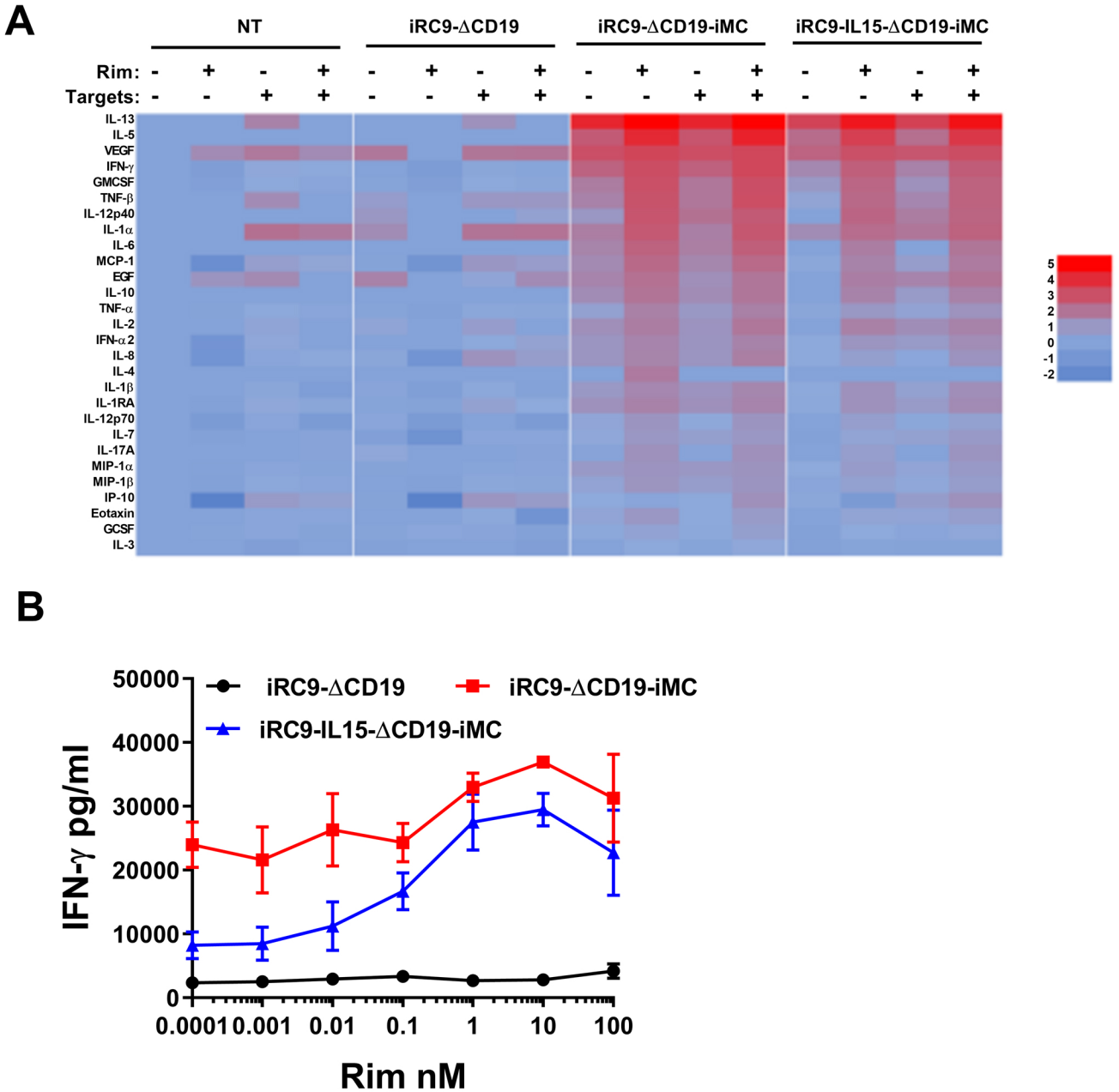
1. Narayanan P, Lapteva N, Seethammagari M, Levitt JM, Slawin KM, Spencer DM. A composite MyD88/CD40 switch synergistically activates mouse and human dendritic cells for enhanced antitumor efficacy. *J Clin Invest*. 2011;121(4):1524-1534.
2. Duong MT, Collinson-Pautz MR, Morschl E, et al. Two-Dimensional Regulation of CAR-T Cell Therapy with Orthogonal Switches. *Mol Ther Oncolytics*. 2019;12:124-137.
3. Foster AE, Mahendravada A, Shinners NP, et al. Regulated Expansion and Survival of Chimeric Antigen Receptor-Modified T Cells Using Small Molecule-Dependent Inducible MyD88/CD40. *Mol Ther*. 2017;25(9):2176-2188.
4. Mardiros A, Dos Santos C, McDonald T, et al. T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. *Blood*. 2013;122(18):3138-3148.
5. Philip B, Kokalaki E, Mekkaoui L, et al. A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. *Blood*. 2014;124(8):1277-1287.
6. Takai A, Nakano M, Saito K, et al. Expanded palette of Nano-lanterns for real-time multicolor luminescence imaging. *Proc Natl Acad Sci U S A*. 2015;112(14):4352-4356.
7. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods*. 2003;281(1-2):65-78.



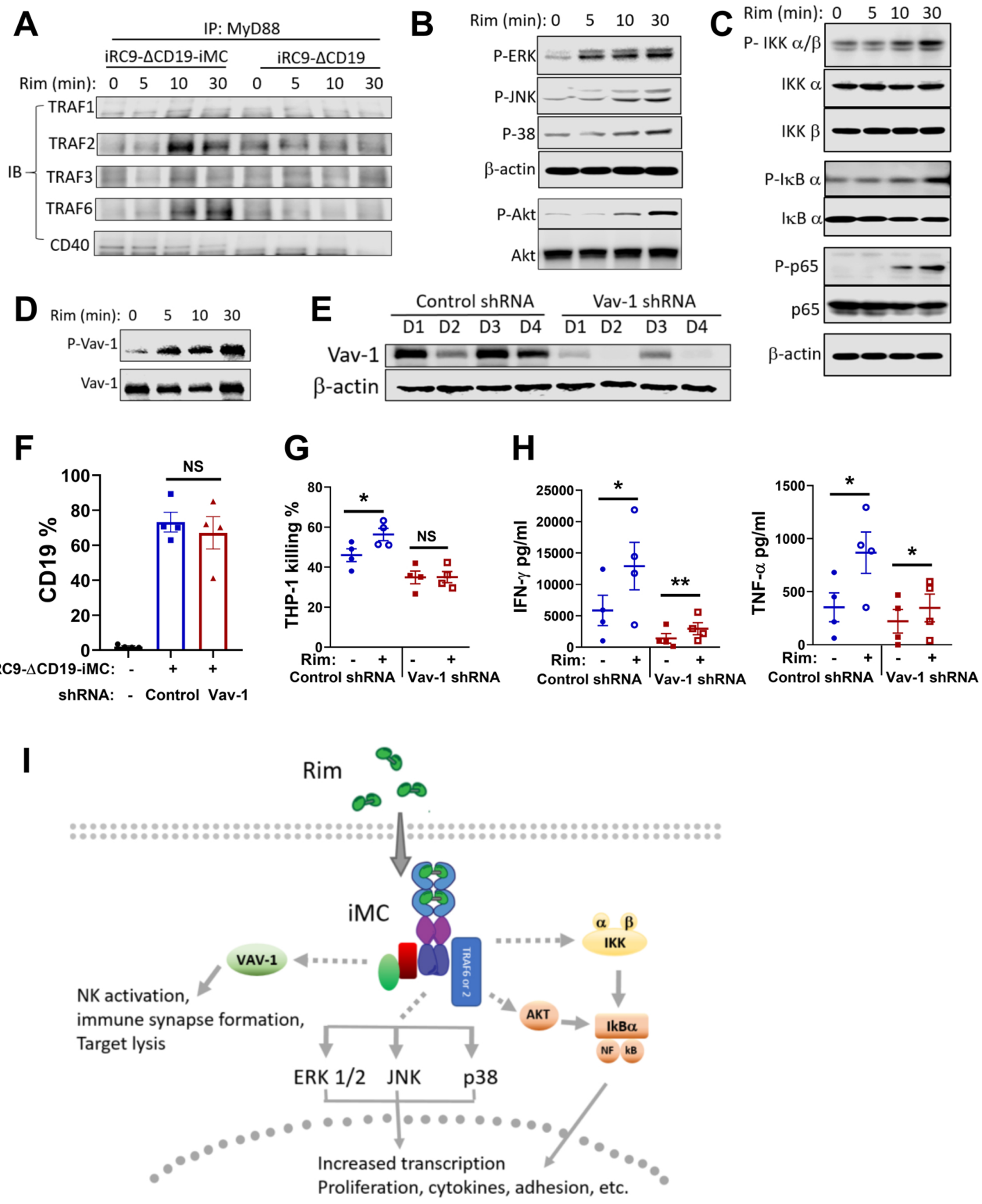
Supplemental figure 1. iMC modified NK cells show enhanced proliferation upon Rim activation. NK cells were labeled with CellTrace dye on the day of transduction. Proliferation was assessed 9 days later by dilution of labeling with each cell doubling. **(A)** Representative histograms demonstrating CellTrace dilution in iRC9-ΔCD19-iMC modified NK cell populations (gated on CD56+CD3-CD19+) with of 1 nM Rim (dark gray) or no Rim (red). **(B)** MFI of CellTrace dye in iRC9-ΔCD19, iRC9-ΔCD19-iMC, or iRC9-IL15-ΔCD19-iMC modified NKs with or without 1 nM Rim. Paired t test was used to compare the indicated groups. NS not significant; * $P < 0.05$; ** $P < 0.01$.



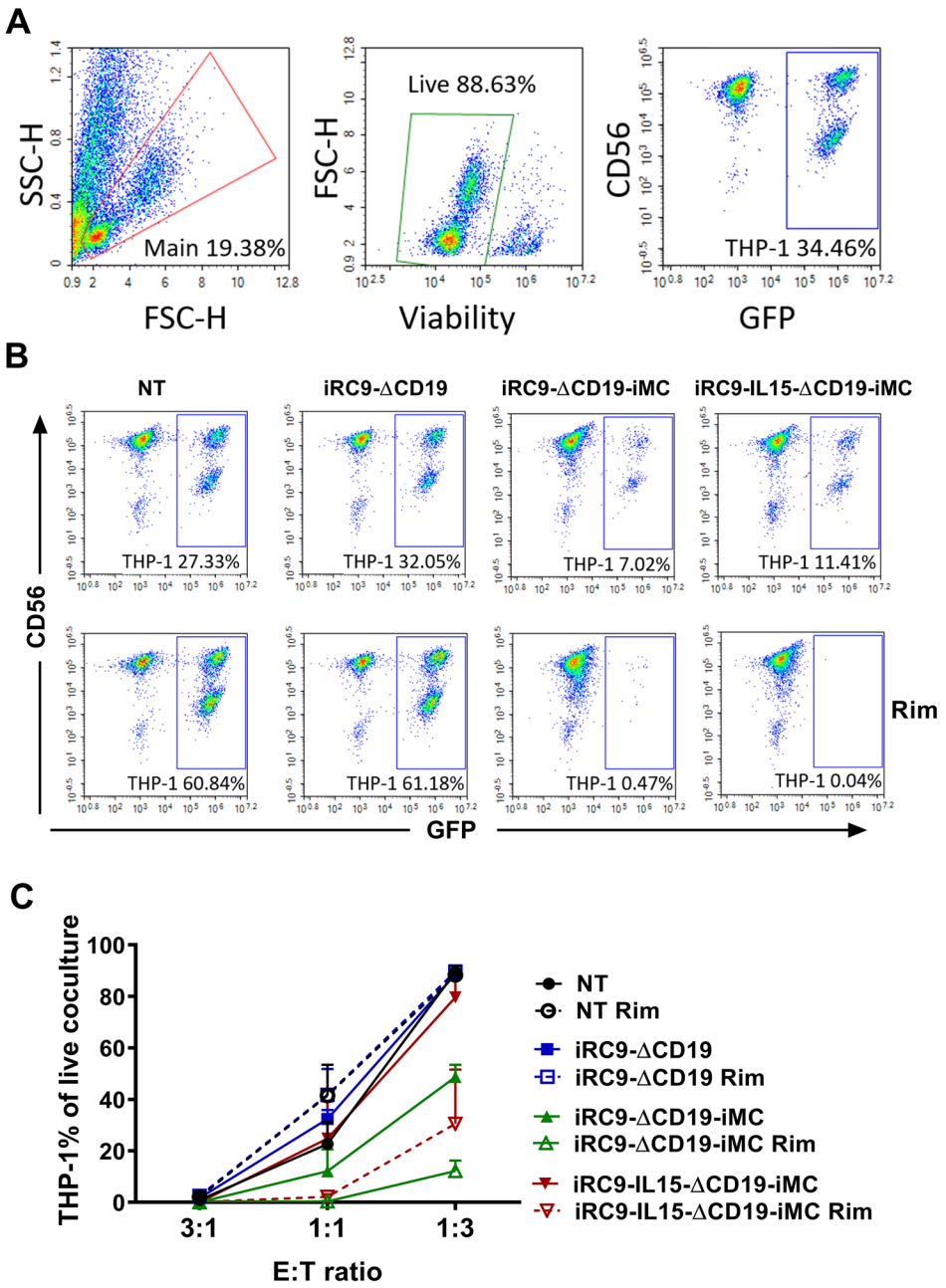
Supplemental figure 2. Phenotype of iMC transduced NK cells. (A) iRC9-ΔCD19, iRC9-ΔCD19-iMC, or iRC9-IL15-ΔCD19-iMC modified NK cell phenotypes based on the average expression of DNAM1, NKP30, NKP44, NKP46, NKG2D, CD16, Fas, FasL, CD69, CCR7, NKG2A, KIR2DL1/S1/S3/S5, KIR2DL2/L3, KIR2DL5, KLRG1, PD-1, TIM-3, LAG-3, CD38, and CD57 were characterized by multiparameter flow cytometry analysis. **(B)** Dual switch CAR NK (CD123.ζ + iRC9-IL15-ΔCD19-iMC) cell phenotype based on the average expression of DNAM1, NKP30, NKP44, NKP46, NKG2D, CD16, NKG2A, KIR2DL1/S1/S3/S5, KIR2DL2/L3, and KIR2DL4 was characterized by multiparameter flow cytometry analysis. MFI or the percentages of positive cells were normalized according the average value of NT NK cells. Paired t test was used to compare indicated groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



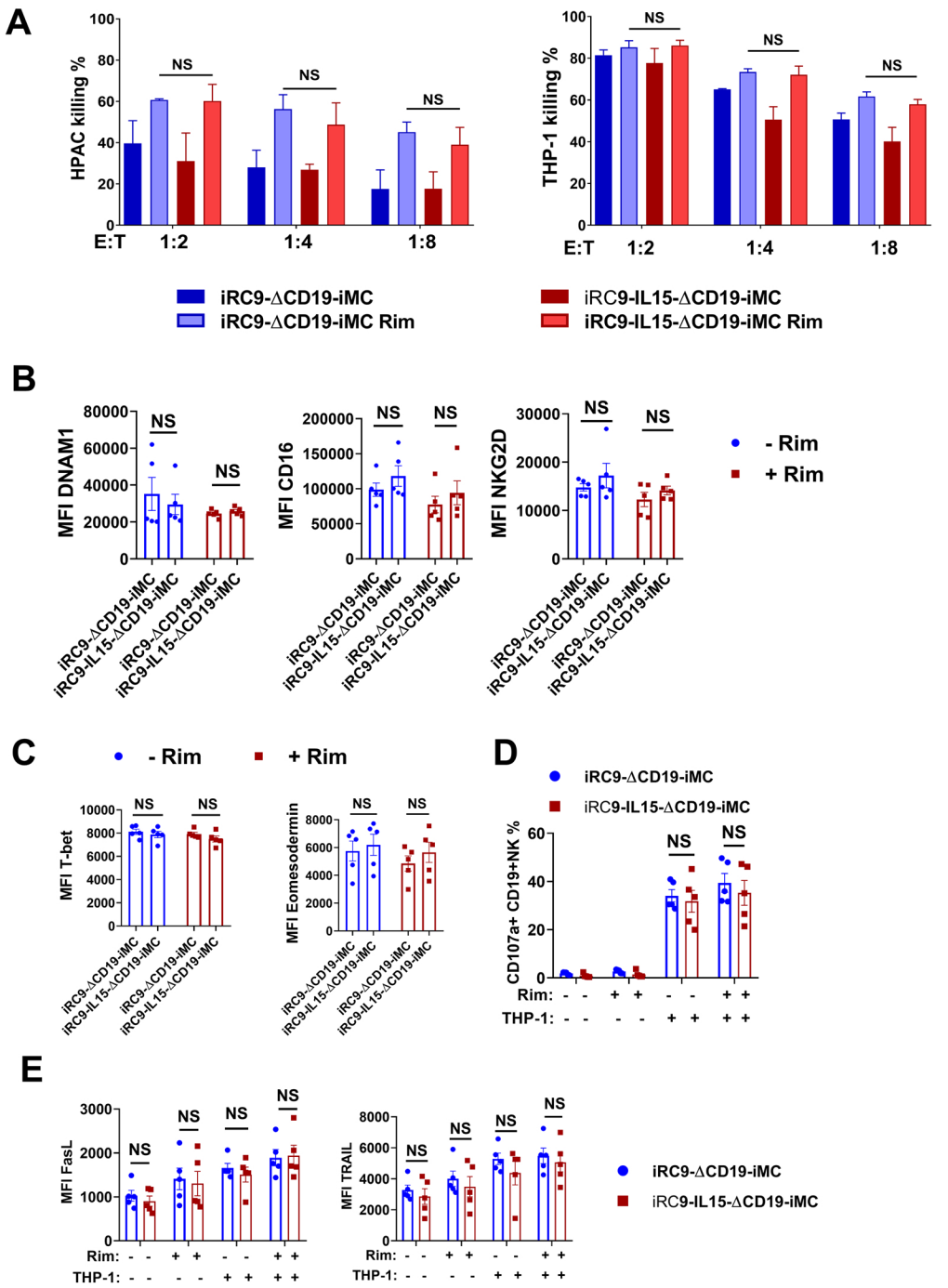
Supplemental figure 3. iMC and iMC-IL15 enhance cytokine production. (A) 28-plex cytokine multiplex analysis of supernatant from NT, iRC9-ΔCD19, iRC9-ΔCD19-iMC, or iRC9-IL15-ΔCD19-iMC modified NKs cocultured with or without THP-1 tumor targets at the E:T ratio of 3:1 in the absence or presence of 1 nM Rim for 48 hrs. Fold changes were in log scale compared with NT cells cultured without Rim or target cells. **(B)** IFN-γ production in supernatants by iRC9-ΔCD19, iRC9-ΔCD19-iMC, or iRC9-IL15-ΔCD19-iMC modified NK cells when co-cultured with K562 cells at an E:T ratio 1:1 for 48 hr in the presence of increasing Rim concentrations (0 – 100 nM) (N = 3). 2-way ANOVA was used for comparisons. $P < 0.0001$.


A
IP: MyD88
iRC9-ΔCD19-iMC
iRC9-ΔCD19
Rim (min): 0 5 10 30 0 5 10 30
IB
TRAF1
TRAF2
TRAF3
TRAF6
CD40
B
Rim (min): 0 5 10 30
P-ERK
P-JNK
P-38
β-actin
P-Akt
Akt
C
Rim (min): 0 5 10 30
P- IKK α/β
IKK α
IKK β
P-IκB α
IκB α
P-p65
p65
β-actin
D
Rim (min): 0 5 10 30
P-Vav-1
Vav-1
E
Control shRNA
Vav-1 shRNA
D1 D2 D3 D4 D1 D2 D3 D4
Vav-1
β-actin
F
CD19 %
NS
iRC9-ΔCD19-iMC: - + +
shRNA: - Control Vav-1
G
THP-1 killing %
*
NS
Rim: - + - +
Control shRNA
Vav-1 shRNA
H
IFN-γ pg/ml
*
**
Rim: - + - +
Control shRNA
Vav-1 shRNA
TNF-α pg/ml
*
*
Rim: - + - +
Control shRNA
Vav-1 shRNA
I
Rim
iMC
TRAF6 or 2
VAV-1
NK activation,
immune synapse formation,
Target lysis
α β
IKK
AKT
IkBα
NF kB
ERK 1/2 JNK p38
Increased transcription
Proliferation, cytokines, adhesion, etc.

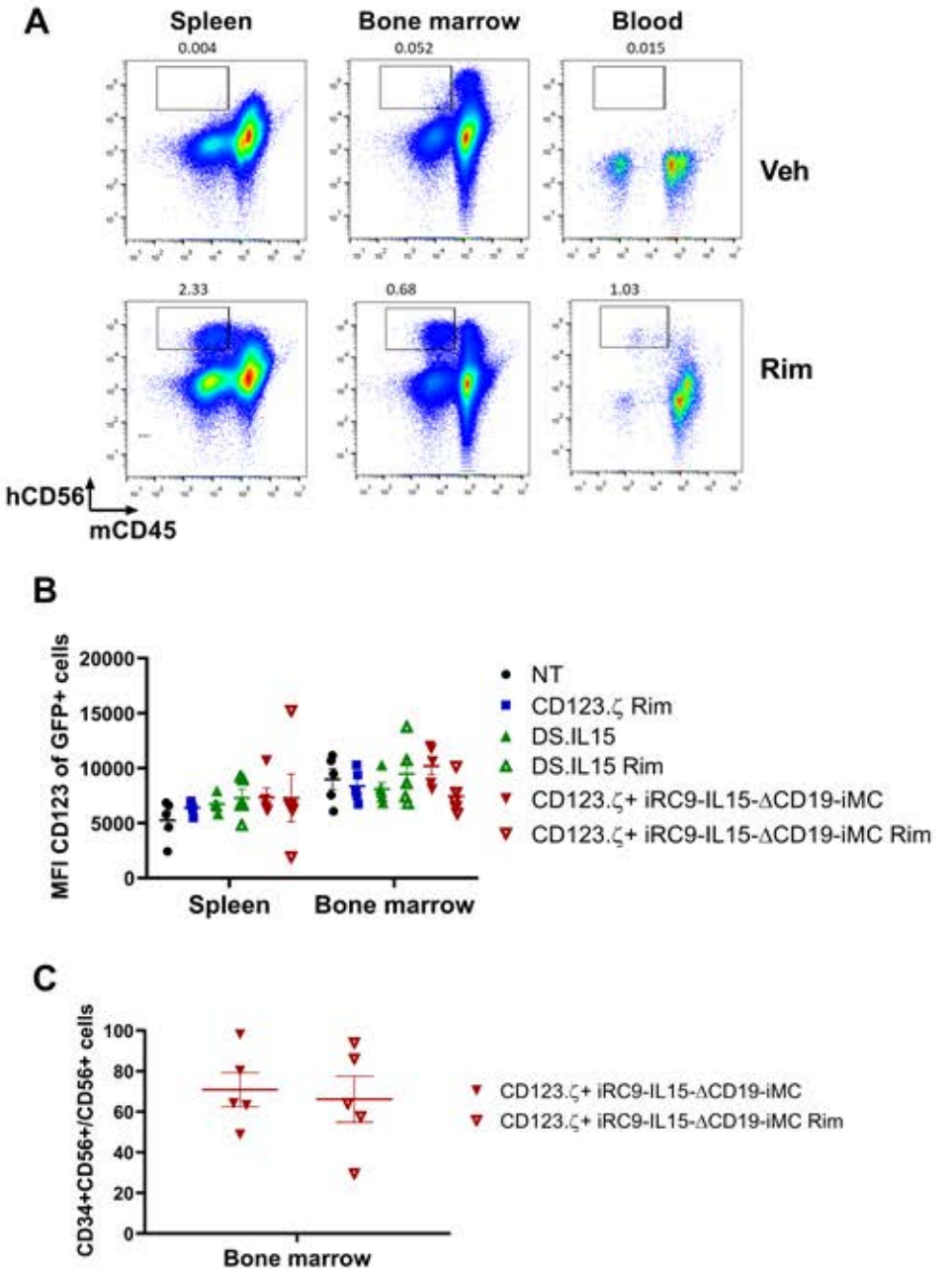
Supplemental figure 4. Rim activation of iMC regulated multiple signaling pathways in NK cells. (**A**) NK cells transduced with either iRC9-ΔCD19-iMC or iRC9-ΔCD19 were treated with 1 nM Rim for 0, 5, 10, 30 min. Cell lysates were subjected to immunoprecipitation with anti-MyD88 and immunoblotted with anti-TRAF1, TRAF2, TRAF3, TRAF6, and CD40. (**B**) iRC9-ΔCD19-iMC modified NK cells treated with 1 nM Rim for the indicated times were lysed and analyzed for phosphorylated (p) p-ERK, p-JNK, p-38, β-actin, p-AKT, and total AKT by Western immunoblotting. (**C**) The cell lysates were also subjected to immunoblotting for p-IKK α/β, IKK α, p-IκB α, IκB α, p-p65, p65 and (**D**) p-vav and total vav1. (**E**) 4 different donors of iRC9-ΔCD19-iMC modified NKs also transfected with control shRNA or shRNA targeting vav-1. Vav-1 knockdown was accessed via immunoblotting. (**F**) iRC9-ΔCD19-iMC transduction was measured by CD19 surface staining and flow analysis. (**G**) Gene modified NKs were co-cultured with THP-1 eGFPFfluc targets overnight. NK cytotoxicity was determined by a luciferase activity assay (E:T 1:16). (**H**) IFN-γ and TNF-α production were measured by ELISA (E:T 1:1). Paired t test was used to compare indicated groups. NS not significant; * $P < 0.05$; ** $P < 0.01$. **(I)** The mechanisms of iMC induced NK cells activation - Rim induced dimerization of iMC molecules recruits adaptor proteins such as TRAF2 and TRAF6, followed by activation of kinases and downstream phosphorylation of MAPK pathways, phosphorylation of VAV-1, and activation of AKT, IKK, and NF-κB pathways, together resulting in non-transcriptional NK cell activation (immune synapse formation, target lysis) and transcriptional regulation and effects (NK cell activation, cytokine production, proliferation).



Supplemental figure 5. iMC enhances NK cell cytotoxicity against THP-1 cells upon Rim activation. Potency of NK cells modified with iRC9-ΔCD19-iMC or iRC9-IL15-ΔCD19-iMC compared with NT or iRC9-ΔCD19 modified NKs cells were tested in a 48-hr coculture assay with THP-1-eGFPFfluc in the presence of 0 or 1 nM Rim. **(A)** THP-1-eGFPFfluc cells were gated out as viability dye negative, GFP+ populations. **(B)** Representative flow plot showing NK cell and tumor cells at an E:T ratio of 1:1. **(C)** THP-1-eGFPFfluc percentage in the co-cultures with 3 different donor's NK cells at E:T ratios of 3:1, 1:1, or 1:3 were plotted. 2-way ANOVA was used for comparisons. $P < 0.0001$.



Supplemental figure 6. Ectopic IL-15 expression does not lead to DS.IL15 NK cell exhaustion. iRC9-ΔCD19-iMC or iRC9-IL15-ΔCD19-iMC gene modified NK cells were cultured in the presence or absence of 1nM Rim for 6 days. **(A)** Potency of NK cells was tested in 24-hr co-culture assays with HPAC-EGFPffluc or THP-1-EGFPffluc at decreasing effector-to-target (E:T) ratios. **(B)** NK activating receptors DNAM1, CD16, and NKG2D cell surface expression, and **(C)** nuclear T-bet and eomesodermin levels were measured by intracellular flow cytometry and indicated by mean fluorescence intensity of CD19+ NK cells. **(D-E)** iRC9-ΔCD19-iMC or iRC9-IL15-ΔCD19-iMC gene modified NK cells were incubated with / without THP-1 targets for 4 hrs (D) or overnight (E) in the presence or absence of 1 nM Rim. Percentages of cells expressing surface (D) CD107a and (E) cell surface FasL and TRAIL were measured by flow cytometry. Paired t tests were used to compare indicated groups. NS not significant.



Supplemental figure 7. In vivo persistence of NK cells modified with DS.IL15 and CD123.ζ (A) Representative flow plots of human NK cells present in spleen, bone marrow and peripheral blood as hCD56+mCD45- populations. **(B)** CD123 surface expression of THP-1-eGFPFfluc tumor cells (GFP+ population) in spleen and bone marrow. Cells were isolated at day 35 post NK cell therapy, excepting the CD123.ζ + DS.IL15 Rim group that was at day 53. 2-way ANOVA was used for comparisons. P = 0.47. **(C)** CD123.ζ CAR expression was determined in bone marrow of iRC9-IL15-ΔCD19-iMC + CD123.ζ groups, indicated as CD34+CD56+/CD56+mCD45- population.