Journal for ImmunoTherapy of Cancer

Enhanced IL-15-mediated NK cell activation and proliferation by an ADAM17 function-blocking antibody involves CD16A, CD137, and accessory cells

Anders W Matson,[1] Rob Hullsiek,[2] Kate J Dixon,[3] Sam Wang,[3] Anders J Lindstedt ,[2,4] Ryan R Friess,[5] Shee Kwan Phung,[1] Tanya S Freedman ,[6,7,8] Martin Felices ,[9] Emily N Truckenbrod,[3] Jianming Wu,[3] Jeffrey S Miller,[6,7,9] Bruce Walcheck [3,6,7]

To cite: Matson AW, Hullsiek R, Dixon KJ, *et al*. Enhanced IL-15-mediated NK cell activation and proliferation by an ADAM17 function-blocking antibody involves CD16A, CD137, and accessory cells. *Journal for ImmunoTherapy of Cancer* 2024;**12**:e008959. doi:10.1136/jitc-2024-008959

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-008959).

Accepted 27 June 2024

For numbered affiliations see end of article.

Correspondence to
Dr Bruce Walcheck; walch003@umn.edu

ABSTRACT

Background Natural killer (NK) cells are being extensively studied as a cell therapy for cancer. These cells are activated by recognition of ligands and antigens on tumor cells. Cytokine therapies, such as IL-15, are also broadly used to stimulate endogenous and adoptively transferred NK cells in patients with cancer. These stimuli activate the membrane protease ADAM17, which cleaves various cell-surface receptors on NK cells as a negative feedback loop to limit their cytolytic function. ADAM17 inhibition can enhance IL-15-mediated NK cell proliferation in vitro and in vivo. In this study, we investigated the underlying mechanism of this process.

Methods Peripheral blood mononuclear cells (PBMCs) or enriched NK cells from human peripheral blood, either unlabeled or labeled with a cell proliferation dye, were cultured for up to 7 days in the presence of rhIL-15±an ADAM17 function-blocking antibody. Different fully human versions of the antibody were generated; Medi-1 (IgG1), Medi-4 (IgG4), Medi-PGLALA, Medi-F(ab′)$_2$, and TAB16 (anti-ADAM17 and anti-CD16 bispecific) to modulate CD16A binding. Flow cytometry was used to assess NK cell proliferation and phenotypic markers, immunoblotting to examine CD16A signaling, and IncuCyte-based live cell imaging to measure NK cell antitumor activity.

Results The ADAM17 function-blocking monoclonal antibody (mAb) Medi-1 markedly increased early NK cell activation by IL-15. By using different engineered versions of the antibody, we demonstrate involvement by CD16A, an activating Fcγ receptor and well-described ADAM17 substrate. Hence, Medi-1 when bound to ADAM17 on NK cells is engaged by CD16A and blocks its shedding, inducing and prolonging its signaling. This process did not promote evident NK cell fratricide or dysfunction. Synergistic signaling by Medi-1 and IL-15 enhanced the upregulation of CD137 on CD16A$^+$ NK cells and augmented their proliferation in the presence of PBMC accessory cells or an anti-CD137 agonistic mAb.

Conclusions Our data reveal for the first time that CD16A and CD137 underpin Medi-1 enhancement of IL-15-driven NK cell activation and proliferation, respectively, with the latter requiring PBMC accessory cells. The use of Medi-1 represents a novel strategy to enhance IL-15-driven NK cell proliferation, and it may be of therapeutic importance by increasing the antitumor activity of NK cells in patients with cancer.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Natural killer (NK) cell therapies are being broadly investigated to treat cancer, and cytokines, such as IL-15, are used to enhance their function and prolong their survival in patients with cancer. Various NK cell stimuli activate the membrane protease ADAM17, which regulates the cell surface density of various receptors on NK cells as a negative feedback mechanism.

WHAT THIS STUDY ADDS

⇒ Treating NK cells with the ADAM17 function-blocking monoclonal antibody Medi-1 markedly enhanced their activation and proliferation by IL-15. Our study reveals that the Fc and Fab regions of Medi-1 function synergistically with IL-15 in NK cell activation. This augmented the upregulation of CD137 in NK cells, which enhanced their proliferation in the presence of peripheral blood mononuclear cell accessory cells.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our study is of translational importance as Medi-1 treatment in combination with IL-15 or other cytokines could potentially augment the proliferation and function of endogenous or adoptively transferred NK cells in patients with cancer.

BACKGROUND

Natural killer (NK) cells are innate lymphocytes that use germline-encoded receptors to interrogate cells of the body for ligands

that are either aberrantly upregulated or downregulated to mediate natural cytotoxicity. Human NK cells also target cells by recognizing cell-bound IgG antibodies via their Fc receptor CD16A (FcγRIIIA), inducing antibody-dependent cell-mediated cytotoxicity (ADCC).[1] Due to their broad antitumor effector functions, there has been a growing emphasis on invoking endogenous NK cells in patients with cancer and the adoptive transfer of autologous or allogeneic NK cells.[2–4] Key challenges in using NK cells for cancer therapies are that they compose a minor population of circulating lymphocytes and their persistence is limited following adoptive transfer.[5 6] Cytokines such as IL-15 are commonly used to promote human NK cell priming, cytotoxicity, and expansion ex vivo and in patients.[7]

NK cell activation induces the activity of the membrane-associated protease ADAM17 (a disintegrin and metalloproteinase-17),[8 9] which has a variety of substrates.[10 11] Their cleavage typically occurs in a cis manner at an extracellular location proximal to the cell membrane, referred to as ectodomain shedding.[10] CD16A on NK cells is a well-described ADAM17 substrate that is rapidly cleaved following NK cell activation as a negative feedback mechanism[10], and upon its engagement of cell-bound antibodies, as a detachment process.[12]

We have reported that blocking ADAM17 function with a monoclonal antibody (mAb) markedly enhanced IL-15-driven NK cell expansion ex vivo and in vivo.[9] In this study, we sought to elucidate the underlying mechanism of this process. We show that Medi-1, a fully human IgG1 mAb that selectively blocks ADAM17 function, is engaged by CD16A while blocking its shedding. This signaling synergizes with IL-15 to enhance CD137 upregulation and NK cell proliferation when in the presence of peripheral blood mononuclear cell (PBMC) accessory cells or an anti-CD137 agonist mAb.

METHODS

Antibodies

The anti-human ADAM17 mAb MEDI3622 has been previously described.[13] The following Fc variants were generated from its nucleotide sequence. Human isotypes IgG1 and IgG4 were generated by Syd Labs (Natick, Massachusetts, USA) and are referred to as Medi-1 and Medi-4, respectively. The mutations Pro329Gly, Leu234Ala, and Leu235Ala (PGLALA) were engineered into the Fc region of Medi-1 (Syd Labs) to impair FcγR binding and is referred to as Medi-PGLALA. The Fc region of Medi-1 was proteolytically removed (SouthernBiotech, Birmingham, Alabama, USA) and is referred to Medi-F(ab′)$_2$. The Trispecific Killer Engager (TriKE) containing a human IL-15 moiety and humanized camelid single-domain antibodies against CD16A and B7H3 (cam16-IL15-camB7H3), as well as a Targeted ADAM17 Blocker-16 (TAB16), containing a single-chain fragment variable (scFv) from Medi-1 and a humanized camelid single-domain antibody against CD16, were generated similar to as previously described.[14] All commercially available antibodies are listed in table 1. Any antibodies used in functional assays that contained a preservative, such as sodium azide (NaN_3), were subjected to a Zeba Spin Desalting Column 40K (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's instructions, and resuspended in PBS buffer without Ca^{+2} and Mg^{+2} (Gibco, Waltham, Massachusetts, USA).

Flow cytometric analyses

NK cell phenotypic analyses were performed as previously described.[9] Briefly, fluorescence minus one (FMO) and appropriate isotype-matched antibodies were used for controls. An FSC-A/SSC-A plot was used to set an electronic gate on leukocyte populations, and an FSC-A/FSC-H plot was used to set an electronic gate on single cells. The cell viability dye Ghost Dye Red 710 (Tonbo Bioscience, San Diego, California, USA) was used to distinguish live vs dead cells per the manufacturer's instructions. Cell samples were acquired on a BD FACSCelesta Cell Analyzer (BD Biosciences, San Jose, California, USA) and analyzed using FlowJo V.10.9.0 Software (BD Bioscience).

Cell isolation and culture

PBMCs were obtained from whole blood, collected from healthy consenting adults at the University of Minnesota (IRB protocol # 9708M00134) in sodium heparin tubes (BD Bioscience), and from plateletpheresis products from Innovative Blood Resources (St. Paul, Minnesota, USA). PBMCs were isolated using Lymphocyte Separation Medium (Promocell, Heidelberg, Germany). NK cells were enriched using a negative selection human NK Cell Isolation Kit (Miltenyi Biotec, Bergisch, Germany) or (StemCell Technologies, Vancouver, BC, Canada). Isolated NK cells were $\geq$ 90% pure, as determined by $CD56^+$ $CD3^-$ staining and flow cytometry. PBMCs or enriched NK cells, either unlabeled or labeled with CellTrace Violet (CTV) Cell Proliferation Dye (ThermoFisher Scientific) as per the manufacturer's instructions, were cultured for up to 7 days at 37°C in 5% CO_2 in RPMI 1640 media (Gibco) supplemented with 10% Heat Inactivated FBS (Gibco) and 1x Anti-Anti (Gibco), in the presence or absence of rhIL-15 or rhIL-2 (R&D Systems, Minneapolis, Minnesota, USA) and the indicated antibodies. NK cell proliferation was analyzed via flow cytometry and a division index was calculated using FlowJo.

SKOV-3 cells (HTB-77), an ovarian adenocarcinoma cell line, K562 cells (CCL-243), a chronic myelogenous leukemia cell line, and NK-92 cells (CRL-2407), a malignant non-Hodgkin's lymphoma NK cell line, were purchased from ATCC (Manassas, Virginia, USA). SKOV-3 cells stably expressing NucLight-Green (SKOV-3/NLG) were generated as previously described.[15] SKOV-3 cells were maintained in McCoy's 5A media (Gibco) supplemented with 10% HI FBS

Table 1 Commercial antibodies

Antibody	Flourochrome conjugate	Animal origin	Species recognized	Antigen/ligand recognized	Source, catalog #
CD56	Pe/Cy7	Mouse	Human	CD56	Biolegend, 318318
CD107a (LAMP-1)	APC	Mouse	Human	CD107a	Biolegend, 328620
CD69	PerCP/Cy5.5	Mouse	Human	CD69	BioLegend, 310927
CD16	BV510	Mouse	Human	CD16	BioLegend, 302048
NKG2D	BV605	Mouse	Human	NKG2D	BioLegend, 320832
NKp46	BV650	Mouse	Human	NKp46	BioLegend, 331927
KIR2DL1	FITC	Mouse	Human	KIR2DL	BioLegend, 339504
NKG2A	PE	Mouse	Human	NKG2A	BioLegend, 375104
CD3	BV785	Mouse	Human	CD3	BioLegend, 300472
CD3	FITC	Mouse	Human	CD3	BioLegend, 300440
KIR2DL2/L3	FITC	Mouse	Human	KIR2DL2/L3	BioLegend, 312604
KIR3DL1	PE	Mouse	Human	KIR3DL1	BioLegend, 312708
NKp44	APC	Mouse	Human	NKp44	BioLegend, 325110
LAG-3	BV510	Mouse	Human	LAG-3	BioLegend, 369318
TIGIT	BV605	Mouse	Human	TIGIT	BioLegend, 372712
TIM-3	BV650	Mouse	Human	TIM-3	BioLegend, 345028
CD25	FITC	Mouse	Human	CD25	BioLegend, 302604
BBK-2 *** NaN_3 removed	NA	Human	Human	CD137	Invitrogen, MA5-13739
CD137	APC	Mouse	Human	CD137	BioLegend, 309810
Human 4-1BBL/ CD137L/TNFSF9, ECD, Fc-fusion (sCD137L/Fc)	NA	Human	Human	CD137	G&P Bioscience, FCL2590B
IFN-γ	PE	Mouse	Human	IFN-γ	BioLegend, 502508
CD4	FITC	Mouse	Human	CD4	BioLegend, 317408
CD8	PE	Mouse	Human	CD8	BioLegend, 344706
Ghost Dye Red 710	Red 710				Tonbo Biosciences, 13-0871
D1(A12)	NA	Human	Human	ADAM17	Merck Millipore Sigma, MABT884
Human IgG1	NA	Human	NA	NA	Merck Millipore Sigma, AG502-M
Urelumab	NA	Human	Human	CD137	Invitrogen, MA5-141939
Daratumumab	NA	Human	Human	CD38	Janssen Biotech, Inc
pZAP-70/pSYK	NA	Rabbit	Human	Phospho-Zap-70 (Tyr319)/ Syk (Tyr352) (65E4)	Cell Signaling Technology, 2717S
Total SYK	NA	Mouse	Human	Syk (4D10)	Cell Signaling Technology, 80460
Anti-Rabbit IgG	IRDye 800CW	Donkey	Rabbit	IgG	LICORbio, 926-32213
Anti-Mouse IgG	IRDye 680RD	Donkey	Mouse	IgG	LICORbio, 926-68072

and 1X Anti-Anti. K562 cells were maintained in 10% RPMI 1640 media, as described above. NK-92 cells were maintained in MEM Alpha media (Gibco) with 12.5% HI FBS (Gibco), 12.5% Horse Serum (Gibco), 1x Anti-Anti, and 0.1 mM 2-Mercaptoethanol (Gibco). All cell lines were routinely tested for mycoplasma with the MycoAlert Mycoplasma test kit (Lonza, Basel, Switzerland).

Cellular assays

PBMCs were cultured with SKOV-3 or K562 cells at an effector:target (E:T) ratio of 1:1 at 37°C and 5% CO_2 for 5 hours. CD107a and IFN-γ staining were determined as previously described.[8] To measure cell cytotoxicity in real-time, SKOV-3/NLG cells were plated at a density of 4×10^3 cells/well in a 96-well flat bottom, tissue culture-treated plate (Falcon, cat# 353072, Corning, NY) 24 hours prior

to starting the assay. Enriched NK cells were added at the indicated E:T ratios in RPMI 1640 media at 37°C in 5% CO_2. Fluorescent images of live cells were obtained hourly for 7 days using an IncuCyte SX3 live cell imaging and analysis system (Sartorius Göttingen, Germany), as previously described.[15]

NK-92 cells expressing human CD16A were stimulated with phorbol 12-myristate 13-acetate (PMA), as described previously.[16] Briefly, NK-92/CD16A cells (3×10^5/mL) were treated with or without 10 ng/mL PMA (Sigma Aldrich, St. Louis, Missouri, USA) and 1 µg/mL ionomycin (AdipoGen Life Sciences, San Diego, California, USA) for 2 hours at 37°C in the presence or absence of Medi-1, Medi-4, Medi-PGLALA, Medi-F(ab′)$_2$, D1(A12), or an isotype-matched negative control antibody at the indicated concentrations. The cells were then washed twice with PBS containing 0.05% FBS, treated with cell viability dye, and stained for CD16A. All samples were fixed with 2% paraformaldehyde in PBS without Ca^{+2} and Mg^{+2} and subjected to flow cytometric analyses.

Immunoblotting

Enriched NK cells were left untreated or treated with Medi-1 or an isotype-matched negative control antibody (5 µg/mL) at 37°C in serum-free RPMI 1640 (Gibco). Cells were then washed, lysed with detergent, and prepared for immunoblotting as previously described.[17] Briefly, ~2.5×10^5 cells were run in each lane of a 7% NuPAGE tris-acetate gel (Invitrogen, Carlsbad, California, USA) and transferred to Immobilon-FL polyvinylidene difluoride membrane (EMD Millipore, Burlington, Massachusetts, USA). REVERT Total Protein Stain (LI-COR Biosciences, Lincoln, Nebraska, USA) was used for normalization to the total protein content of each sample. Membranes were treated with Intercept (TBS) Blocking Buffer (LI-COR Biosciences), incubated with the indicated primary antibodies, and then incubated with the appropriate near-infrared secondary antibodies. Blots were visualized using an Odyssey CLx near-infrared imager (LI-COR Biosciences), and signals were background-subtracted using ImageStudio Software (LI-COR Biosciences). After correction for whole-lane protein content, samples were normalized to the t=0 signal.

Statistical analysis

Data are presented as the mean±SD or, for immunoblotting, SEM. Compiled in vitro data are from at least three independent experiments using separate donors. Statistical analysis was conducted, and significance was determined using GraphPad Prism Software V.10.0.3 for IOS or V.10.2.3 (403) for Windows (La Jolla, California, USA). Comparison between two treatments was computed using the paired two-tailed Student's t-test. Comparison among three or more treatments was made using one-way or two-way analysis of variance, as appropriate, followed by the indicated post hoc test. All data presented as geometric mean fluorescent intensity (MFI) were log-transformed before statistical analysis. Outlier analyses were performed on immunoblotting data using unbiased ROUT with Q=1% followed by a mixed-effects analysis approach with correction for multiple comparisons/Fisher's least significant difference test. The area under the curve was calculated to compare the killing kinetics of the live imaging in vitro killing assays. The data generated in this study are available on request from the corresponding author.

RESULTS

Medi-1 treatment selectively amplifies NK cell proliferation in the presence of IL-15

It is well established that IL-15 promotes the proliferation of NK cells and T cells, especially in the presence of monocytes which transpresent IL-15.[18] To determine the effects of the ADAM17 function-blocking mAb Medi-1 on IL-15-driven proliferation of these populations, PBMCs labeled with CTV were cultured with IL-15 at 10 ng/mL, a standard concentration used for ex vivo lymphocyte expansion,[9 19] ±Medi-1 (5 µg/mL) for up to 7 days. As shown in figure 1A, IL-15 stimulation along with Medi-1 markedly enhanced NK cell proliferation, which was dose-dependent (online supplemental figure 1A), whereas Medi-1 treatment alone had a negligible effect on NK cell proliferation (figure 1A). An isotype-matched control for Medi-1 did not augment IL-15-driven NK cell proliferation (online supplemental figure 1B). Medi-1 treatment also increased NK cell proliferation at a low concentration of IL-15 (1 ng/mL) (online supplemental figure 1C). Enhanced IL-15-driven NK cell proliferation by Medi-1 caused a corresponding increase in the proportion of these cells in the PBMC population after 7 days of culture (figure 1B), resulting in a 4.5-fold expansion, with no effect on cell viability, compared with a 1.5-fold expansion exhibited by cells stimulated with IL-15 alone (figure 1C). In contrast, T cells within the IL-15-stimulated PBMC population were only marginally affected by Medi-1 treatment, with NKT cells showing minor augmentation of proliferation while CD4 and CD8 T cells showed no benefit (figure 1D). Hence, the Medi-1 effect was primarily NK cell-specific and it increased their sensitivity to IL-15 stimulation.

D1(A12) is another human IgG1 mAb that blocks ADAM17 function[20] (online supplemental figure 2). Interestingly, D1(A12) treatment of PBMCs marginally, but not significantly, enhanced NK cell proliferation by IL-15 stimulation (online supplemental figure 3). This could be due to distinct aspects of the Fab and/or Fc regions of D1(A12) or its targeted epitopes. The variable heavy and light chains of this engineered mAb were designed to recognize distinct epitopes in different regions of ADAM17.[20] Moreover, the strength of CD16A binding to IgG1 is affected by glycosylation and amino acid polymorphisms in its Fc region,[21] which could vary between D1(A12) and Medi-1.

IL-15 is used therapeutically in various forms, including its incorporation into multiengager complexes, such as a TriKE.[22] To assess whether Medi-1 treatment enhanced

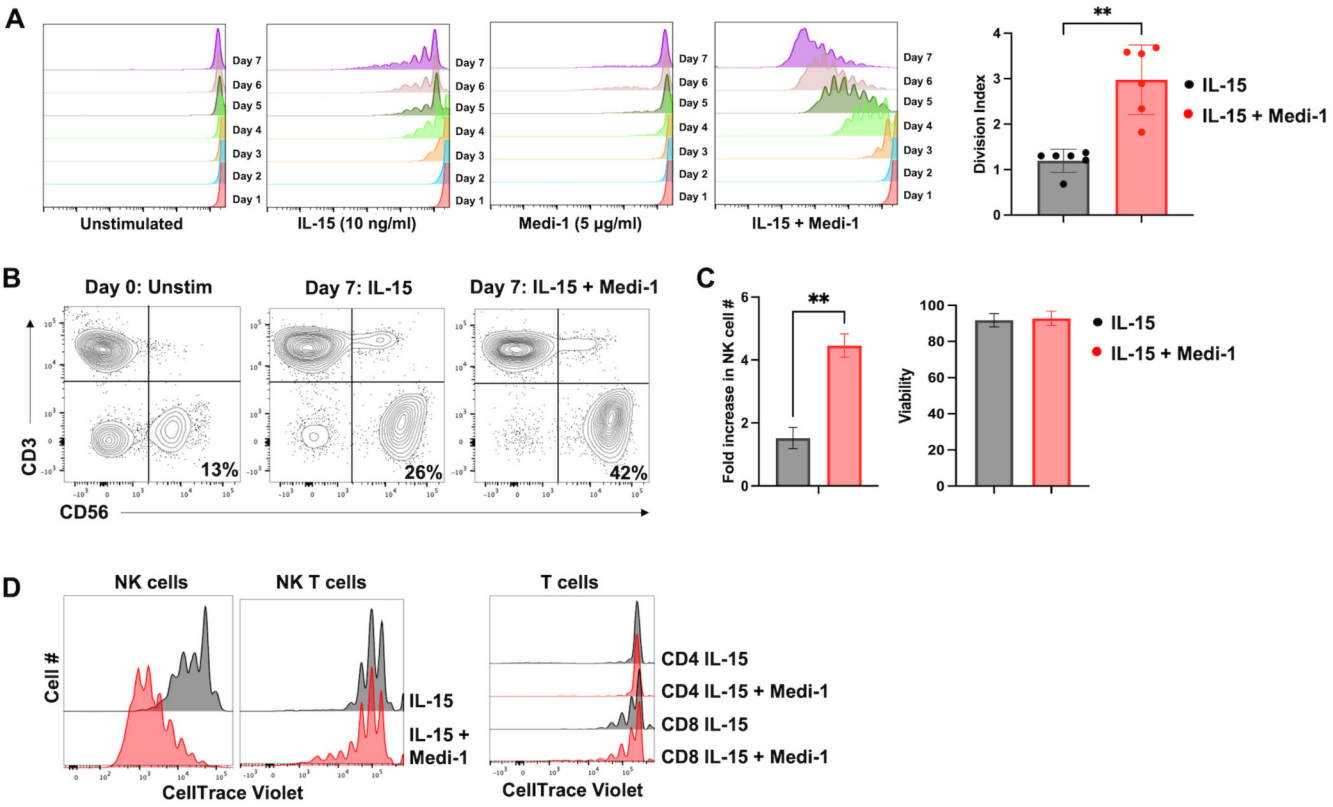

Figure 1 Medi-1 treatment selectively increases NK cell proliferation by IL-15. Freshly isolated PBMCs were labeled with CellTrace Violet (CTV) to track cell division and cultured for 7 days±IL-15 (10 ng/mL)±Medi-1 (5 µg/mL). (A) CD56$^+$ CD3$^-$ NK cells were analyzed for CTV dilution by flow cytometry. Representative data are shown (left panels). Division index (right panel), mean±SD, n=6 donors. (B) Percentage of NK cells for the indicated conditions and time points. Data are representative of 6 independent experiments. (C) Fold increase in NK cells and viability after 7 days of expansion for the indicated conditions. Mean±SD, n=6 donors. (D) The indicated lymphocyte populations were analyzed for CTV dilution by flow cytometry. NK cells (CD56$^+$ CD3$^-$), NK T cells (CD56$^+$ CD3$^+$), CD8 T cells (CD56$^-$ CD3$^+$ CD8$^+$) and CD4 T cells (CD56$^-$ CD3$^+$ CD4$^+$). Data comparing NK cells to T cells are representative of at least three independent experiments using leukocytes from separate donors. **p<0.01; ns, not significant. Statistical significance was determined by paired two-tailed Student's t-tests. NK, natural killer; PBMC, peripheral blood mononuclear cell.

NK cell proliferation by IL-15 in other contexts, we used it in conjunction with a TriKE consisting of two humanized camelid nanobodies against CD16 and the tumor-associated antigen B7-H3 attached to IL-15, referred to as cam16-IL15-camB7H3. We observed that Medi-1 treatment enhanced NK cell proliferation mediated by the TriKE as well (online supplemental figure 1D). Moreover, the effects of Medi-1 were not restricted to IL-15, as the presence of Medi-1 also enhanced IL-2-driven NK cell proliferation (online supplemental figure 1E).

NK cells undergoing IL-15-driven proliferation in the presence or absence of Medi-1 express similar functional markers

Overstimulation of NK cells with IL-15 or other stimuli can cause changes in their phenotypic profile, including the downregulation of activating receptors and the upregulation of inhibitory receptors.[23] Stimulation of NK cells with a single 10 ng/mL dose of IL-15 for 7 days does not induce their exhaustion.[19] To assess whether this is altered by Medi-1 treatment, PBMCs were stimulated in this manner for 7 days±Medi-1 and then NK cells were compared for their staining levels of well-described activating and inhibitory receptors by flow cytometry. As shown in figure 2A, we observed no statistical differences in the percentage of NK cells expressing NKp44, NKp46, NKG2D, KIR2DL1, KIR2DL2/L3, KIR3DL1, or NKG2A when stimulated with IL-15±Medi-1. The percentage of NK cells expressing CD16A, an ADAM17 substrate,[10] was slightly but significantly increased in the presence of Medi-1 (figure 2A). The geometric MFI of positively staining NK cells for NKG2D, KIR2DL1, KIR2DL2/L3, and NKG2A was also marginally increased for IL-15-stimulated NK cells in the presence of Medi-1 (figure 2A). The inhibitory receptors LAG-3, TIGIT, and TIM-3 have been reported to be upregulated in expression and activity in exhausted T cells.[23 24] These markers can be upregulated on activated NK cells, but whether their expression signifies exhaustion is less clear.[23] LAG-3, TIGIT, and TIM-3 staining levels on resting NK cells varied; therefore, we included FMO controls in the flow cytometric plots to better distinguish changes in their staining during NK cell activation. When NK cells were cultured in the presence of IL-15 and Medi-1, the percentage of LAG-3$^+$ cells

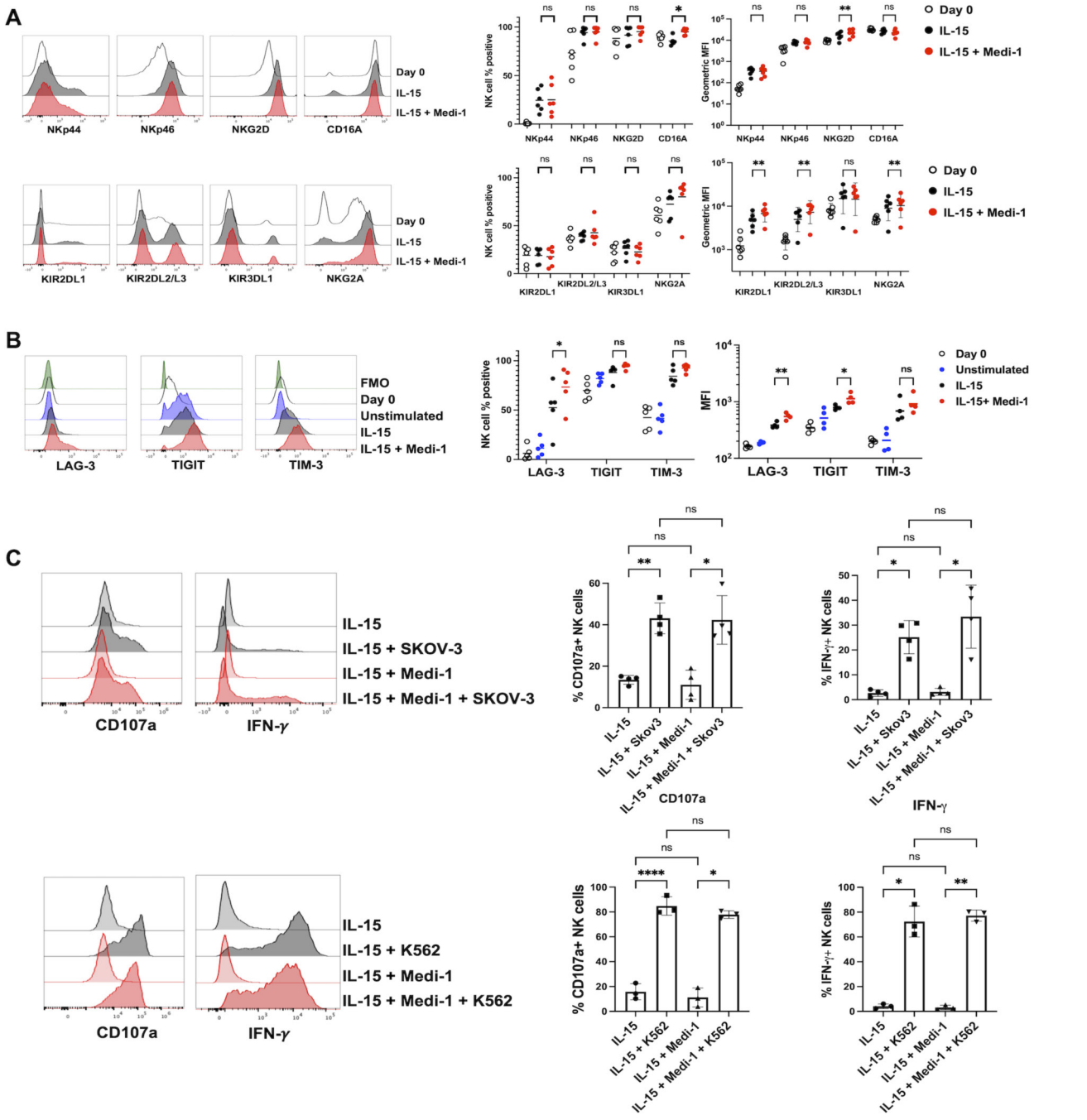

Figure 2 Proliferating NK cells treated with IL-15±Medi-1 express similar functional markers. Freshly isolated PBMCs were cultured in the presence or absence of IL-15 (10 ng/mL) and Medi-1 (5 µg/mL), as indicated. At day 0 or day 7, $CD56^+$ $CD3^-$ NK cells were analyzed for their expression levels of the indicated markers by flow cytometry. (A) Activating receptors (top panels) and inhibitory receptors (bottom panels). Representative histogram plots (left panels). y-axis=cell number. Cumulative data showing the percentage of NK cells positively staining for each marker (mean is indicated by the horizontal line) and their geometric MFI (right panels). For the latter, data were log-transformed prior to analysis. n=6 donors. (B) Exhaustion markers. Representative histograms are shown in the left panel. Unstimulated=unstimulated cells stained at day 7. Fluorescence minus one (FMO) of cells at day 0. Cumulative data are shown as the percentage of NK cells positively staining for each marker and the geometric MFI of staining. For the latter, data were log-transformed prior to analysis. n=4–5 donors. (C) PBMCs were cultured in IL-15±Medi-1 for 7 days, washed, and then incubated with K562 or SKOV-3 cells for 5 hours (effector:target ratio=1:1). PBMCs were stained for CD56, CD3, CD107a, and IFN-γ and examined by flow cytometry ($CD56^+$ $CD3^-$ cells are shown). Representative histogram plots (left panels) and cumulative data (right panels) are shown, n=3–4 donors. *p<0.05; **p<0.01; ****p<0.0001; ns, not significant. Statistical significance was determined by a one-way ANOVA with a Dunnett post hoc test. ANOVA, analysis of variance; MFI, mean fluorescent intensity; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

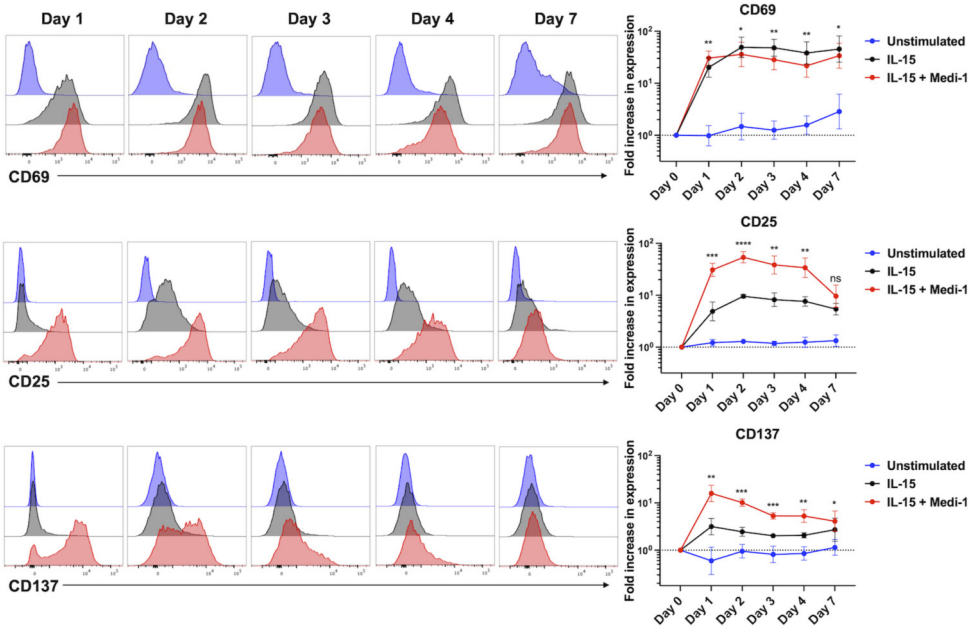

Figure 3 Medi-1 treatment augments the upregulation of early activation markers on NK cells. Freshly isolated PBMCs were treated as described in figure 2. $CD56^{+}$ $CD3^{-}$ NK cells were analyzed for their CD69, CD25, and CD137 expression levels by flow cytometry on days 0, 1–4, and 7 of culture. Representative histogram plots (left panels). y-axis=cell number. Cumulative data (right panels) are shown as an averaged log-transformed geometric MFI fold increase from day 0. Mean±SD, n=4 donors. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns, not significant. Statistical significance was determined by a two-way ANOVA with Tukey post-hoc test. ANOVA, analysis of variance; MFI, mean fluorescent intensity; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

was significantly increased, as was the geometric MFI of cells positively staining for LAG-3 and TIGIT (figure 2B).

To assess the significance of the modest increase in expression of certain activating and inhibitory receptors by NK cells treated with IL-15 and Medi-1, we evaluated the functional readouts of degranulation and cytokine production via CD107a and IFN-γ detection, respectively, which are diminished upon NK cell exhaustion.[23] PBMCs were stimulated with IL-15±Medi-1 for 7 days and then co-cultured with the ovarian cancer cell line SKOV-3 or the myelogenous leukemia cell line K562 for 5 hours. With both tumor cell targets, NK cells expanded by IL-15±Medi-1 upregulated CD107a and IFN-γ at equivalent levels (figure 2C), though their response to K562 cells was much more robust. Hence, though Medi-1 treatment augmented IL-15-driven NK cell proliferation and increased the expression of certain inhibitory markers, this did not lead to broad changes in well-recognized functional phenotypic markers.

Early activation markers distinguish IL-15-stimulated NK cells treated with Medi-1

We next examined the expression of the early activation markers CD69, CD25 (IL-2Rα), and CD137 (4-1BB) on NK cells following the stimulation of PBMCs with IL-15±Medi-1. Upon IL-15 stimulation, NK cells rapidly and uniformly upregulated CD69, which remained elevated for the 7-day culture (figure 3). IL-15 stimulation along with Medi-1 increased CD69 upregulation on day 1, though its expression modestly decreased at the remaining time points (figure 3). CD25 levels on NK cells following IL-15 stimulation were increased at all time points compared with unstimulated NK cells, and this was markedly enhanced in the presence of Medi-1 on days 1–4 (figure 3). Similarly, Medi-1 treatment significantly enhanced CD137 upregulation on NK cells by IL-15 (figure 3). Hence, CD25 and CD137, but not CD69, demonstrated distinct expression patterns on NK cells treated with IL-15 and Medi-1 when compared with IL-15 treatment alone, which was apparent by day 1 of stimulation (figure 3).

The kinetics of CD69, CD25, and CD137 upregulation were also examined using enriched NK cells treated with IL-15±Medi-1 for up to 7 days. Again, CD25 and CD137 demonstrated a distinct pattern of upregulation by IL-15-stimulated NK cells treated with Medi-1 (figure 4A). Since CD137 achieved peak expression by day 1, we examined earlier time points of stimulation at 4, 8, and 18 hours. CD25 and CD69 demonstrated significantly higher levels of upregulation as early as 4 hours after NK cell treatment with IL-15 and Medi-1 compared with NK cells stimulated with IL-15 alone (figure 4B). Medi-1 treatment significantly increased CD137 as early as 8 hours after stimulation relative to NK cells treated with IL-15 alone (figure 4B). These data demonstrate that Medi-1 treatment played a direct role in enhancing NK cell activation by IL-15 (ie, independent of other PBMC populations).

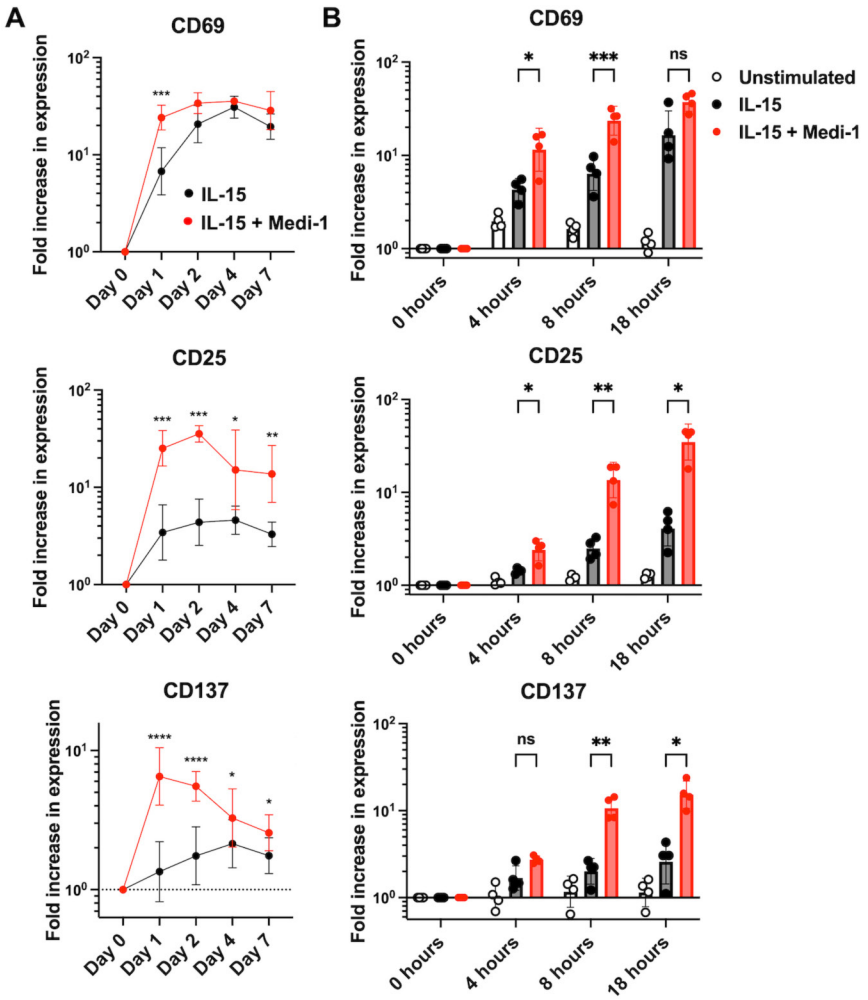

Figure 4 Direct effects of Medi-1 on enriched NK cells. Freshly isolated NK cells (>90% enriched) were treated and analyzed as described in figure 2. (A) CD56$^+$ CD3$^-$ NK cells were analyzed for their expression levels of CD69, CD25, and CD137 by flow cytometry on days 0, 1–4, and 7 of culture. (B) CD56$^+$ CD3$^-$ NK cells were similarly examined at hours 0, 4, 8, and 18. Data are shown as the averaged log-transformed geometric MFI fold increase from day 0 (A) hour 0 (B). Mean±SD, n=4 donors. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Statistical significance was determined by a two-way ANOVA with a Šídák (A) or Tukey post hoc test (B). ANOVA, analysis of variance; MFI, mean fluorescent intensity; NK, natural killer.

The Fc region of Medi-1 enhances NK cell activation by IL-15

The distinct expression patterns of CD25 and CD137 by IL-15-stimulated NK cells when treated with Medi-1 were indicative of its synergetic effect. CD16A is the main IgG Fc activating receptor expressed by human NK cells and it exclusively mediates ADCC.[10] As Medi-1 is a human IgG1 mAb, it can potentially be engaged by CD16A on binding NK cells. To test whether the Fc region of Medi-1 contributed to its synergistic effect, we generated several other Fc variants of the mAb, including Medi-F(ab′)$_2$ in which its Fc region was removed; Medi-PGLALA containing the mutations Pro329Gly, Leu234Ala, and Leu235Ala that also abolish FcγR binding, but with less potential effects on antibody structure and function[25]; and Medi-4 containing the Fc region of IgG4, which binds to CD16A with~10 fold lower affinity than IgG1.[26] The predicted rank order of CD16A binding affinity for the generated Medi Fc variants was Medi-1>Medi-4> Medi-PGLALA>/=Medi F(ab′)$_2$. All versions of Medi, but not a human IgG control mAb, equivalently blocked CD16A downregulation on cell activation (online supplemental figure 2), demonstrating that their ability to block ADAM17 function was not altered. PBMCs were stimulated with IL-15±a Medi Fc variant at the same molar concentration for 24 hours. The expression of CD25 and CD137 was then determined by flow cytometry. As shown in figure 5A, IL-15-stimulated NK cells treated with Medi-1 versus the other Fc variants demonstrated significantly higher levels of these activation markers. The same effects were observed upon IL-15-driven NK cell proliferation (figure 5B).

The above findings indicate that CD16A engages the Fc region of Medi-1 when bound to ADAM17 on NK cells. CD16A non-covalently associates with the signaling adaptors FcRγ and CD3ζ.[27 28] These immunoreceptor tyrosine-based activation motif (ITAM)-containing signaling adaptors become phosphorylated and associate with the ZAP-70 and SYK protein tyrosine kinases, which are also phosphorylated, leading to downstream signaling cascades for cell activation.[29] We observed that treating NK cells with Medi-1 alone led to significantly higher

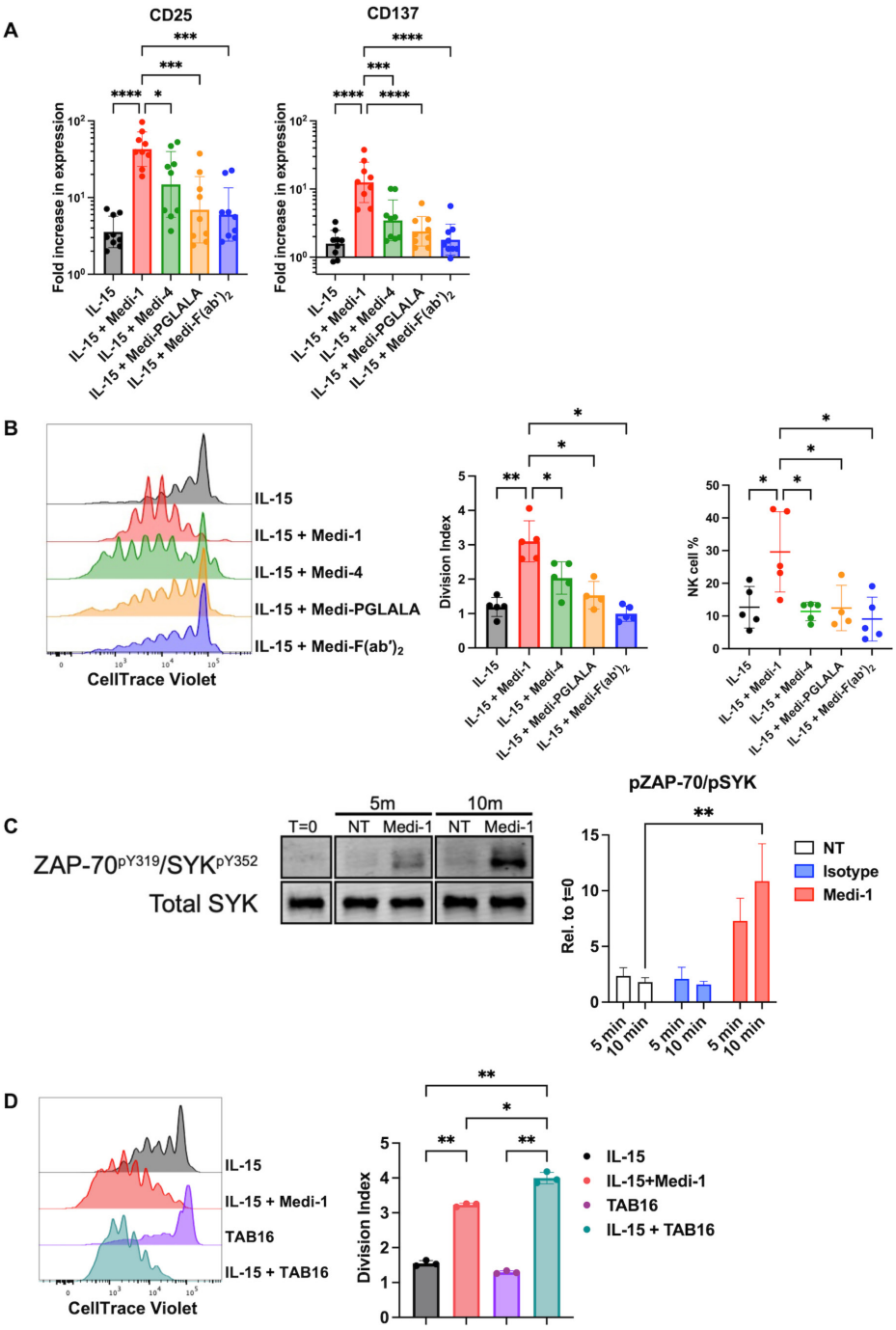

Figure 5 Medi-1 synergizes with IL-15 stimulation through its Fc region. (A) Freshly isolated PBMCs were cultured with IL-15 (10 ng/mL)±molar equivalents of Medi-1, Medi-4, Medi-PGLALA, or Medi-F(ab′)$_2$, as indicated. CD56$^+$ CD3$^-$ NK cells were analyzed for their expression levels of CD25 and CD137 by flow cytometry at 18 hours of culture. Data are shown as the averaged log-transformed geometric MFI fold increase from time 0. Mean±SD, n=9 donors. (B) PBMCs were labeled with CTV and cultured for 7 days with IL-15±Medi-1, Medi-4, Medi-PGLALA, or Medi-F(ab′)$_2$, as indicated. CD56$^+$ CD3$^-$ NK cells were analyzed for CTV dilution by flow cytometry. Representative histograms (left panel) and division index (middle panel) are shown. Percentage of NK cells for the indicated conditions (right panel). Mean±SD, n=5 donors. (C) Enriched NK cells were cultured with no further treatment (NT), with Medi-1, or with isotype-matched negative control antibody for the indicated times. Immunoblotting shows pZAP-70 (Y319)/pSYK (Y352), direct targets of Src-family kinases on ligation of ITAM-coupled receptors[50]; total SYK protein is shown as a loading control. Representative (left panel) and cumulative data (right panel) are shown. Mean±SEM, three donors (two for isotype) used for a total of nine independent treatments (six for isotype). (D) PBMCs were treated as described in panel B with IL-15±Medi-1 or TAB16 at equivalent molarity (33 nM). Representative histograms (left panel) and division index (right panel) are shown. Mean±SD, n=3 donors. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Statistical significance was determined by a one-way ANOVA with a Dunnett (A, D) or Tukey (B) post hoc test, or a two-way mixed-effects analysis with a Šídák post-hoc test (C). ANOVA, analysis of variance; CTV, CellTrace Violet; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

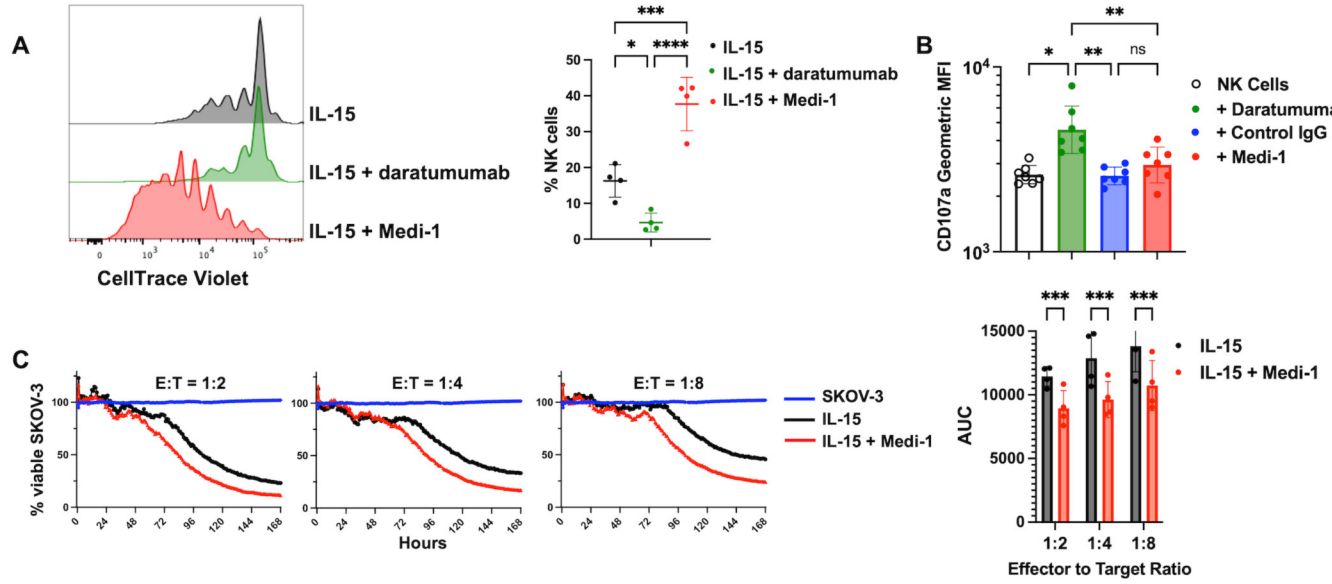

Figure 6 Medi-1 treatment of IL-15-stimulated NK cells does not induce fratricide or impair natural cytotoxicity. (A) PBMCs were labeled with CTV and cultured for 7 days with IL-15 (10 ng/mL)±Medi-1 or daratumumab at 5 µg/mL, as indicated. CD56$^+$ CD3$^-$ NK cells were analyzed for CTV dilution by flow cytometry. Representative data are shown (left panel) as well as the percentage of NK cells for the indicated conditions (right panel). Mean±SD, n=4 donors. (B) PBMCs cells were cultured for 24 hours in IL-15±an isotype-matched negative control mAb (human IgG1), Medi-1 (5 µg/mL), or daratumumab at (5 µg/mL), as indicated. CD56$^+$ CD3$^-$ NK cells were analyzed for their expression levels of CD107a by flow cytometry. Cumulative data are shown as geometric MFI±SD, n=7 donors. (C). Enriched NK cells were co-cultured with SKOV-3-NLG cells at the indicated E:T ratios for 7 days in the presence of IL-15 (10 ng/mL)±Medi-1 (5 µg/mL). Cytotoxicity was assessed by IncuCyte-based live cell imaging. The percentage of viable SKOV-3 cells was double normalized to SKOV-3 cells alone (left panel). The area under the curve (AUC) of the remaining SKOV-3 cells was calculated for each condition. Mean±SD, n=4. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Statistical significance was determined by a one-way ANOVA with Tukey post hoc test (A, B) or two-way ANOVA with Šídák post-hoc test (C). ANOVA, analysis of variance; CTV, CellTrace Violet; E:T, effector:target; MFI, mean fluorescent intensity; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

levels of ZAP-70/SYK phosphorylation than NK cells treated with an isotype-matched negative control antibody or no antibody (figure 5C).

As an approach to directly engage CD16A and simultaneously block ADAM17 function, we generated a bispecific antibody engager that consists of a humanized camelid nanobody against CD16 and a scFv from Medi-1, referred to as a Targeted ADAM17 Blocker-16 (TAB16). PBMCs were labeled with CTV and cultured with IL-15 alone or with molar equivalents of Medi-1 or TAB16 for 7 days. TAB16+IL-15 induced significantly higher levels of NK cell proliferation than IL-15 alone and, interestingly, higher levels of proliferation than IL-15+Medi-1 (figure 5D).

A potential adverse effect of CD16A engagement of the Fc region Medi-1 would be the induction of NK cell degranulation and fratricide. For example, daratumumab, a human IgG1 mAb that recognizes CD38 on NK cells, has been shown to induce fratricide.[30] Indeed, we observed impaired NK cell proliferation and a greatly decreased representation within the PBMC population when CTV-labeled PBMCs were cultured for 7 days with IL-15 and daratumumab, which was not seen with Medi-1 treatment (figure 6A), indicating it did not induce a critical level of fratricide or clearance by macrophage present in the culture. In other assays, enriched NK cells cultured with daratumumab for 24 hours demonstrated a significant upregulation in cell surface levels of CD107a, a sensitive marker of NK cell degranulation upon CD16A signaling,[8] compared with control cells. This was not observed for NK cells cultured with Medi-1 (figure 6B). To assess the effects of Medi-1 treatment on the functional state of NK cells, we examined their natural cytotoxicity against tumor cells. For this assay, enriched NK cells were used to exclude antitumor activity by other leukocytes. NK cells were cultured with SKOV-3/NLG cells at various E:T ratios in the presence of IL-15±Medi-1, and tumor cell lysis was assessed by IncuCyte live cell imaging for 7 days. Interestingly, in the presence of Medi-1, NK cells mediated significantly higher levels of SKOV-3 killing (figure 6C). Taken together, our results show that the Fc region of Medi-1 contributed to the activation and proliferation of IL-15-stimulated NK cells while not inducing apparent adverse effects.

Enhanced IL-15-driven NK cell proliferation by Medi-1 involves CD137 and PBMC accessory cells

Circulating NK cells are composed of CD56 bright and dim populations, and CD16A is primarily expressed by the latter cells.[31] It was mainly these cells that upregulated CD137 after stimulation with Medi-1 and IL-15 (online supplemental figure 4).

It is well established that CD137 stimulation in T cells promotes their activation and proliferation.[32] However, much less is known about the biological importance of CD137 in NK cells. Engagement of this coactivation receptor by agonistic antibodies or by CD137L-expressing artificial feeder cells induces NK cell proliferation.[33 34] To investigate the role of CD137 in the augmented proliferation of Medi-1+IL-15-stimulated NK cells, we blocked its function. Others have reported that the addition of soluble CD137L to PBMC cultures can inhibit T cell proliferation by acting as a competitive inhibitor.[35 36] We attempted this for NK cells using a recombinant human CD137L extracellular domain-Fc fusion protein (sCD137L-Fc). We found that sCD137L-Fc diminished NK cell proliferation by IL-15 as well as IL-15+Medi-1 (figure 7A), which occurred in a dose-dependent manner (online supplemental figure 5). A caveat of this approach is that sCD137L-Fc, when attached to NK cells, might be engaged by CD16A, reducing its interaction with Medi-1. However, the presence of sCD137L-Fc did not affect the enhanced upregulation of CD25 by NK cells treated with IL-15 and Medi-1 (figure 7B), indicating that Medi-1 engagement by CD16A was not affected. We could not assess CD137 staining since sCD137L-Fc competed with our anti-CD137 mAb used for flow cytometry (data are not shown), demonstrating its attachment to CD137.

We also examined the effects of a function-blocking anti-human CD137 mAb on NK cell proliferation by IL-15. Anti-CD137 mAbs that inhibit its attachment to CD137L can also be agonistic and induce cell activation, as is the case for utomilumab.[37] To our knowledge, the best characterized blocking anti-human CD137 mAb that is not agonistic is BBK-2, which partially neutralizes CD137 function.[38] PBMCs were stimulated with IL-15±Medi-1 in the presence or absence of BBK-2 or an appropriate isotype control mAb. As shown in figure 7A, BBK-2, but not the isotype control mAb, significantly reduced NK cell proliferation by IL-15 alone and in combination with Medi-1. Taken together, the above findings support CD137 upregulation as a mechanism by which Medi-1 treatment augments IL-15-mediated NK cell proliferation. CD137L is primarily expressed by monocytes, macrophages, and B cells.[39] Interestingly, we observed that only IL-15-stimulated NK cells in PBMCs and not enriched NK cells (≥90% NK cell purity) underwent enhanced proliferation in the presence of Medi-1 (figure 7C), demonstrating an important role for PBMC accessory cells. We previously reported enhanced IL-15-driven proliferation of enriched NK cells by an ADAM17 function-blocking mAb when performed in 96-well cell culture plates.[9] However, media conditioning, nutrient exhaustion, and pH changes were more variable under these conditions. For our current study, NK cell expansion was performed in 48-well plates with more growth medium and appreciable expansion of enriched NK cells was not observed.

To address whether CD137 activation could enhance NK cell proliferation by IL-15 in the absence of PBMC accessory cells, we used an anti-CD137 agonist mAb. Enriched NK cells were labeled with CTV and cultured for 7 days with IL-15±Medi-1±urelumab, a well-described CD137 agonistic mAb.[40] Interestingly, in the presence of urelumab, enriched NK cells underwent a marked increase in proliferation by IL-15, and this was increased further by the addition of Medi-1 (figure 7D).

DISCUSSION

We show that the ADAM17 function-blocking mAb Medi-1 markedly enhanced NK cell activation and proliferation by IL-15, and that the latter required the presence of PBMC accessory cells. In this study, we investigated the mechanisms by which Medi-1 augmented these events. IL-15 stimulated NK cells treated with Medi-1, but not IL-15 or Medi-1 alone, distinctly upregulated high levels of CD25 and CD137, indicating a synergistic activation process. Medi-1 is a human IgG1 antibody and when bound to NK cells, it could be engaged by their activating IgG Fc receptor CD16A. Indeed, NK cell treatment with various Medi Fc variants in which the Fc region was removed (Medi-F(ab')$_2$), mutated (Medi-PGLALA), or exchanged with the Fc region of IgG4 to prevent or diminish CD16A binding resulted in significantly reduced NK cell activation and proliferation in the presence of IL-15 when compared with Medi-1. In addition, we show CD16A signaling in NK cells treated with Medi-1. To directly coengage ADAM17 and CD16A, we generated the unique bispecific antibody TAB16 consisting of an scFv from Medi-1 and a camelid antibody specific to CD16. TAB16 treatment also significantly enhanced IL-15-driven NK cell proliferation, more so than Medi-1 and IL-15. This could be due to a higher affinity interaction between the anti-CD16 camelid antibody and CD16A than CD16A binding to the Fc region of Medi-1, inducing increased CD16A signaling. Signaling by CD16A when binding to NK cell-attached Medi-1 will likely vary by NK cells from separate individuals due to CD16A polymorphisms distributed within the normal population that affect its binding affinity.[41]

An issue with CD16A engagement of NK cell-bound antibodies is the induction of fratricide, which has been reported for the anti-CD38 mAb daratumumab.[30] We show, however, that Medi-1 treatment did not induce degranulation by enriched NK cells nor their elimination in PBMC cultures, whereas daratumumab treatment caused both events. CD16A is a low-affinity FcγR and its signaling upon binding antibody-opsonized cells is affected by antigen density, mobility, and accessibility. For instance, low antigen density is ADCC limiting, as a threshold of CD16A signaling is required to induce degranulation by NK cells.[21] ADAM17 can be constitutively expressed at low levels on the surface of cells,[42] including hematopoietic cells,[43] and so the surface density of ADAM17 on NK cells may be low enough that Medi-1 engagement by CD16A does not achieve the signaling threshold necessary for

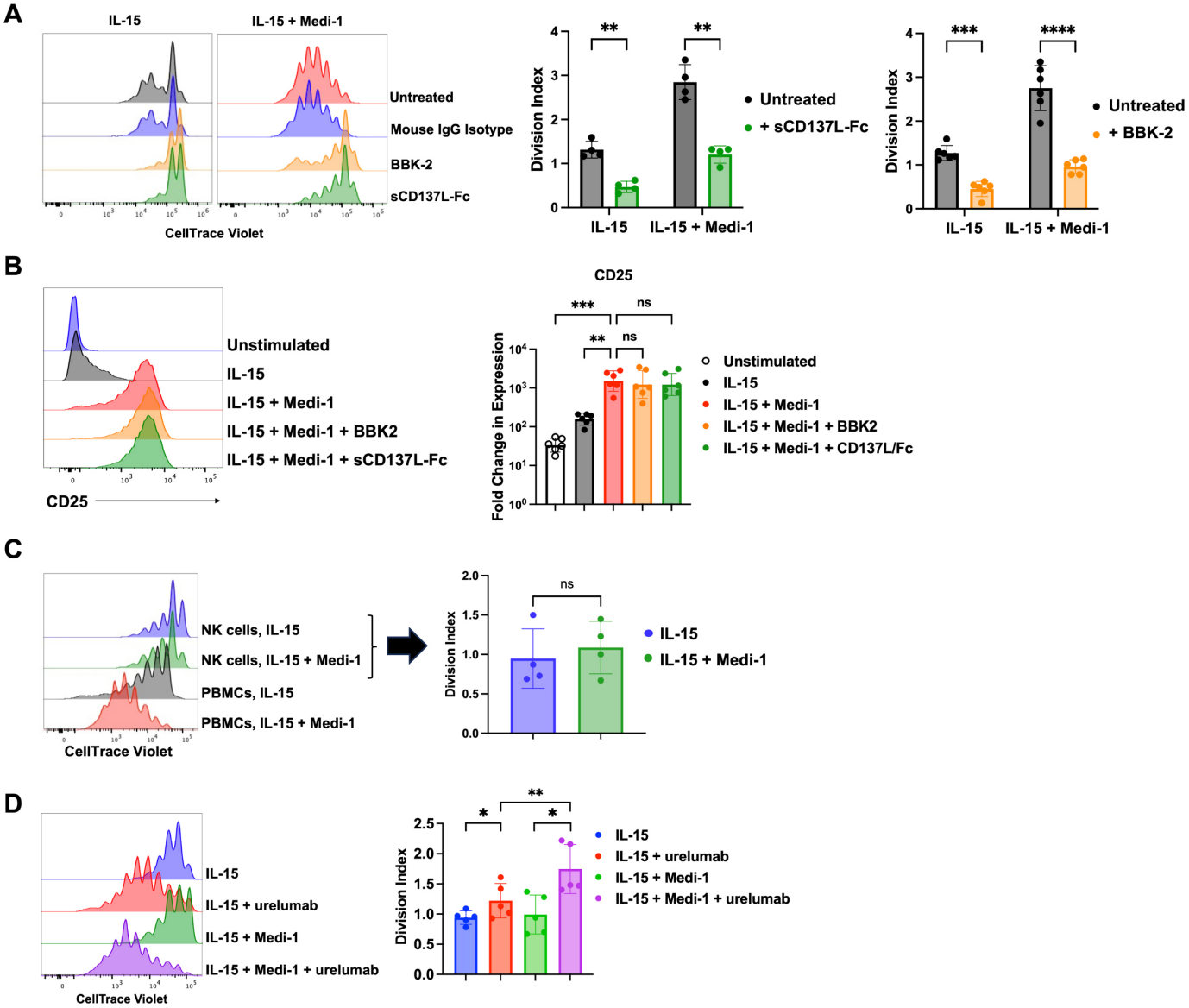

Figure 7 Medi-1-enhanced NK cell proliferation by IL-15 involves CD137. PBMCs were labeled with CTV and cultured for 7 days with IL-15 (10 ng)±Medi-1 (5 µg/mL)±BBK-2 (5 µg/mL), an isotype-matched negative control mAb (mouse IgG1, 5 µg/mL), or sCD137L-Fc fusion protein (1 µg/mL). (A) $CD56^+$ $CD3^-$ NK cells were analyzed for CTV dilution by flow cytometry (left panel). Division index of NK cells in the presence or absence of BBK-2 or sCD137L-Fc fusion protein (right panels). Mean±SD, n=4–6 donors. (B) PBMCs were treated with IL-15±the indicated reagents for 24 hours and the expression of CD25 was determined on NK cells. Representative CD25 expression on NK cells (left panel). The right panel shows the log-transformed fold change in CD25 geometric MFI from baseline. Mean±SD, n=6. (C) CTV-labeled PBMCs or enriched NK cells from matched donors were cultured for 7 days with IL-15 (10 ng)±Medi-1 (5 µg/mL). $CD56^+$ $CD3^-$ NK cells were analyzed for CTV dilution by flow cytometry (left panel). Division index (right panel) for enriched NK cells, mean±SD n=4 donors. (D) CTV-labeled enriched NK cells were cultured for 7 days with IL-15 (10 ng)±Medi-1 (5 µg/mL)±urelumab (1 µg/mL). $CD56^+$ $CD3^-$ NK cells were analyzed for CTV dilution by flow cytometry (left panel). Division index (right panel), mean±SD n=5 donors. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Statistical significance was determined by a two-way ANOVA with Šídák post hoc test (A), one-way with Dunnentt's post-hoc test (B) or paired two-tailed Student's t-tests (C, D). ANOVA, analysis of variance; CTV, CellTrace Violet; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

degranulation in a critical mass of cells. NK cell effector activities induced by CD16A may vary depending on its signaling level, akin to T cell effector functions modulated by TCR signaling strength.[44]

Despite the enhanced activation and proliferation of IL-15-stimulated NK cells by Medi-1, we did not observe a striking change in expression of several functional markers when compared with NK cells stimulated with IL-15 alone which would indicate exhaustion or dysfunction. To the contrary, we observed that NK cells stimulated with IL-15 in the presence of Medi-1 mediated significantly higher levels of tumor cell killing at various E:T ratios. Currently, we do not know the cause of this increased antitumor activity. It is possible that CD16A

signaling induced by Medi-1 synergized with other activating receptors on NK cells to enhance their cytolytic activity. Addressing this and the broader antitumor effects of Medi-1 in vivo are important future studies.

D1(A12), another human IgG1 ADAM17 function-blocking mAb,[20] did not enhance NK cell activation and proliferation by IL-15. There are a few explanations that may account for the different effects of D1(A12) and Medi-1 on NK cells, including epitope differences, binding affinity, and Fc glycosylation and/or polymorphisms that could affect CD16A engagement.[21] It was beyond the scope of our study to determine the underlying reason for this difference. Our findings show, however, that not all ADAM17 function-blocking mAbs may be equivalent in enhancing IL-15-driven NK cell proliferation.

Of interest was the robust upregulation of CD137 by NK cells treated with IL-15 and Medi-1. CD137 is a potent coactivating receptor that has mainly been studied in T cells. CD8 T cells undergoing antigen activation and CD137 engagement demonstrate enhanced proliferation, reversal of exhaustion, apoptosis protection, and increased cytokine secretion and other effector functions.[32] In contrast, the biological function of CD137 in NK cells is not well understood. IL-15 stimulation or CD16A engagement can induce CD137 upregulation in NK cells.[34 45] Nuclear factor κB (NF-κB) and nuclear factor of activated T cells (NFAT) are involved in the transcriptional induction of CD137,[46 47] and they are activated by IL-15 and CD16A signaling, respectively.[46 48] Though the molecular mechanisms underlying the effects of Medi-1 and IL-15 on CD137 expression by NK cells are not currently known, it is possible that NF-κB and NFAT work in synergy to drive this process.

PBMC accessory cells are well known to enhance NK cell proliferation by transpresenting IL-15.[18 49] CD137L is primarily expressed by monocytes, macrophages, and B cells.[39] We demonstrate for the first time that NK cell proliferation by IL-15 in the presence of PBMC accessory cells can be diminished by blocking CD137, as was their enhanced proliferation mediated by Medi-1. Therefore, CD137 upregulation by IL-15-stimulated NK cells may establish a positive feedback loop to increase NK cell proliferation by PBMC accessory cells, which can be further augmented by Medi-1 treatment. Of interest is that the induction of CD137 was targetable by the agonistic CD137 antibody urelumab. This approach to amplify NK cell expansion could have applications in patients and ex vivo prior to their adoptive transfer as a feeder cell-free method.

In summary, we have demonstrated that Medi-1 treatment synergizes with IL-15 in activating NK cells and enhancing their proliferation. IL-2 is also broadly used for NK cell stimulation and proliferation ex vivo and in patients with cancer,[7] and we show that Medi-1 treatment of NK cells similarly enhanced NK cell proliferation by this cytokine. The mechanisms underpinning Medi-1's effects involve its engagement by CD16A and blocking ADAM17, which induce and presumably prolong its signaling. This increased the upregulation of CD137 expression, promoting NK cell expansion in the presence of PBMC accessory cells expressing CD137L (online supplemental graphical abstract). Our study is of translational importance as Medi-1 treatment in combination with cytokine therapies could augment the proliferation and function of endogenous or adoptively transferred NK cells in patients with cancer.

Author affiliations
[1]Graduate Program in Comparative and Molecular Biosciences, University of Minnesota, Saint Paul, Minnesota, USA
[2]Graduate Program in Microbiology, Immunology, and Cancer Biology, University of Minnesota, Minneapolis, Minnesota, USA
[3]Department of Veterinary and Biomedical Sciences, University of Minnesota, Minneapolis, Minnesota, USA
[4]Medical Scientist Training Program, University of Minnesota, Minneapolis, Minnesota, USA
[5]Graduate Program in Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota, USA
[6]Center for Immunology, University of Minnesota, Minneapolis, Minnesota, USA
[7]Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota, USA
[8]Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota, USA
[9]Department of Medicine, Division of Hematology, Oncology, and Transplantation, University of Minnesota, Minneapolis, Minnesota, USA

X Tanya S Freedman @tfreedmanlab

Contributors AM designed and performed the experiments, analyzed the data and wrote the paper. RH, KJD, SW, AJL, RF, ET and SKP performed the experiments and analyzed the data. JW provided reagents. AM, TSF, MF, ET and JSM analyzed the data and wrote the paper. BW designed the experiments, analyzed the data, and wrote the paper. BW is the guarantor.

Funding This work was supported by NIH R01CA203348 (to BW and JW), NIH P01CA111412 (to BW, MF and JSM), NIH R01AR073966 (to TSF), NIH T32HL007741 (to AJL), and a Basic/Clinical Pilot Research Award, Department of Pharmacology, University of Minnesota (to TSF).

Competing interests MF, JSM and BW are inventors on the patent application WO2022266341A1 (Targeted adam17 blocker compounds, anti-adam17 antibodies, methods of making, and methods of using).

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the University of Minnesota Institutional review board (IRB ID: 9708M00134). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data sharing not applicable as no datasets generated and/or analyzed for this study. Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as online supplemental information.

ORCID iDs
Anders J Lindstedt http://orcid.org/0000-0003-1988-5163
Tanya S Freedman http://orcid.org/0000-0001-5168-5829
Martin Felices http://orcid.org/0000-0002-5945-0634
Bruce Walcheck http://orcid.org/0000-0003-1933-3887

REFERENCES

1 Miller JS, Lanier LL. Natural killer cells in cancer immunotherapy. *Annu Rev Cancer Biol* 2019;3:77–103.
2 Lamers-Kok N, Panella D, Georgoudaki A-M, *et al*. Natural killer cells in clinical development as non-engineered, engineered, and combination therapies. *J Hematol Oncol* 2022;15:164.
3 Laskowski TJ, Biederstädt A, Rezvani K. Natural killer cells in antitumour adoptive cell immunotherapy. *Nat Rev Cancer* 2022;22:557–75.
4 Wolf NK, Kissiov DU, Raulet DH. Roles of natural killer cells in immunity to cancer, and applications to immunotherapy. *Nat Rev Immunol* 2023;23:90–105.
5 Kennedy PR, Felices M, Miller JS. Challenges to the broad application of allogeneic natural killer cell immunotherapy of cancer. *Stem Cell Res Ther* 2022;13:165.
6 Berrien-Elliott MM, Jacobs MT, Fehniger TA. Allogeneic natural killer cell therapy. *Blood* 2023;141:856–68.
7 Yang Y, Lundqvist A. Immunomodulatory effects of IL-2 and IL-15; implications for cancer immunotherapy. *Cancers (Basel)* 2020;12:3586.
8 Romee R, Foley B, Lenvik T, *et al*. NK cell CD16 surface expression and function is regulated by a disintegrin and Metalloprotease-17 (ADAM17). *Blood* 2013;121:3599–608.
9 Mishra HK, Dixon KJ, Pore N, *et al*. Activation of ADAM17 by IL-15 limits human NK cell proliferation. *Front Immunol* 2021;12:711621.
10 Wu J, Mishra HK, Walcheck B. Role of ADAM17 as a regulatory checkpoint of CD16A in NK cells and as a potential target for cancer immunotherapy. *J Leukoc Biol* 2019;105:1297–303.
11 Wang K, Xuan Z, Liu X, *et al*. Immunomodulatory role of metalloproteinase ADAM17 in tumor development. *Front Immunol* 2022;13:1059376.
12 Srpan K, Ambrose A, Karampatzakis A, *et al*. Shedding of CD16 disassembles the NK cell immune synapse and boosts serial engagement of target cells. *J Cell Biol* 2018;217:3267–83.
13 Rios-Doria J, Sabol D, Chesebrough J, *et al*. A monoclonal antibody to ADAM17 inhibits tumor growth by inhibiting EGFR and non-EGFR-mediated pathways. *Mol Cancer Ther* 2015;14:1637–49.
14 Vallera DA, Ferrone S, Kodal B, *et al*. NK-cell-mediated targeting of various solid tumors using a B7-H3 tri-specific killer engager in vitro and in vivo. *Cancers (Basel)* 2020;12:2659.
15 Snyder KM, Dixon KJ, Davis Z, *et al*. iPSC-derived natural killer cells expressing the Fcgammar fusion CD64/16A can be armed with antibodies for multitumor antigen targeting. *J Immunother Cancer* 2023;11:e007280.
16 Jing Y, Ni Z, Wu J, *et al*. Identification of an ADAM17 cleavage region in human CD16 (FcgammaRIII) and the engineering of a non-cleavable version of the receptor in NK cells. *PLoS ONE* 2015;10:e0121788.
17 Brian BF 4th, Guerrero CR, Freedman TS. Immunopharmacology and quantitative analysis of tyrosine kinase signaling. *CP in Immunology* 2020;130:e104.
18 Ma S, Caligiuri MA, Yu J. Harnessing IL-15 signaling to potentiate NK cell-mediated cancer immunotherapy. *Trends Immunol* 2022;43:833–47.
19 Felices M, Lenvik AJ, McElmurry R, *et al*. Continuous treatment with IL-15 exhausts human NK cells via a metabolic defect. *JCI Insight* 2018;3:e96219.
20 Tape CJ, Willems SH, Dombernowsky SL, *et al*. Cross-domain inhibition of TACE Ectodomain. *Proc Natl Acad Sci U S A* 2011;108:5578–83.
21 Temming AR, de Taeye SW, de Graaf EL, *et al*. Functional attributes of antibodies, effector cells, and target cells affecting NK cell-mediated antibody-dependent cellular cytotoxicity. *J Immunol* 2019;203:3126–35.
22 Phung SK, Miller JS, Felices M. Bi-specific and tri-specific NK cell engagers: the new avenue of targeted NK cell immunotherapy. *Mol Diagn Ther* 2021;25:577–92.
23 Roe K. NK-cell exhaustion, B-cell exhaustion and T-cell exhaustion-the differences and similarities. *Immunology* 2022;166:155–68.
24 Judge SJ, Murphy WJ, Canter RJ. Characterizing the dysfunctional NK cell: assessing the clinical relevance of exhaustion, anergy, and senescence. *Front Cell Infect Microbiol* 2020;10:49.
25 Schlothauer T, Herter S, Koller CF, *et al*. Novel human IgG1 and IgG4 FC-engineered antibodies with completely abolished immune effector functions. *Protein Eng Des Sel* 2016;29:457–66.
26 Bruhns P, Iannascoli B, England P, *et al*. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. *Blood* 2009;113:3716–25.
27 Lanier LL, Yu G, Phillips JH. Co-association of CD3 Zeta with a receptor (CD16) for IgG FC on human natural killer cells. *Nature New Biol* 1989;342:803–5.
28 Letourneur O, Kennedy IC, Brini AT, *et al*. Characterization of the family of dimers associated with FC receptors (FC epsilon RI and FC gamma RIII). *J Immunol* 1991;147:2652–6.
29 Chen Y, Lu D, Churov A, *et al*. Research progress on NK cell receptors and their signaling pathways. *Mediators Inflamm* 2020;2020:6437057.
30 Naeimi Kararoudi M, Nagai Y, Elmas E, *et al*. CD38 deletion of human primary NK cells eliminates Daratumumab-induced Fratricide and boosts their effector activity. *Blood* 2020;136:2416–27.
31 Michel T, Poli A, Cuapio A, *et al*. Human CD56Bright NK cells: an update. *J Immunol* 2016;196:2923–31.
32 Chester C, Sanmamed MF, Wang J, *et al*. Immunotherapy targeting 4-1Bb: mechanistic rationale, clinical results, and future strategies. *Blood* 2018;131:49–57.
33 Vidard L, Dureuil C, Baudhuin J, *et al*. CD137 (4-1BB) engagement fine-tunes synergistic IL-15- and IL-21-driven NK cell proliferation. *J Immunol* 2019;203:676–85.
34 Wilcox RA, Tamada K, Strome SE, *et al*. Signaling through NK cell-associated CD137 promotes both helper function for CD8+ cytolytic T cells and responsiveness to IL-2 but not cytolytic activity. *J Immunol* 2002;169:4230–6.
35 Rabu C, Quéméner A, Jacques Y, *et al*. Production of recombinant human Trimeric CD137L (4-1BBL). Cross-linking is essential to its T cell co-stimulation activity. *J Biol Chem* 2005;280:41472–81.
36 Shuford WW, Klussman K, Tritchler DD, *et al*. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J Exp Med* 1997;186:47–55.
37 Fisher TS, Kamperschroer C, Oliphant T, *et al*. Targeting of 4-1BB by monoclonal antibody PF-05082566 enhances T-cell function and promotes anti-tumor activity. *Cancer Immunol Immunother* 2012;61:1721–33.
38 Lee UH, Son JH, Lee JJ, *et al*. Humanization of antagonistic anti-human 4-1BB monoclonal antibody using a Phage-displayed combinatorial library. *J Immunother* 2004;27:201–10.
39 Choi BK, Lee HW. The murine CD137/CD137 ligand Signalosome: a signal platform generating signal complexity. *Front Immunol* 2020;11:553715.
40 Chin SM, Kimberlin CR, Roe-Zurz Z, *et al*. Structure of the 4-1BB/4-1BBL complex and distinct binding and functional properties of Utomilumab and Urelumab. *Nat Commun* 2018;9:4679.
41 Wu J, Edberg JC, Redecha PB, *et al*. A novel polymorphism of FcgammaRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. *J Clin Invest* 1997;100:1059–70.
42 Black RA, Rauch CT, Kozlosky CJ, *et al*. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature New Biol* 1997;385:729–33.
43 Ebsen H, Schröder A, Kabelitz D, *et al*. Differential surface expression of ADAM10 and ADAM17 on human T lymphocytes and tumor cells. *PLoS ONE* 2013;8:e76853.
44 Tubo NJ, Jenkins MK. TCR signal quantity and quality in CD4(+) T cell differentiation. *Trends Immunol* 2014;35:591–6.
45 Lin W, Voskens CJ, Zhang X, *et al*. Fc-dependent expression of CD137 on human NK cells: insights into 'agonistic' effects of anti-CD137 monoclonal antibodies. *Blood* 2008;112:699–707.
46 Aramburu J, Azzoni L, Rao A, *et al*. Activation and expression of the nuclear factors of activated T cells, NFATp and NFATc, in human natural killer cells: regulation upon CD16 ligand binding. *J Exp Med* 1995;182:801–10.
47 Kim J-O, Kim HW, Baek K-M, *et al*. NF-kappaB and AP-1 regulate activation-dependent CD137 (4-1BB) expression in T cells. *FEBS Lett* 2003;541:163–70.
48 McDonald PP, Russo MP, Ferrini S, *et al*. Interleukin-15 (IL-15) induces NF-kappaB activation and IL-8 production in human neutrophils. *Blood* 1998;92:4828–35.
49 Koka R, Burkett PR, Chien M, *et al*. Interleukin (IL)-15R[Alpha]-deficient natural killer cells survive in normal but not IL-15R[Alpha]-deficient mice. *J Exp Med* 2003;197:977–84.
50 Greene JT, Brian BF IV, Senevirathne SE, *et al*. Regulation of myeloid-cell activation. *Curr Opin Immunol* 2021;73:34–42.