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Meeting Article

The CD16 and CD32b Fc-gamma receptors regulate antibody-mediated responses in mouse natural killer cells

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Abstract

Natural killer (NK) cells are innate lymphocytes capable of mediating immune responses without prior sensitization. NK cells express Fc-gamma receptors (FcγRs) that engage the Fc region of IgG. Studies investigating the role of FcγRs on mouse NK cells have been limited due to lack specific reagents. In this study, we characterize the expression and biological consequences of activating mouse NK cells through their FcγRs. We demonstrate that most NK cells express the activating CD16 receptor, and a subset of NK cells also expresses the inhibitory CD32b receptor. Critically, these FcγRs are functional on mouse NK cells and can modulate antibody-mediated responses. We also characterized mice with conditional knockout alleles of *Fcgr3* (CD16) or *Fcgr2b* (CD32b) in the NK and innate lymphoid cell (ILC) lineage. NK cells in these mice did not reveal any developmental defects and were responsive to cross-linking activating NK receptors, cytokine stimulation, and killing of YAC-1 targets. Importantly, CD16-deficient NK cells failed to induce antibody-directed cellular cytotoxicity of antibody-coated B-cell lymphomas in in vitro assays. In addition, we demonstrate the important role of CD16 on NK cells using an in vivo model of cancer immunotherapy using anti-CD20 antibody treatment of B-cell lymphomas.

Keywords: natural killer cells, NK, Fc receptor, FcγRIII, CD16, FcγRIIb, CD32b, antibodies

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; IACUC, Institutional Animal Care and Use Committee; ILC, innate lymphoid cell; ITAM, immunoreceptor tyrosine-based activating motif; MHC, major histocompatibility complex; MCMV, mouse cytomegalovirus; NK, natural killer; NKR, natural killer receptor; NFAT, nuclear factor of activated T-cells; p.i., postinfection; PI, propidium iodide; PBS, phosphate-buffered saline; RNA-seq, RNA sequencing; WT, wild type.

1 Introduction

Natural killer (NK) cells are a subset of innate lymphocytes that serve as sentinels of the innate immune system. They patrol the body and seek and destroy pathogenic cells. These harmful cells can be virally infected, cancerous, or otherwise "stressed."[1,2] They are equipped with a myriad of receptors, which interact with ligands on targets that relay information about the fitness of a target cell. Consequently, healthy cells are left intact but pathogenic ones get eliminated using preformed cytotoxic granules.

Although all NK receptors (NKRs) are germline encoded and lack the antigen specificity acquired through VDJ recombination, they also express Fcγ receptors (FcγRs) that harness the specificity of antibodies generated by the B-cell repertoire. FcγRs interact with the Fc portion of IgGs and proceed to modulate downstream immune functions.[3,4] Humans encode 5 low-affinity FcγRs, 1 inhibitory (CD32B,/FcγRIIB/*FCGR2B*) and 4 stimulatory FcγRs (CD32A/FcγRIIA/*FCGR2A*, CD32C/FcγRIIC/*FCGR2C*, CD16A/FcγRIIIA/*FCGR3A*, and CD16B/FcγRIIIB/*FCGR3B*),[3–5] whereas mice encode 3 low-affinity FcγRs, with CD16 (FcγRIII/*Fcgr3*) and CD16.2 (FcγRIV/*Fcgr4*) being activating and CD32b being inhibitory.[3–5] NK cells in both humans and mice have only been shown to express to the low-affinity receptors CD16 and CD32 (CD16A, CD32A, CD32B, and CD32C in humans; CD16 and CD32b in mice).[6–8] In humans, CD16A was one of the earliest markers discovered to identify NK cells and is expressed on the mature subset of NK cells ($CD56^{dim}CD16^{+}$).[9,10] Similarly, CD16 is expressed on 90% of NK cells in mice; however, CD16 is expressed at a relatively early stage of development—following acquisition of NK1.1 and around the same time as NKp46.[11]

Similar to most activating NKRs, the CD16 receptor lacks the ability to signal on its own and, therefore, requires an adaptor molecule that contains an immunoreceptor tyrosine-based activating motif (ITAM) to mediate signaling cascades. In human NK cells, CD16 can associate with FcεR1γ or CD3ζ, whereas mouse CD16 can only associate with FcεR1γ.[6,12,13] In NK cells, cross-linking of the CD16 receptor results in phosphorylation of the ITAM(s) that initiate signaling cascades, which ultimately lead to the release of cytotoxic granules containing granzymes and perforin, a process known as antibody-dependent cellular cytotoxicity (ADCC), resulting in killing of target cells.[2] Alternatively,

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the inhibitory CD32b has an intracellular tail that encodes an immunoreceptor tyrosine-based inhibitory motif capable of recruiting phosphatases upon cross-linking and results in the suppression of ITAM-mediated signals.[14]

In humans, CD16 has long been appreciated as being integral to the arsenal of NKRs that can be harnessed to mediate the therapeutic potential of NK cells.[3,15–17] In fact, it is thought that NK cell–mediated ADCC of antibody-coated B-cell lymphomas is an important therapeutic function of rituximab.[18] However, studies elucidating the importance of CD16 in mouse NK cells have been limited due to unavailability of reagents. For example, the extracellular domains of mouse CD16 and CD32b receptors are so similar that monoclonal antibodies raised against these proteins cross-react with the other Fc receptors (clones 2.4G2 and 93).[19] In addition, because CD16 is expressed on a number of other immune cells, in particular myeloid cells, it has been difficult to assess the importance of NK cell–mediated ADCC in in vivo experiments.[20–22] However, recently, novel antibodies were generated capable of distinguishing between mouse CD16 and CD32b,[23] thus allowing the assessment of distinct functions of these receptors. In addition, Prof. Jeffrey Ravetch's group has generated mice with floxed alleles of FcγR, which are instrumental in assessing the importance of these receptors on different immune cell types.[24]

In this study, we phenotypically and functionally characterized the biological roles that CD16 and CD32b play in wild-type (WT) NK cell responses. We also evaluated mice with floxed alleles of *Fcgr3* and *Fcgr2b* crossed to NKp46^{Cre+} mice to generate NK- and innate lymphoid cell (ILC)-specific conditional knockout mice. Using these mice, we interrogated the impact of both the stimulatory CD16 and the inhibitory CD32b Fcγ receptors. We assessed the impact of these receptors on development, maturation, and function of NK cells. Specifically, we assessed the role of these Fc receptors in NK cells during mouse cytomegalovirus (MCMV) infection and in a model of cancer immunotherapy using an anti-CD20 antibody bound to a B-cell lymphoma, akin to rituximab treatment in patients.

2 Materials and methods

2.1 Animals

C57BL/6 (B6) and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6.SJL-Ptprc^{a}Pepc^{b}/BoyCrl (CD45.1 congenic) mice were purchased from Charles River Laboratories (Wilmington, MA). B6.*Fcgr3*$^{fl/fl}$ and B6.*Fcgr2b*$^{fl/fl}$ mice were generously provided by Prof. Jeffrey Ravetch as previously described.[24] B6.*Ncr1*^{iCre} (NKp46^{Cre+}, WT^{Cre+}) mice were generously provided by Prof. Eric Vivier as previously described.[11] NKp46^{Cre+} mice were bred with mice containing floxed alleles of CD16 (*Fcgr3*$^{fl/fl}$) or CD32b (*Fcgr2b*$^{fl/fl}$) to generate CD16$^{\Delta NK}$ and CD32b$^{\Delta NK}$ conditional knockout mice, respectively.[24] B6.*Klra8*$^{-/-}$ (Ly49H$^{-/-}$) mice were obtained from Prof. Silvia Vidal (McGill University).[25] All mice were bred and housed, and experiments were conducted according to protocols approved by the UCSF Institutional Animal Care and Use Committee (IACUC) based on guidelines set by the National Institutes of Health (NIH).

2.2 Cells

BWZ.36 (BWZ) cells were generously provided by Dr. N. Shastri (UC Berkeley).[26] YAC-1 were obtained from the American Type and Culture Collection (ATCC, Manassas, VA). The C57BL/6-derived BL3750 B-cell lymphoma line was generously provided by Dr. Ronald Levy (Stanford University).[27] Cells were cultured in complete RPMI-1640 (RPMI) or DMEM-HG supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin, 110 µg/mL sodium pyruvate, 50 µM 2-mercaptoethanol, 10 mM HEPES, and 10% fetal bovine serum (FBS). Mouse NK cells were harvested from mouse spleens and cultured in completed 10% RPMI with 1,000 U/mL recombinant human IL-2 (rhIL-2, teceleukin, generously provided by NCI Biological Resources Branch, Bethesda, MD).

2.3 Virus and infections

Smith strain of MCMV was prepared by homogenizing salivary glands harvested from infected BALB/c mice, as previously described.[28–30] MCMV virus was titered on mouse embryonic fibroblasts. Mice were infected by intraperitoneal injection of 1,000 plaque-forming units of MCMV. Spleens from mice were harvested at different time points and analyzed by flow cytometry.

2.4 NK enrichment and adoptive transfer

Splenic NK cells were enriched from WT/B6, WT CD45.2^{+} NKp46^{Cre+}, WT CD45.1^{+} NKp46^{Cre+}, CD45.2^{+} CD16$^{\Delta NK}$, and CD32b$^{\Delta NK}$ mouse spleens using MojoSort mouse NK cell isolation kit according to the manufacturer's protocol (BioLegend, San Diego, CA). Cells were assessed for enrichment of Ly49H^{+}NK1.1^{+}NKp46^{+}CD3ε^{-}CD19^{-} NK cells using flow cytometry. Equal numbers of Ly49H^{+} NK cells were adoptively transferred into Ly49H$^{-/-}$ (*Klra8*$^{-/-}$) mice by intravenous injection. One day posttransfer, mice were infected with MCMV by intraperitoneal infection. Blood was collected at various time points and analyzed for expansion of Ly49H^{+} NK cells.

2.5 Cancer immunotherapy of B-cell lymphoma

For in vivo cancer immunotherapy treatments, mice were injected with 1×10^{5} BL3750 in 200 µL unsupplemented RPMI-1640 subcutaneously. The following day, mice were injected with 100 µg anti-mouse CD20 antibody (clone 5D2, mouse IgG2a isotype) or isotype-matched control antibody (MOPC-173) in 100 µL. Tumor volumes were calculated by measuring tumor width and length using vernier calipers. Mice were measured until an end point of 2.0 cm^{3}, which was approved by UCSF IACUC protocols.

2.6 Polymerase chain reaction and cloning

The extracellular domains of mouse CD16 (*Fcgr3*), CD32b (*Fcgr2b*), and CD16.2 (*Fcgr4*) were polymerase chain reaction amplified using gene-specific primers (CD16: forward: 5′–gaa ttc ctc gag gcc gcc ATG TTT CAG AAT GCA CAC TC–3′, reverse: 5′–ata aga atg cgg ccg cAG TGT GGT ACC AGA CTA GAG–3′; CD32b: forward: 5′–gaa ttc ctc gag gcc gcc ATG GAG AGC AAC TGG ACT GTC CAT G–3′, reverse: 5′–ata aga atg cgg ccg cAA TTG TCA ATA CTG GTA AAG ACC–3′; CD16.2: forward: 5′–gaa ttc ctc gag gcc gcc ATG TGG CAG CTA CTA CTA CCA ACA GC–3′, reverse: 5′–ata aga atg cgg ccg cTT GAT GCC ACG GTG GAA ACA TGG–3′) using Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA). These constructs were subcloned into pMSCV2.2-IRES-EGFP vector expressing a type I CD3ζ fusion cassette to generate vectors expressing CD3ζ-CD16, CD3ζ-CD32b, and CD3ζ-CD16.2 chimeric receptors that were used to generate reporters, as previously described.[31] All vectors were sequences for validation of desired inserts.

2.7 Transfections and transductions

For transfections, HEK293T cells were plated 1 d prior to transfection in 6-well plates (6×10^{5}/well). Transfections were performed using Lipofectamine 2000 according to the manufacturer's

protocol (Thermo Fisher Scientific, Waltham, MA). Retroviruses were generated by cotransfecting pMSCV2.2-IRES-EGFP vectors into HEK293T along with packaging plasmids, and viral supernatant was then used to transduce BWZ cells. Cells were then sorted for expression of enhanced green fluorescent protein (EGFP) prior to flow cytometric sorting for Fcγ receptor expression.

2.8 BWZ reporter assays

Plate-bound stimulations were conducted using high binding EIA/RIA plates (Corning, New York, NY) precoated with purified antibodies [10 to 30 μg/mL in phosphate-buffered saline (PBS)] overnight. The next day, wells were thoroughly washed with PBS, and then BWZ reporters (5×10^4) were added to the wells and plates were incubated overnight. Stimulations using 30.2 nM phorbol-12-myristate-13-acetate (PMA) and 0.5 μM ionomycin served as positive controls, while media alone served as negative controls. The following day, cells were pelleted by centrifugation, washed with PBS, and resuspended in 150 μL CPRG buffer [90 mg/L chlorophenol-red-β-D-galactopyranoside (Roche, Basel, Switzerland), 9 mM $MgCl_2$, 0.1% NP-40 in PBS], and assays were developed at room temperature. Readings were recorded using a microplate reader (Tecan Life Sciences, Männedorf, Switzerland) at OD 595 to 655 nm. Data were normalized to control values using the following formula: % receptor-specific stimulation = (treatment − negative control)/(positive control − negative control) × 100%.

For cocultures with stimulators, BL3750 cells (5×10^4) were added into 96 flat-well plates with BWZ cells (5×10^4) in the presence of antibodies at a range of concentrations and incubated overnight at 37 °C. The following day, cells were washed, resuspended in CPRG buffer, and analyzed as described above.

2.9 Antibodies and flow cytometry

Cells were stained with primary mAbs in FACS buffer (2.5% FBS, 1 mM EDTA, and 0.03% NaN_3 in PBS) on ice for 25 min, washed, incubated with secondary antibodies for another 25 min, and then analyzed using a LSRII conventional flow cytometer (BD Biosciences, Franklin Lakes, NJ) or a Cytek Aurora Spectral cytometer (Cytek Biosciences, Fremont, CA). Cells were gated by forward and side light-scatter properties and then for cell viability using propidium iodide (PI), DAPI, or Zombie Red (BioLegend) exclusion. Data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR). The anti-mouse CD20 mouse IgG2a antibody (clone 5D2) was generously provided by Dr. Jeffrey Venstrom (Genentech, Inc., South San Francisco, CA).[32,33] The anti-mouse Clr-b antibody (clone 4A6) was a kind gift from Dr. James Carlyle (University of Toronto).[34] The anti-mouse NKR-P1B^{B6} antibody (clone 2D12) was generously provided by Dr. Wayne Yokoyama (Washington University in St. Louis).[35] Anti-mouse CD16 (S17014E or AT154-2), mouse CD16/32 (93 or 2.4G2), mouse CD32b (AT130-2), NK1.1/NKR-P1C^{B6} (PK136), NKp46 (29A1.4), mouse CD3ϵ (145-2C11 or 17A2), CD19 (clone 6D5), Ly49H (clone 3D10), CD27 (LG.3A10), CD11b (M1/70), Ly49D (4E5), Ly49C/I (5E6), Ly49G2 (4D11), Ly49A (YE1/48.10.6), NKG2A/C/E (20D5), DNAM-1 (TX42.1), CD90.2 (30-H12), KLRG1 (2F1/KLRG1), Ly6C (HK1.4), CD69 (H1.2F3), CD62L (MEL-14), CD20 (SA275A11), IgM (AF6-78), IgD (11-26c.2a), B220 (RA3-6B2), ICAM-1 (YN1/1.7.4), H-2D^b (KH95), H-2K^b (AF6-88.5), Qa-1^b (6A8.6F10.1A6), pan-Rae-1 (186107), Mult-1 (237104), CD48 (HM48-1), CD155/PVR (TX56), Nectin-2 (829038), 4-1BBL (clone TKS-1), CD107a (1D4B), IFN-γ (XMG1.2), mouse IgG1 isotype-matched control (MOPC-21), mouse IgG2a isotype-matched control (MOPC-173), and rat IgG2a isotype-matched control (RTK2758) were purchased from BioLegend, BD Biosciences, Thermo Fisher Scientific, or Bio-Rad (Hercules, CA). All secondary PE- or APC-conjugated streptavidin reagents were purchased from BioLegend. Unless otherwise stated in figure legends, NK cells are defined as NK1.1$^+$NKp46$^+$CD49b$^+$CD3ϵ$^-$CD19$^-$ lymphocytes.

2.10 NK-cell stimulation assays

Plate-bound antibody stimulations were performed by culturing fresh mouse splenocytes in 96-well high-binding EIA/RIA plates (Corning) precoated with purified antibodies (30 μg/mL in PBS overnight incubation) for 5 h in the presence of anti-CD107a antibody and monensin (1 μL anti-CD107a mAb/well and 2 μM monensin) (BioLegend). Cytokine stimulations were performed by culturing fresh mouse splenocytes in the presence of mouse IL-12 (10 ng/mL) and IL-18 (5 ng/mL) for 5 h in the presence of monensin. After incubations, cells were stained for surface markers and live/dead, fixed, permeabilized, stained for intracellular IFN-γ, and then analyzed using flow cytometry. Stimulations using 30.2 nM PMA and 0.5 μM ionomycin served as positive controls, while media alone served as negative controls.

2.11 Cytotoxicity assays

Splenic NK cells were enriched from mice spleens using a MojoSort mouse NK-cell isolation kit according to the manufacturer's protocol (BioLegend). Cells were cultured in complete 10% RPMI supplemented with 1,000 U/mL rhIL-2. Cells were used as effectors after 5 to 7 days of culturing in rhIL-2.

Target cells (BL3750 or YAC-1) were labeled with CellTrace Violet (Thermo Fisher Scientific) and then used as targets in cytotoxicity assays with NK-cell effectors in the presence of antibodies (10 μg/mL). These cells were cocultured for 4 h at 37 °C and then washed, resuspended in FACS buffer containing PI (0.5 μg/mL), and transferred to cluster tubes for analysis by flow cytometry. Cell counts were obtained by adding CountBright Absolute Counting Beads (Thermo Fisher Scientific) to samples according to the manufacturer's protocol. Percentage of specific lysis (% Specific lysis) was calculated by determining the number of live cells in experimental samples relative to control samples as follows:

$$\%\ \text{Specific lysis} = \frac{(\#\ \text{of live cells in experimental} - \#\ \text{of live cells in spontaneous control})}{(\#\ \text{of live cells in maximal control} - \#\ \text{of live cells in spontaneous control})} \times 100$$

2.12 Bioinformatic analysis

RNA sequencing (RNA-seq) data previously generated by H. Yoshida and colleagues at the Immunological Genome Project and by Prof. Joseph Sun's laboratory were used for this study.[36–38] Briefly, mapped read counts deposited in the NCBI by authors (GEO Accession #: GSE109125, GSE106138, and GSE140035) were plotted or calculated for the $\log_2$ fold change difference and reported in this study. Microarray data previously generated by N. Bezman and colleagues at the Immunological Genome Project (GEO Accession #: GSE15907) were also analyzed and reported in this study.[39]

2.13 Statistical analysis

Data were analyzed using Prism 9 (GraphPad Software, La Jolla, CA), employing 1-way or 2-way ANOVA analysis. Graphs show mean ± SEM or mean ± SD; $^*P < 0.033$, $^{**}P < 0.002$, $^{***}P < 0.001$. All data are representative of at least 2 independent experiments or 3 to 4 biological replicates. See figure legends for details.

3 Results

3.1 Fcγ receptor expression by mouse NK cells

We first characterized the expression of the CD16 and CD32b Fcγ receptors on NK cells from WT B6 mice. Consistent with previous reports, CD16 was detected on 90% of NK cells, whereas CD32b was found on 20% to 40% of NK cells (Fig. 1A). Upon closer inspection, we observed that CD32b was exclusively coexpressed on CD16$^+$ NK cells. We also observed CD16 expression on most NK1.1$^+$NKp46$^+$Lin$^-$ cells in the bone marrow, suggesting that it is expressed early during development (data not shown), consistent with previous studies.[11] In splenic NK cells, CD16 was expressed at the highest levels on the CD27$^+$CD11b$^+$ subset of NK cells (Fig. 1B–D), whereas CD32b appeared to accumulate during maturation, with the highest frequency on CD27$^-$CD11b$^+$ and the lowest frequency on CD27$^+$CD11b$^-$ NK cells (Fig. 1B and E). This was reflected at the transcript level as determined by RNA-seq measurements of the different NK cell subsets in the spleen and bone marrow using data generated by the Immunological Genome Project (Fig. 1F).[38]

3.2 The Fcγ receptors are functional on mouse NK cells

Plate-bound stimulations using cross-linking antibodies are an effective method to measure receptor-mediated NK-cell activation, especially given that redirected lysis assays are challenging to interpret CD16 or CD32b function because P815 are FcγR$^+$ targets, and using anti-CD16 or anti-CD32b antibodies could induce ADCC or block FcγRs on both effector and target. We have previously shown that cross-linking the CD16 receptor on NK cells using plate-bound antibody stimulation yields weak yet significant activation, as measured by degranulation (CD107a) and cytokine production (IFN-γ).[6] Therefore, we performed a more thorough analysis of NK activation using anti-NK1.1 and anti-NKp46 antibodies as positive controls side-by-side with anti-CD16 and anti-CD32b antibodies. Because NK1.1 is a potent activator of NK cells, we also performed stimulations using anti-NK1.1 and anti-CD32b antibodies to determine if CD32b could suppress anti–NK1.1-induced activation. We observed potent degranulation with NK1.1 and NKp46 cross-linking, although less IFN-γ production with NKp46, whereas with CD32b, CD16, and isotype-matched Ig controls, we observed low levels of CD107a at the cell surface (Fig. 2). This suggested that IgGs on the surface of the plate could cross-link the activating CD16 receptor through their exposed Fc regions. We also included CD32b in our flow cytometry panel to determine if there were functional differences between CD32b$^-$ and CD32b$^+$ NK cells (Fig. 2A). Here, we observed that upon NK1.1 cross-linking, the CD32b$^-$ subset of NK cells had higher activation than CD32b$^+$ NK cells, suggesting that CD32b-mediated recognition of IgG-Fc was inhibiting NK cells. This was more pronounced in stimulations using anti-NK1.1 with anti-CD32b antibodies. Interestingly, inhibition by CD32b was more evident in the dampening of CD107a degranulation on CD32b$^+$ NK cells, as CD32b$^+$ NK cells were only weakly capable of producing IFN-γ (Fig. 2A–C). Notably, CD32b$^+$ and CD32b$^-$ NK cells were equally capable of being activated with PMA+

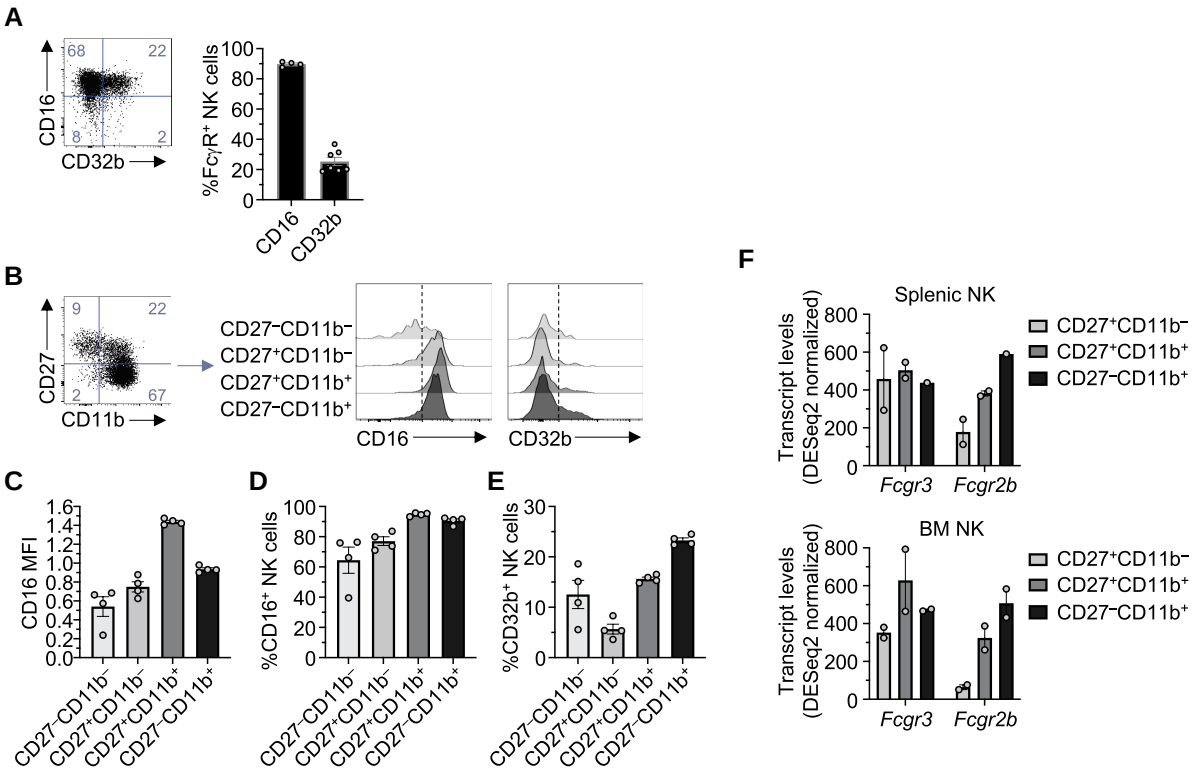

Fig. 1. Mouse natural killer (NK) cells express the stimulatory CD16 and the inhibitory CD32b Fcγ receptors. (A) Splenic NK cells (NK1.1$^+$NKp46$^+$CD49b$^+$CD3e$^-$CD19$^-$) were stained for expression of CD16 and CD32b receptors and analyzed using flow cytometry. Numbers in blue represent the percentage of cells in the gate. Graph on the right shows mean ± SEM of data in A. Experiments are representative of at least 3 independent experiments and include $n = 4$ to 7 biological replicates per experiment. (B) NK cells were analyzed for CD16 and CD32b expression on the different NK-cell subsets during maturation, as defined by CD27 and CD11b expression (CD27$^-$CD11b$^+$ → CD27$^+$CD11b$^-$ → CD27$^+$CD11b$^+$ → CD27$^-$CD11b$^+$). Dashed lines represent the percentage FcγR$^+$ cells in the gate. Data in B are quantified as (C) normalized CD16 mean fluorescent intensity (MFI), (D) percentage of CD16$^+$ NK cells, and (E) percentage of CD32b$^+$ NK cells. (F) RNA sequencing analysis of *Fcgr3* and *Fcgr2b* transcripts in different subsets of NK cells in the spleen and bone marrow (BM). Data were obtained from data sets deposited by the Immunological Genome Project GSE109125.

ionomycin treatments, confirming that this difference was antibody mediated. Collectively, these data demonstrate that although CD16 does not induce signals as strong as NK1.1 or NKp46, the receptor is still functional. These data also demonstrate that CD32b is functional on mouse NK cells and capable of inhibiting antibody-mediated responses.

NK cells activated with IL-2 or IL-15 typically have stronger functional responses; therefore, we tested if this was also the case with CD16. We enriched NK cells and cultured them in rhIL-2 for 6 d and then assessed their ability to be activated using plate-bound antibody stimulations. We observed that rhIL-2–cultured NK cells were much more responsive to CD16 stimulation (Fig. 3A and B). We also observed NK-cell activation in wells with isotype-matched IgG controls, suggesting that immobilized antibodies on the plates were responsible for stimulating CD16. Interestingly, cross-linking activating receptors on IL-2–activated NK cells resulted in both degranulation and IFN-γ production; however, treatments with PMA + ionomycin only resulted in IFN-γ production but not degranulation. In addition, we also observed that rhIL-2–activated NK cells downregulated expression of CD32b, while they upregulated CD16 (Fig. 3C). Interestingly, a recent study from Prof. J. Sun's laboratory detected downregulation of both *Fcgr3* and *Fcgr2b* transcripts following a 3-h incubation of NK cells in IL-2 with IL-15 or IL-12 with IL-18 (Fig. 3D).[37] These results suggest that cytokine-activated NK cells have enhanced CD16 responses.

To confirm that the observed activation with isotype-matched antibody controls was due to recognition of the Fc region of IgG on immobilized antibodies, we constructed reporter cells bearing the low-affinity Fcγ receptors by fusing intracellular CD3ζ with the extracellular domains of CD16, CD32b, or CD16.2 and transduced these into BWZ cells. Briefly, BWZ cells encode an NFAT-*LacZ* cassette that produce β-galactosidase upon NFAT activation, events that are triggered by cross-linking the chimeric receptor.[26,40] These BWZ.ζ-FcγR cells were confirmed to express the chimeric Fcγ receptors on their cell surface (Supplementary Fig. 1A). Importantly, coating mouse IgG1 antibodies onto plates, irrespective of antigen specificity, cross-linked and activated the CD16 and CD32b chimeric receptors. However, consistent with literature, CD16.2 could not recognize mouse IgG1 isotype antibodies (Supplementary Fig. 1B–F).[41]

3.3 Expression of Fc receptors on NK cells during MCMV infection

Although antibodies can be found as early as day 7 postinfection (p.i.), they accumulate to a maximum at day 20,[42] and thus we hypothesized that perhaps FcγR expression would reveal evidence of

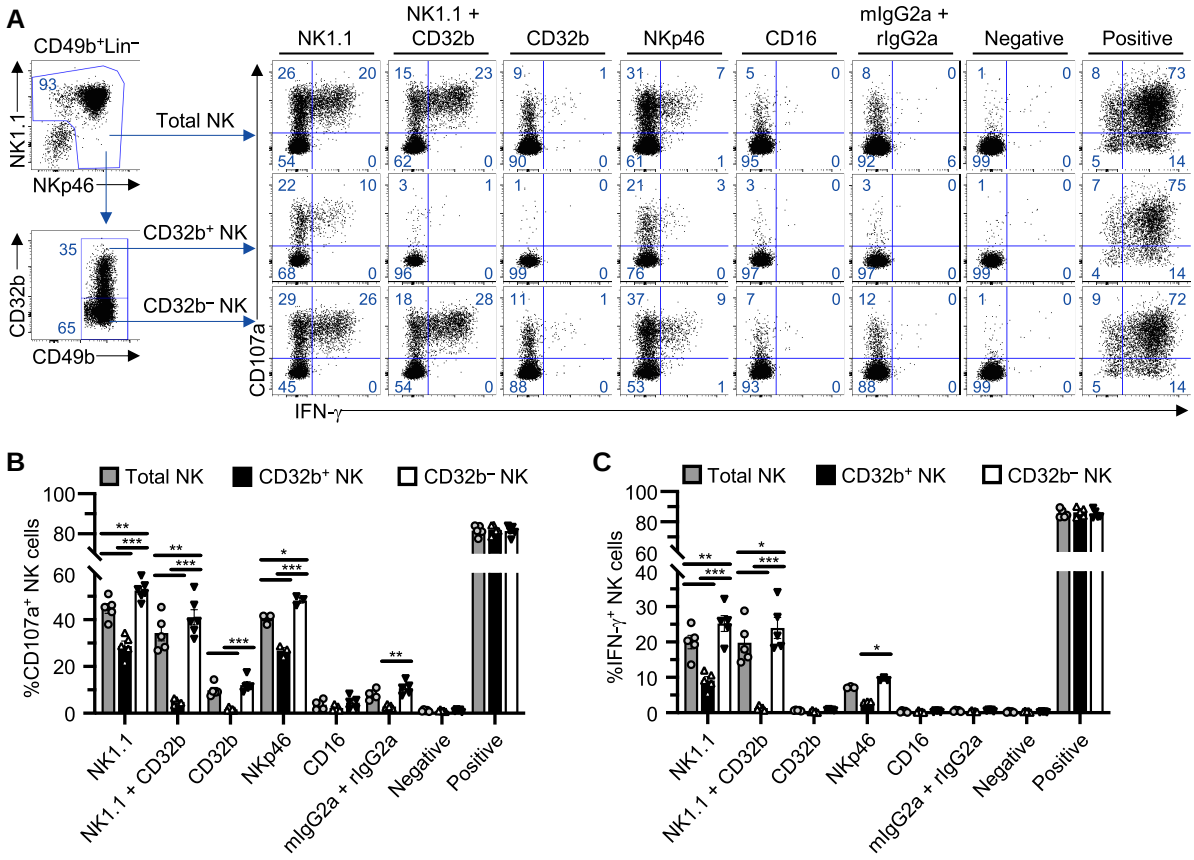

Fig. 2. CD16 and CD32b Fcγ receptors are functional on ex vivo mouse natural killer (NK) cells. (A) Splenocytes from wild-type C57BL/6 mice were stimulated by receptor-specific antibodies in plate-bound stimulation assays and then 5 h later analyzed by flow cytometry. NK cells were identified as $CD49b^+Lin^-$ (Lin: $CD3\varepsilon^-CD19^-$), and NK activation was measured in terms of degranulation ($CD107a^+$) and cytokine production ($IFN\text{-}\gamma^+$). $CD32b^+$ and $CD32b^-$ NK cell subsets were also assessed. Receptor labels on top of the plots correspond to antibody specificity. Media and PMA + ionomycin were used as negative and positive controls, respectively. Numbers in blue represent the percentage of cells in the gate. Data in A are shown graphically in terms of (B) percentage of $CD107a^+$ and (C) percentage of $IFN\text{-}\gamma^+$ NK cells. Graph shows mean ± SEM of data in A. Data are representative of 2 independent experiments performed with $n = 2$ to 3 biological replicates per experiment. $^*P < 0.033$, $^{**}P < 0.002$, and $^{***}P < 0.001$ represent statistical significance from treatments demonstrated as determined by 2-way ANOVA.

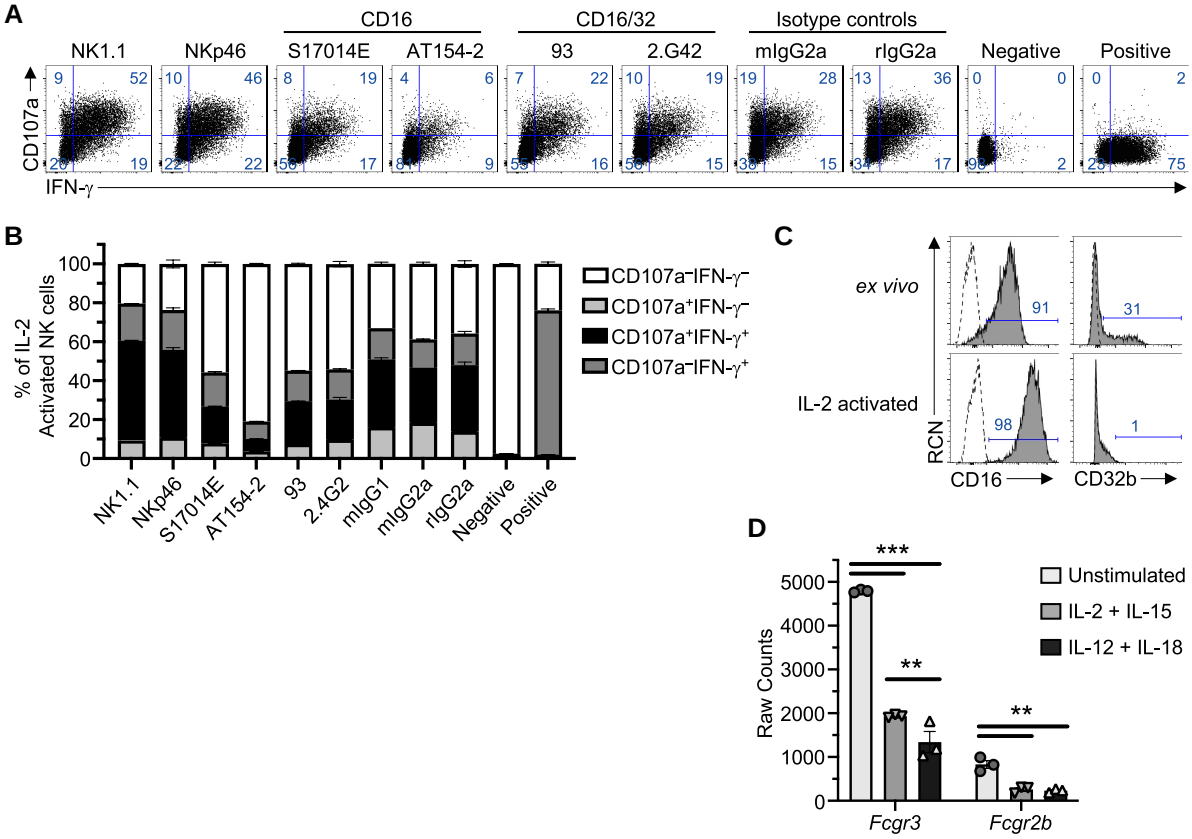

Fig. 3. IL-2–activated mouse natural killer (NK) cells have enhanced CD16 responses. (A) Splenic NK cells from wild-type C57BL/6 mice were negatively enriched and cultured in rhIL-2 for 5 d and then activated using plate-bound antibody stimulations. NK-cell activation was measured as expression of CD107a and IFN-γ production. Receptor labels on top of the plots correspond to antibody specificity and clone names. Media and PMA + ionomycin were used as negative and positive controls, respectively. Numbers in blue represent the percentage of cells in the gate. (B) Graphical representation of data in A. (C) Ex vivo NK cells and IL-2–activated NK cells on day 6 were stained for expression of CD16 or CD32b and analyzed using flow cytometry. Shaded histograms represent stained cells, whereas dashed lines represent fluorescence minus one (FMO) control. Data are representative of 2 independent experiments performed in triplicates. (D) RNA sequencing analysis of *Fcgr3* and *Fcgr2b* transcript levels (raw counts) in resting NK cells or NK cells treated with cytokines (IL-2 + IL-15 or IL-12 + IL-18) for 3 h. Data were obtained from data sets deposited by C. Lau and colleagues, GSE140035. Graphs show mean ± SEM; $^{**}P < 0.002$, and $^{***}P < 0.001$ represent statistical significance as determined by 2-way ANOVA.

their involvement in NK cell–mediated controlling virus. We infected WT mice with MCMV and measured the expression of Fcγ receptors on NK cells. We observed that early during infection, CD16 was upregulated on Ly49H$^+$ NK cells and Ly49H$^+$KLRG1$^+$Ly6C$^+$ NK cells, which have been characterized as memory-like NK cells,[43,44] suggesting that this may be activation induced rather than antibody mediated (Fig. 4A and B). Of note, these Ly49H$^+$KLRG1$^+$Ly6C$^+$ NK cells were approximately 70% CD27$^+$CD11b$^+$ at day 4, but by day 7 p.i., they were back to around 5%, as the majority of Ly49H$^+$KLRG1$^+$Ly6C$^+$ NK cells are CD27$^-$CD11b$^+$ by then (data not shown). However, although CD16 mean fluorescent intensity peaked at day 7 p.i., there was a steady decline in the frequency of CD16$^+$ NK cells in Ly49H$^-$, Ly49H$^+$, and memory-like Ly49H$^+$ NK-cell subsets (Fig. 4A). This decrease in frequency of CD16 expression was more pronounced on memory-like NK cells. CD32b frequency on NK cells dropped as early as day 4 to a lowest point at day 7 and remained low until day 28, suggesting that activation may downregulate expression of CD32b or that CD32b$^-$ NK cells are the ones that expand during infection (Fig. 4A and C).

Infection with MCMV is known to drive transcriptomic gene regulation in NK cells.[36,37,39] To determine if genes associated with the Fcγ receptor complexes were modulated during MCMV infection, we analyzed RNA-seq data previously generated by Prof. J. Sun's laboratory.[36] Here we observed a sharp decrease in *Fcgr3* at day 2 p.i. followed by a peak on day 7 p.i. but then appeared to be stabilized (Fig. 4D). *Fcgr2b* transcripts also dropped dramatically 2 d p.i. but recovered at a slower rate than *Fcgr3* (Fig. 4D). These data are in agreement with previous microarray studies from our laboratory performed by the Immunological Genome Project (Fig. 4E).[39] These data suggest that NK-cell activation or inflammatory conditions during MCMV infection contribute to the majority of changes observed in FcγR expression.

3.4 Characterization of NK cells from conditional Fc receptor-deficient mice

To conclusively interrogate the role of these receptors, we generated conditional Fc receptor deficiency in NK cells and ILCs by crossing *Fcgr3*$^{fl/fl}$ or *Fcgr2b*$^{fl/fl}$ with *Ncr1*^{iCre} mice to generate CD16 conditional KO (CD16$^{\Delta NK}$) and CD32b conditional KO (CD32b$^{\Delta NK}$), respectively. As shown in Fig. 5A, NK cells in these mice had similar NK-cell frequencies in the spleen and demonstrated no deficiency in development or maturation, as measured by CD27 and CD11b expression (Fig. 5A and B). These mice were also confirmed to be deficient in CD16 and CD32b in the NKp46$^+$ compartment of ILCs but not other immune cell types known to express these

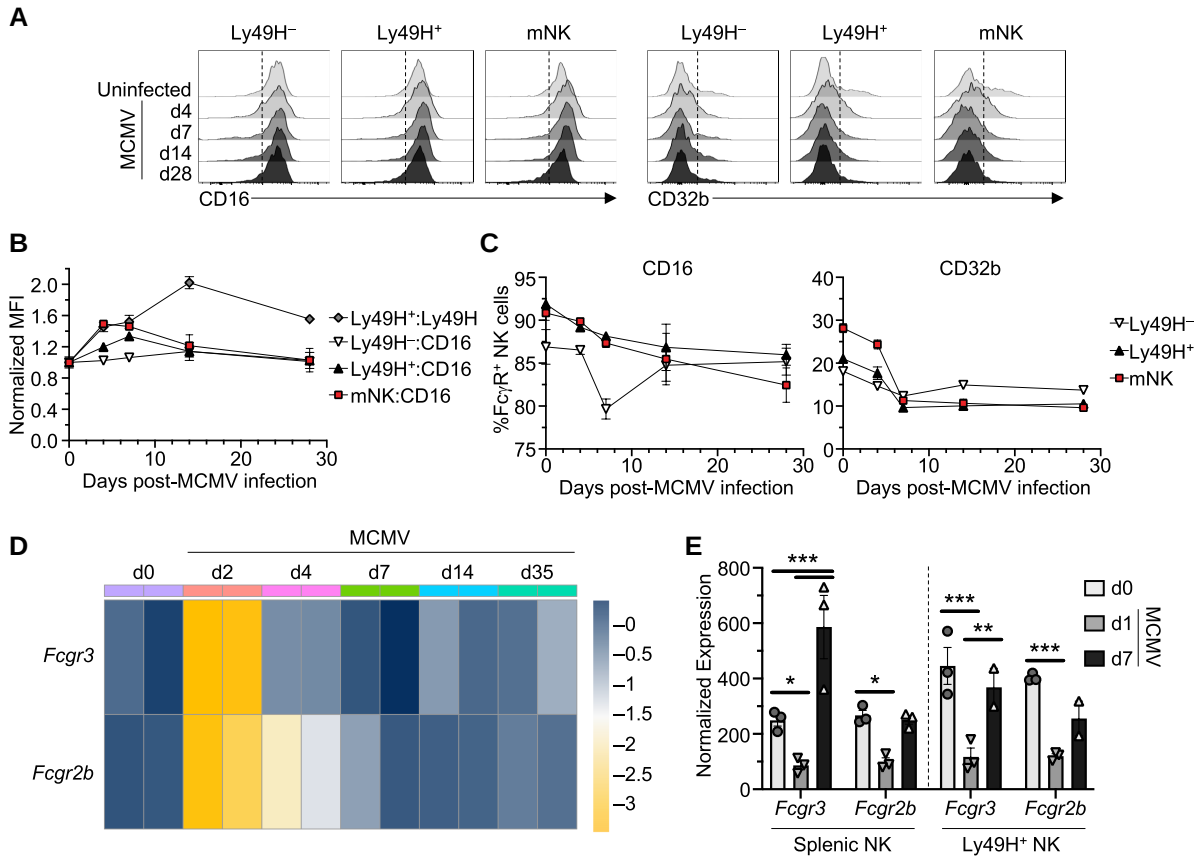

Fig. 4. Modulation of Fcγ receptors on natural killer (NK) cells during mouse cytomegalovirus (MCMV) infection. (A) Wild-type C57BL/6 mice were infected with MCMV on different days to align with a time course, and on the final day, splenic NK cells were analyzed for their expression of CD16 and CD32b by flow cytometry. (B) Normalized mean fluorescent intensity (MFI) of Ly49H and CD16 on Ly49H$^+$, Ly49H$^-$, and memory NK cells (Ly49H$^+$KLRG1$^+$Ly6C$^+$) during MCMV infection. (C) Frequency of CD16 and CD32b expression on NK-cell subsets during MCMV infection. Data are representative experiments performed with $n = 4$ to 5 biological replicates. (D) RNA sequencing analysis of *Fcgr3* and *Fcgr2b* during MCMV infection in Ly49H$^+$ NK cells. Data were obtained from data sets deposited by C. Lau and colleagues, GSE106138. (E) Microarray data of *Fcgr3* and *Fcgr2b* transcript levels during early time points of MCMV infection. Data were obtained from data sets deposited by the Immunological Genome Project GSE15907. Graphs show mean ± SEM; $^*P < 0.033$, $^{**}P < 0.002$, and $^{***}P < 0.001$ represent statistical significance as determined by 2-way ANOVA.

receptors (Fig. 5D–F, data not shown). Ablation of CD16 or CD32b did not affect expression of NK1.1, NKp46, or CD16 in CD32b$^{\Delta NK}$ NK cells, although we did observe a slight decrease in NKp46 expression on *Ncr1*$^{iCre+}$ mice in comparison to WT (Fig. 5D and E, data not shown). Importantly, the lack of any of the FcγR did not affect expression of the other FcγR on NK cells, indicating that CD16 and CD32b expression is regulated independently of the other (Fig. 5D and F). We also assessed NKR expression in these mice to ensure that genetic ablation of these receptors did not affect expression of other NKRs. As seen in Fig. 5F, we did not detect any alteration in NKRs other than the Fc receptors intended to be deleted, other than a slight decrease in frequency of Ly49H expression on NK cells from CD32b$^{\Delta NK}$ mice. Therefore, CD16$^{\Delta NK}$ and CD32b$^{\Delta NK}$ mice do not have any NK-cell development or maturation defect and only lack the Fc receptors that were intentionally genetically ablated.

3.5 NK cells from conditional Fc receptor-deficient mice reveal Fc receptor function

To determine if Fc receptor deficiency had any impact on NK-cell functionality, we assessed NK-cell function in different settings. First, we performed plate-bound antibody stimulations using antibodies against NKRs, as performed above (Fig. 2). NK cells from CD16$^{\Delta NK}$ and CD32b$^{\Delta NK}$ cells were strongly stimulated by cross-linking their NK1.1 and NKp46 receptors (Fig. 6A); however, consistent with our previous results, CD16 cross-linking failed to generate strong responses.[6] Interestingly, although NK cells from CD16$^{\Delta NK}$ mice were activated by NK1.1 and NKp46 cross-linking, it was slightly lower than WT^{Cre+} NK cells, suggesting that despite not capable of being activated on its own, the CD16 receptor likely synergizes with activating receptors to enhance NK-cell function (Fig. 6A–C). CD32b$^{\Delta NK}$ cells had slightly higher responses than WT, most evident when cross-linked with anti-NKp46 mAb, confirming that the inhibitory CD32b on NK cells could suppress NK-cell function. In agreement with previous observations, the activation observed with isotype-matched antibodies is a consequence of CD16 stimulation, as NK cells from CD16$^{\Delta NK}$ mice failed to elicit this response (Fig. 6A and B).

Second, we assessed the impact that FcγR deficiency had on cytokine production by NK cells. To this end, we stimulated fresh ex vivo NK cells with IL-12 and IL-18 and measured IFN-γ production. As shown in Fig. 6D and E, CD16$^{\Delta NK}$ and CD32b$^{\Delta NK}$ cells mounted robust IFN-γ production as effectively as WT NK cells in response to cytokine stimulation. Therefore, cytokine-mediated IFN-γ production remains functional in CD16 and CD32b-deficient NK cells.

Last, we determined the ability of FcγR-deficient NK cells in mediating cytotoxicity. Here, we performed cytotoxicity assays using IL-2–cultured NK cells as effectors against the prototypical

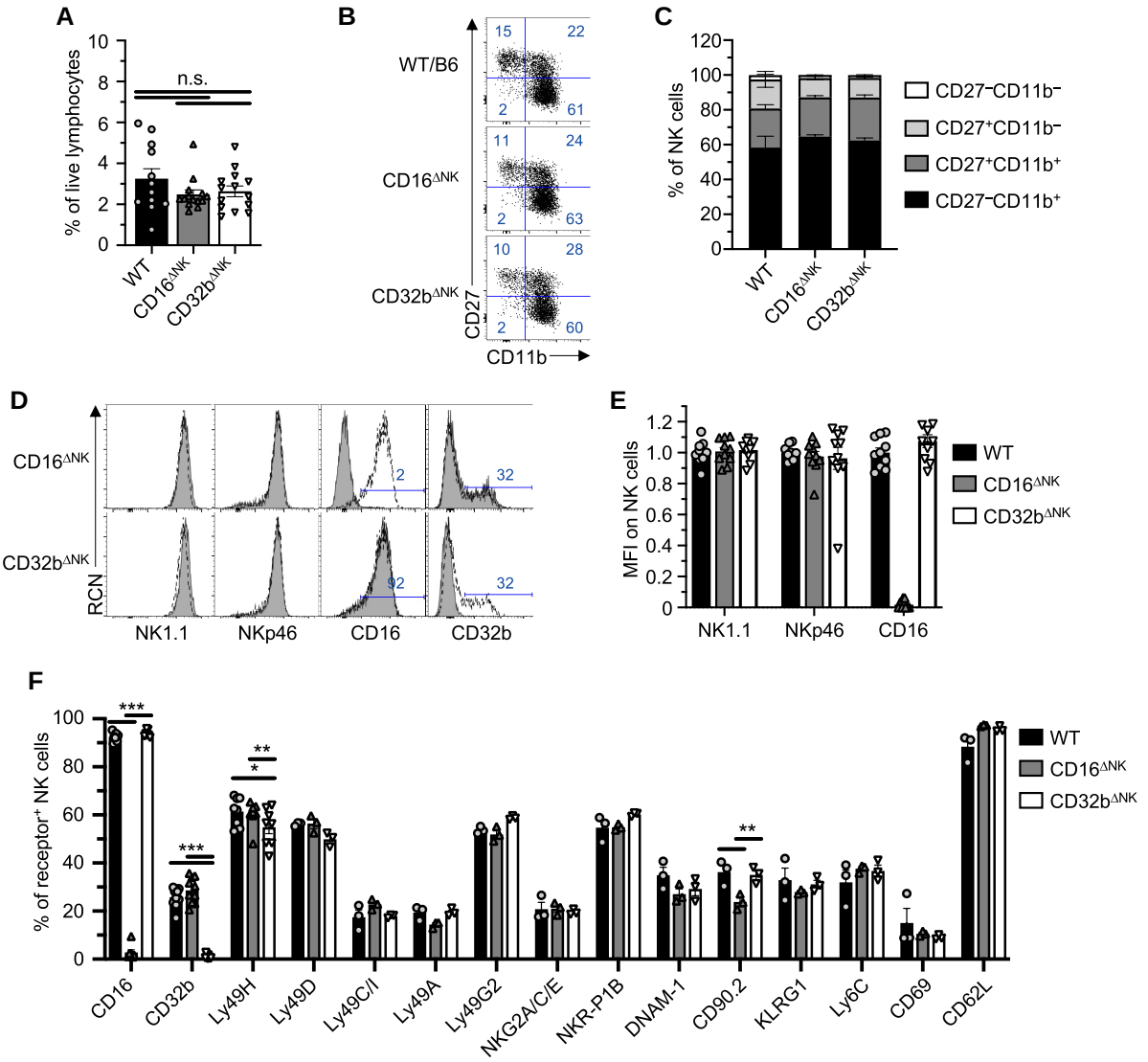

Fig. 5. Characterization of natural killer (NK) cells from mice with conditionally deleted Fcγ receptors. (A) Frequency of NK cells in wild-type (WT) (NKp46^{iCre+}), CD16 conditional knockout (KO) (CD16$^{\Delta NK}$), and CD32b conditional KO (CD32b$^{\Delta NK}$) C57BL/6 mice as a percentage of live lymphocytes. (B) Analysis of NK development as defined by CD27 and CD11b expression. (C) Quantitation of data in B are shown as mean ± SEM. (D) Expression of NK1.1, NKp46, CD16, and CD32b in NK cells from WT, CD16$^{\Delta NK}$, and CD32b$^{\Delta NK}$ mice. Shaded histograms represent NK cells from conditional KO mice, whereas dashed lines represent WT NK cells. Blue numbers above the gate represent percentage of marker$^+$ NK cells. (E) Quantitation of data in D as normalized mean fluorescent intensity (MFI) shown as mean ± SEM. (F) Percentage of NK receptor expression on NK cells from WT, CD16$^{\Delta NK}$, and CD32b$^{\Delta NK}$ mice. Graph shows mean ± SEM and is a summary of $n = 3$ to 10 biological replicates; $^*P < 0.033$, $^{**}P < 0.002$, and $^{***}P < 0.001$ represent statistical significance from negative control treatments as determined by 2-way ANOVA.

NK-cell target cell line, YAC-1. There was no difference in the ability of Fc receptor–deficient NK cells to kill YAC-1 targets (Fig. 6F). Collectively, these data reveal that NK cells from conditional Fc receptor–deficient mice do not have any impaired function in killing targets or responding to NK receptor stimulation but do display variability in responding to antibody recognition.

3.6 The BL3750 B-cell lymphoma is susceptible to NK cell–mediated killing and ADCC

CD16 is the receptor responsible for mediating ADCC; therefore, we hypothesized that CD16-deficient NK cells would not be capable of killing antibody-coated cells. To test this, we used the CD20$^+$ BL3750 B-cell lymphoma cell line.[6,27] Because NK-cell activation is dictated by the engagement of ligands on targets with receptors on NK cells, we first assessed the ligand expression of BL3750. Consistent with previous work, BL3750 cells are CD19$^+$IgM$^+$IgD$^-$, indicative of an immature B-cell lymphoma, and importantly are CD20$^+$ (Fig. 7A).[27] In addition, they are ICAM-1$^+$, MHC class I$^+$ (H-2D^b and H-2K^b), CD48$^+$, and CD155/PVR$^+$, and they have low levels of Qa-1, Clr-b, and Mult-1 but do not express Rae-1, Nectin-2, or 4-1BBL (Fig. 7B).

Before conducting ADCC assays with the BL3750 cells, we first confirmed that the anti-CD20 antibody (clone 5D2, mouse IgG2a) could be recognized on antibody-coated BL3750 cells by CD16. We used BWZ.ζ-CD16 reporter cells cocultured with BL3750 in the presence of anti-CD20 antibody or isotype-matched antibody control (Fig. 7C). These experiments confirmed that the 5D2 antibody could be engaged by CD16. To determine if this was confirmed in ADCC assays and to validate our findings from

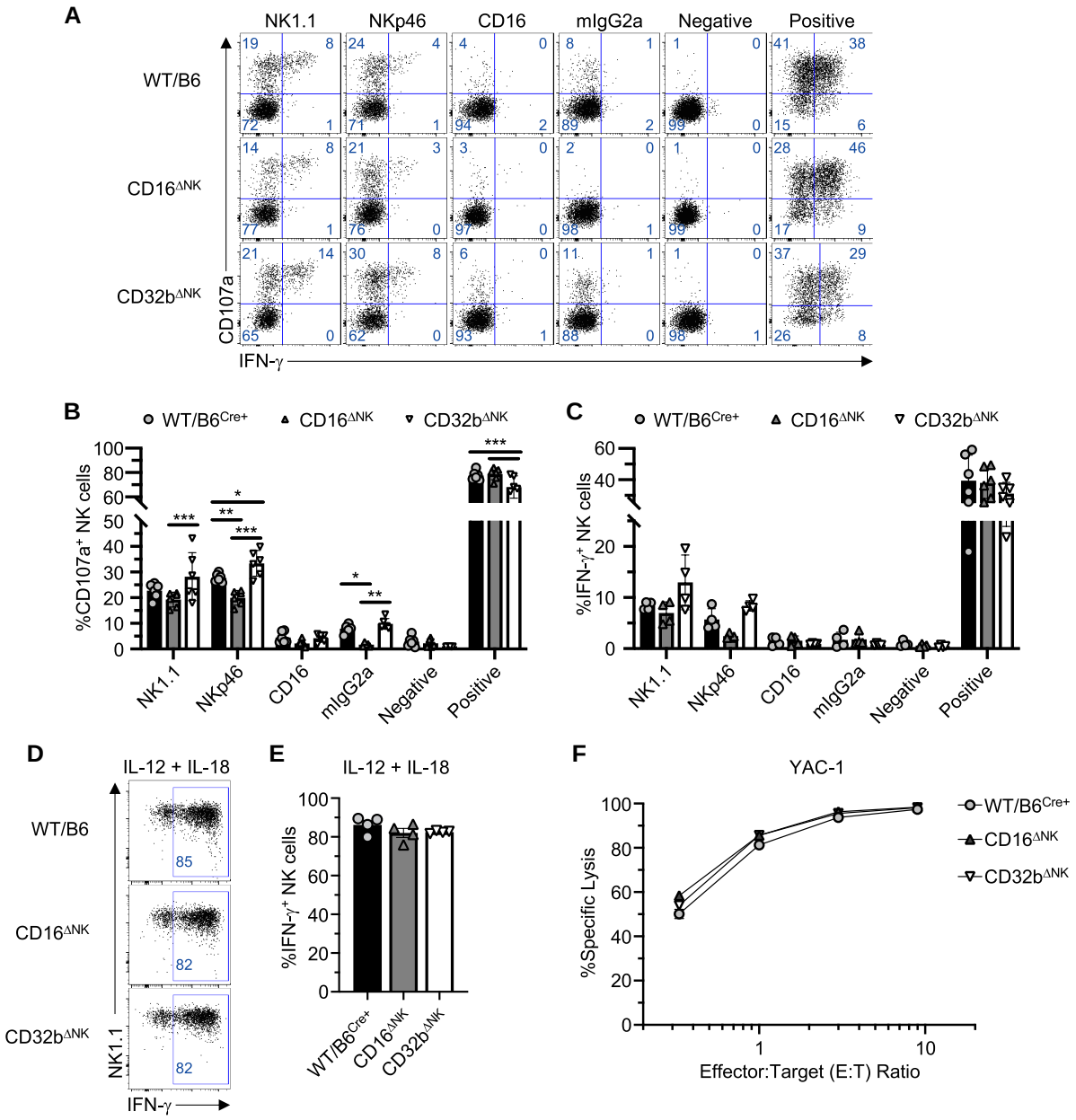

Fig. 6. Functional characterization of natural killer (NK) cells from mice with conditionally deleted Fcγ receptors. (A) Splenic NK cells from wild-type (WT) (NKp46^{iCre+}), CD16 conditional knockout (KO) (CD16$^{\Delta NK}$), and CD32b conditional KO (CD32b$^{\Delta NK}$) C57BL/6 mice were assayed in plate-bound antibody stimulation assays and assessed for NK-cell activation using flow cytometry. Receptor labels on top of the plots correspond to antibody specificity. Media and PMA + ionomycin were used as negative and positive controls, respectively. (B) Quantitation of data in A graphed as percentage of CD107a$^+$ NK cells from each genotype. (C) Quantitation of data in A shown graphically as percentage of IFN-γ$^+$ NK cells from each genotype. (D) NK cells from different genotypes were stimulated using IL-12 and IL-18, and the percentage of NK cell activation after 5 h was measured using flow cytometry. (E) Quantitation of data in D shown graphically as percentage IFN-γ$^+$ NK cells. All graphs show data as mean ± SEM. Data are representative of 2 independent experiments analyzed with $n = 3$ to 4 biological replicates. (F) Cytotoxicity assays using IL-2–activated NK cells from WT, CD16$^{\Delta NK}$, and CD32b$^{\Delta NK}$ mice as effectors and YAC-1T cell lymphomas as targets. *$P < 0.033$, **$P < 0.002$, and ***$P < 0.001$ represent statistical significance from negative control treatments as determined by 2-way ANOVA. Data are representative of 2 independent experiments analyzed in triplicates.

plate-bound antibody stimulations (Figs. 2, 3, and 6), we used ex vivo–enriched splenic NK cells and splenic NK cells that were cultured in rhIL-2 for 6 d as effectors against BL3750 lymphomas. Here we found that ex vivo NK cells were weak at killing antibody-coated BL3750, but IL-2–activated NK cells were efficient (Fig. 7D). To determine the roles of CD16 and CD32b, we then performed ADCC assays using enriched splenic NK cells cultured in IL-2 from conditional FcγR-deficient mice as effectors against BL3750. NK cells from WT and CD32b$^{\Delta NK}$ spleens killed anti–CD20-coated BL3750 targets more efficiently than isotype-matched antibody controls, whereas no increase was observed with CD16$^{\Delta NK}$ NK cells in the presence of anti-CD20 Ab (Fig. 7E). These data confirm that CD16 is responsible for mediating ADCC by NK cells and that IL-2 priming enhances CD16 function in mouse NK cells. Importantly, there was no detectable increase in ADCC in NK cells from CD32b$^{\Delta NK}$ cells, relative to WT NK cells. This is likely because IL-2–activated NK cells downregulate CD32b (Fig. 3B). These data conclusively

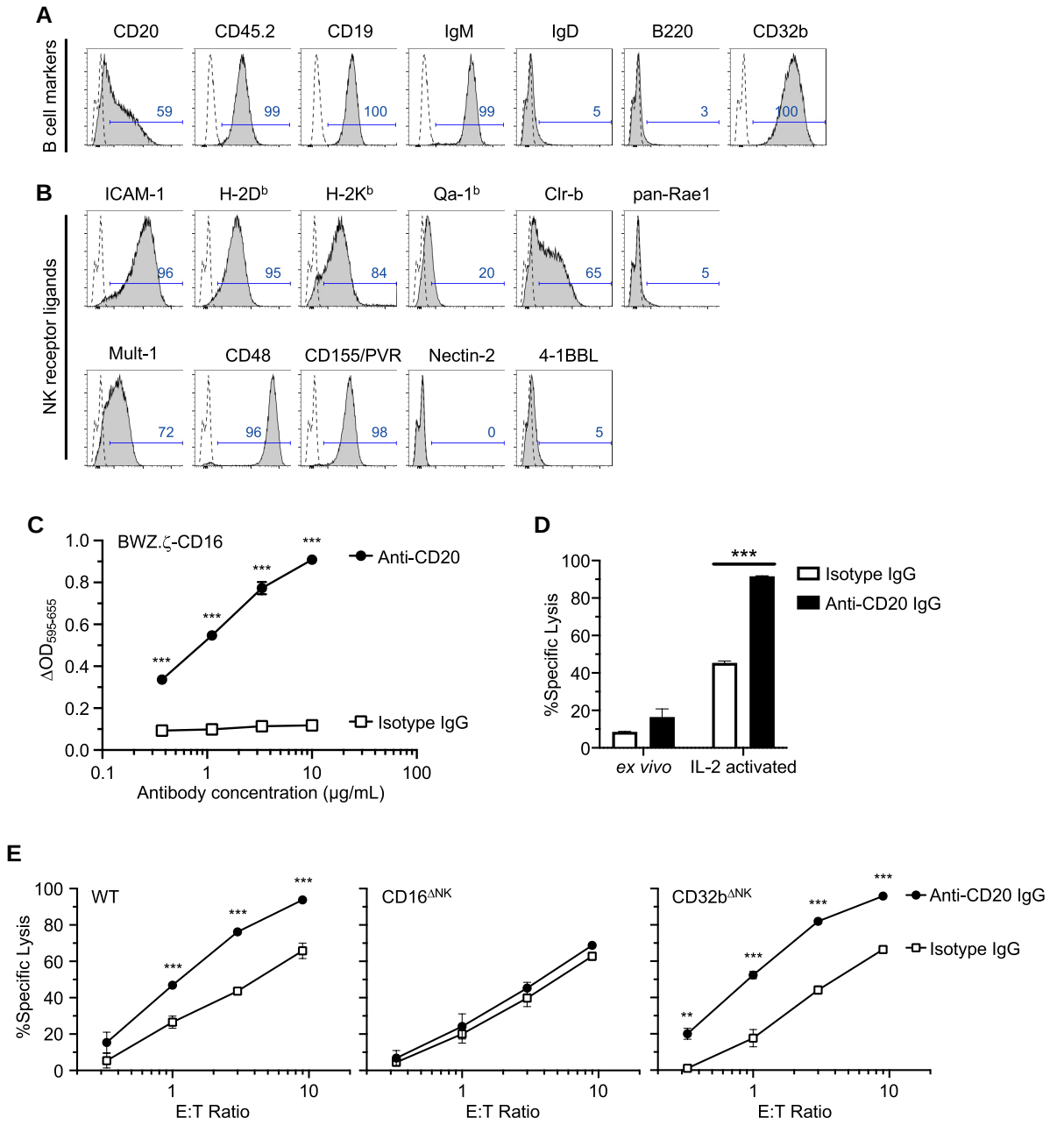

Fig. 7. The BL3750 B-cell lymphoma is susceptible to natural killer (NK) cell–mediated killing and antibody-dependent cellular cytotoxicity (ADCC). BL3750 B-cell lymphomas were analyzed using flow cytometry for the expression of (A) B-cell markers and (B) ligands for NK receptors. (C) BWZ.ζ-CD16 reporters were cocultured with BL3750 cells in the presence of anti-CD20 antibody or isotype-matched control antibody at different antibody concentrations. Cells were cocultured for 18 h and then assessed for production of β-galactosidase using the colorimetric substrate CPRG. (D) Cytotoxicity assays using ex vivo NK cells and IL-2–activated NK cells (day 6) at an effector/target (E:T) ratio of 9:1 against BL3750 in the presence of anti-CD20 antibody or isotype-matched control antibody (10 μg/mL). (E) ADCC assays using WT, CD16-deficient ($CD16^{\Delta NK}$), or CD32b-deficient ($CD32b^{\Delta NK}$) IL-2–activated NK cells as effectors and BL3750 lymphoma as targets in the presence of anti-CD20 antibody or isotype-matched control antibody (10 μg/mL). All graphs show mean ± SEM; $^{**}P < 0.002$ and $^{***}P < 0.001$ represent statistical significance from negative control treatments as determined by 2-way ANOVA. Data are representative of 2 independent experiments analyzed in triplicates.

demonstrate that IL-2–activated mouse NK cells are capable of mediating ADCC, which is dependent on CD16.

3.7 Mice with CD16-deficient NK cells have impaired control of B lymphomas treated with anti-CD20 mAb

Because we observed functional differences upon characterizing FcγR-deficient mice in vitro, we hypothesized that an in vivo model would reveal their importance. Because we observed that CD16 is the only FcγR expressed by NK cells at early time points during MCMV infection, we first infected $CD16^{\Delta NK}$ mice with MCMV and tracked viral titers and expansion of $Ly49H^{+}$ NK cells. We did not detect a difference in NK-cell expansion or viral titers between CD16-deficient and WT mice (data not shown). In addition, we performed competitive adoptive transfer of NK cells into Ly49H-deficient mice to determine if FcγRs were involved in the Ly49H-mediated expansion or formation of memory NK cells; however, we also did not detect any difference with the FcγR-deficient NK cells in this setting (data not shown). We then tested $CD16^{\Delta NK}$

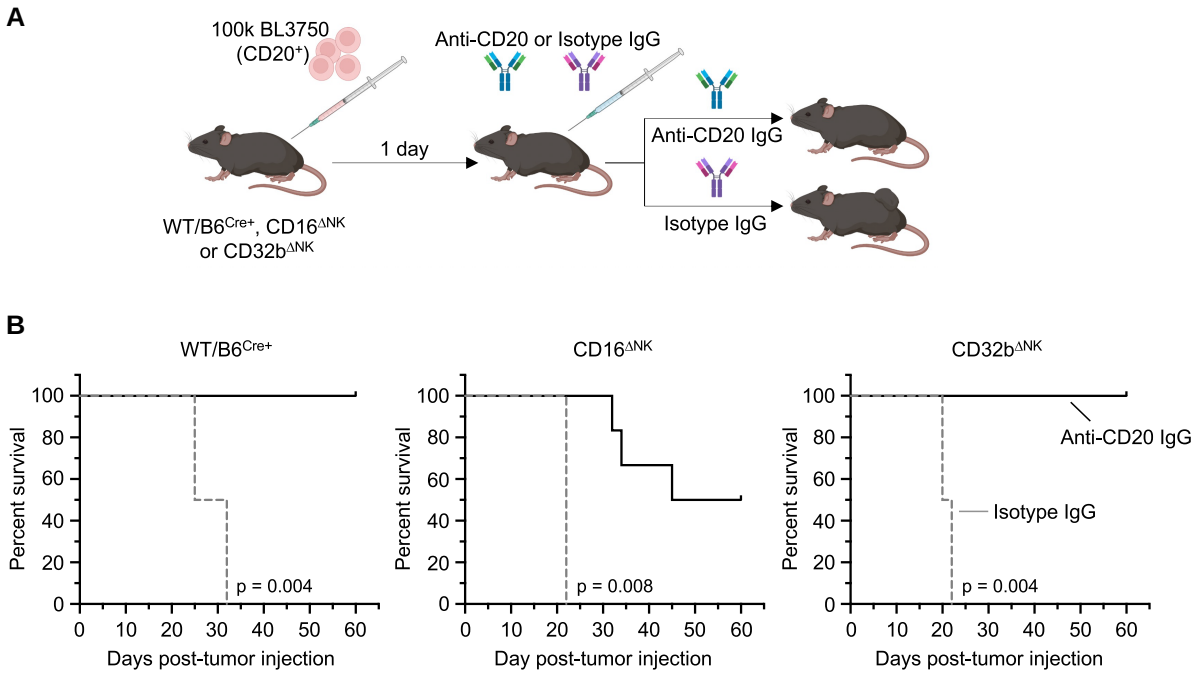

Fig. 8. CD16 plays a significant role in control of transplanted B-cell lymphomas treated with anti-CD20 antibody. (A) Experimental strategy demonstrating injection of 100,000 BL3750 subcutaneously followed by treatment using a single intraperitoneal injection of anti-CD20 antibody or isotype-matched control antibody (100 μg) the following day. Mice were tracked for development and growth of tumors. Panel A was created using BioRender.com. (B) Survival curve of mice injected with BL3750 lymphomas treated with anti-CD20 antibody or isotype-matched control antibody. Experiment was conducted with $n = 6$ mice for the CD20-treated group and $n = 2$ for the control group.

and CD32b$^{\Delta NK}$ mice in a model of cancer immunotherapy using the BL3750 B-cell lymphoma cells treated with an anti-CD20 antibody to model rituximab in patients.[27] Specifically, we subcutaneously injected BL3750 cells into WT, CD16$^{\Delta NK}$, and CD32b$^{\Delta NK}$ mice and treated with anti-CD20 mAb or mIgG2a isotype-matched antibody control the following day, following previously described methods.[27] We tracked tumor growth across each group until an ethical end point of 2 cm^3 tumor volume (Fig. 8). Importantly, injection of BL3750 cells followed by isotype-matched antibody control resulted in growth of tumors to their end point for all mice at a similar time after tumor injection. By contrast, anti-CD20 mAb-treated mice prevented tumor growth in 100% of CD32b$^{\Delta NK}$ and WT mice. Interestingly, anti-CD20 mAb treatment in CD16$^{\Delta NK}$ mice prolonged survival compared to isotype-matched antibody control–treated mice; however, 50% of the mice succumbed to the tumor by day 47 (Fig. 8B). These data conclusively demonstrate that although CD16 on NK cells does not completely control tumor growth during targeting antibody treatment, NK cell–mediated ADCC does play a significant role in the control of cancer cells using targeting antibody therapies.

4 Discussion

The CD16 Fc receptor has long been appreciated to play a critical role in the clearance of antibody-coated cells in humans.[3,16,17,45] However, due to lack of reagents, the role that CD16 plays in mouse has been lacking. Here, we investigated the functional role that the stimulatory CD16 and inhibitory CD32b Fcγ receptors play in NK-cell biology. We demonstrate that consistent with previous reports, the CD16 receptor in mice mediates weak responses in ex vivo NK cells[6,46]; however, this is significantly enhanced upon IL-2 activation. Importantly, we also demonstrate that the CD32b receptor is capable of dampening IgG-mediated NK-cell activation. To our knowledge, this is the first report to definitively demonstrate inhibition by CD32b upon recognition of IgG-Fc by CD32b on NK cells.

The ability of mouse NK cells to acquire enhanced CD16 function after IL-2 activation is similar to other NKRs.[47–49] Priming NK cells in IL-2 has a myriad of consequences, including metabolic changes,[37,47–49] but even a short 1-h priming by IL-2 can render NK cells more lytic toward targets, likely by increasing cellular adhesion.[50] However, with respect to FcγR function, it should be noted that downregulation in CD32b might also contribute to improved ADCC, as we have shown that CD32b is capable of dampening FcγR responses. The downregulation of CD32b during MCMV infection similarly suggests that this may be due to NK-cell activation, either through direct engagement with virally infected cells or as a consequence of inflammatory cytokines rather than being antibody mediated. In fact, downregulation of *Fcgr2b* can be observed as early as 3 h after treatment of mouse NK cells with IL-2, IL-15, or IL-12 and IL-18.[37] Therefore, it is unlikely that the loss of CD32b observed on NK cells during MCMV infection is due to the expansion of CD32b$^-$ NK cells. Further studies are required to unravel these questions and will shed light on the mechanisms at play during inflammation.

We have recently demonstrated that mouse CD16 responses are limited by the inability of CD16 to associate with CD3ζ, thereby relying on FcεR1γ. This is a result of polymorphisms in the transmembrane domain of CD3ζ that prevent efficient receptor assembly[6] and, therefore, suggest that WT CD3ζ on mouse NK cells actually acts as a negative regulator of CD16 function.[51,52] This is contrary to what is observed in human NK cells, whereby CD3ζ facilitates potent CD16 responses.[13] Thus, it is tempting to speculate how much improvement would be observed if CD3ζ-mediated CD16 responses were intact in mouse NK cells and how these now would reflect

human NK-cell biology. Of note, stimulation of mouse NK cells through NK1.1 or NKp46 yields robust activation, a phenotype that is drastically different from CD16, yet these receptors all rely solely on FcεR1γ in mouse NK cells. Therefore, why ex vivo CD16 function is so weak in comparison to NK1.1 and NKp46 may include additional factors in addition to the inability to associate with CD3ζ.

Despite mouse NK cells having diminished CD16 responses, our data clearly demonstrate that the receptor is functional and effective in protecting from pathogenic cells. NK cells are a small minority of immune cells in blood and most tissues of mice (2% to 5% in the blood, 1% to 3% in the spleen, and 1% in the bone marrow), yet, in our studies involving a $CD20^+$ B-cell lymphoma treated with anti-CD20 antibody, 50% of mice with CD16 deficiency on their NK cells succumbed to the tumors. This demonstrates that although NK cell–mediated ADCC is not the only mechanism at play, it does contribute significantly to the protection of the host. Consequently, these results clearly highlight the importance of CD16 in human NK cells as they have potent CD16 function in resting NK cells, which is significantly improved upon activation, and NK cells comprise a substantial proportion of the blood lymphocytes (10% to 20% of human peripheral blood mononuclear cells).[53]

Previous studies investigating B-cell depletion using anti-CD20 Abs in mice deficient in FcγR genes have demonstrated that FcεR1γ, CD64, CD16, and CD16.2 are critical (in particular, FcεR1γ and CD16.2), while CD32b deficiency improves B-cell clearance.[27,54] These studies also demonstrated that macrophages played a significant role in clearing antibody-coated cells. Our results show that CD32b inhibits ADCC responses on NK cells; however, we did not observe a phenotype in our in vivo experiments, although this does not rule out a potential involvement. Further studies perhaps using different target cells and antibodies or antibody dosing may reveal the impact of CD32b deficiency on NK-cell contributions. It is also possible that we did not observe a difference due to downregulation of CD32b upon NK-cell activation in tumor-infiltrating NK cells, similar to what is observed during MCMV infection and priming with cytokines.

Human NK cells have been reported to express CD32 receptors, and although CD32B and CD32C have been confirmed to be expressed on NK cells of some individuals,[7,8] their function remains elusive. The diversity of CD32 receptor expression therefore merits a thorough investigation to understand their involvement in antibody-mediated responses, especially for developing methods to improve NK cell–mediated responses in immunotherapies. In conclusion, our study reveals that both the activating CD16 receptor and the inhibitory CD32b Fcγ receptors are capable of modulating functional antibody-mediated responses of C57BL/6 mouse NK cells.

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Authorship

O.A.A. and L.L.L. designed the research. O.A.A., M.D.R.G.-H., J.S.A.-H., D.G., and T.N. performed the experiments. O.A.A., M.D.R.G.-H., A.J.M., D.G., and L.L.L. contributed to data analysis and interpretation. O.A.A., M.D.R.G.-H., and L.L.L. wrote the manuscript. O.A.A. and L.L.L. critically reviewed and approved the final version of the manuscript.

Supplementary material

Supplementary material is available at *Journal of Leukocyte Biology* online.

Disclosures

The authors declare no conflict of interest.

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