Expression of a Recombinant High Affinity IgG Fc Receptor by Engineered NK Cells as a Docking Platform for Therapeutic mAbs to Target Cancer Cells

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Anti-tumor mAbs are the most widely used and characterized cancer immunotherapy. Despite having a significant impact on some malignancies, most cancer patients respond poorly or develop resistance to this therapy. A known mechanism of action of these therapeutic mAbs is antibody-dependent cell-mediated cytotoxicity (ADCC), a key effector function of human NK cells. CD16A on human NK cells has an exclusive role in binding to tumor-bound IgG antibodies. Though CD16A is a potent activating receptor, it is also a low affinity IgG Fc receptor (FcyR) that undergoes a rapid downregulation in expression by a proteolytic process involving ADAM17 upon NK cell activation. These regulatory processes are likely to limit the efficacy of tumor-targeting therapeutic mAbs in the tumor environment. We sought to enhance NK cell binding to anti-tumor mAbs by engineering these cells with a recombinant FcyR consisting of the extracellular region of CD64, the highest affinity FcyR expressed by leukocytes, and the transmembrane and cytoplasmic regions of CD16A. This novel recombinant FcyR (CD64/16A) was expressed in the human NK cell line NK92 and in induced pluripotent stem cells from which primary NK cells were derived. CD64/16A lacked the ADAM17 cleavage region in CD16A and it was not rapidly downregulated in expression following NK cell activation during ADCC. CD64/16A on NK cells facilitated conjugation to antibody-treated tumor cells, ADCC, and cytokine production, demonstrating functional activity by its two components. Unlike NK cells expressing CD16A, CD64/16A captured soluble therapeutic mAbs and the modified NK cells mediated tumor cell killing. Hence, CD64/16A could potentially be used as a docking platform on engineered NK cells for therapeutic mAbs and IgG Fc chimeric proteins, allowing for switchable targeting elements and a novel cancer cellular therapy.

Keywords: FcR, ADCC, NK cell, immunotherapy, antibody
INTRODUCTION

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that target stressed, infected, and neoplastic cells (1). In contrast to the diverse array of receptors involved in natural cytotoxicity, human NK cells mediate ADCC exclusively through the IgG Fc receptor CD16A (FcγRIIIA) (2–4). This is a potent activating receptor and its signal transduction involves the association of the transmembrane and cytoplasmic regions of CD16A with FcγRIγ and/or CD3ζ (4–9). Unlike other activating receptors expressed by NK cells, the cell surface levels of CD16A undergo a rapid downregulation upon NK cell activation during ADCC and by other stimuli (10–14). CD16A downregulation also occurs in the tumor environment of patients and contributes to NK cell dysfunction (15–19). A disintegrin and metalloproteinase-17 (ADAM17) expressed by NK cells plays a key role in its downregulation by cleaving CD16A in a cis manner at a specific location proximal to the cell membrane upon NK cell activation (13, 14, 20).

There are two allelic variants of CD16A that have either a phenylalanine or valine residue at position 176 (position 158 in CD3ζ). Unlike other activating receptors expressed by NK cells, the cell surface levels of CD16A undergo a rapid downregulation upon NK cell activation during ADCC and by other stimuli (10–14). CD16A downregulation also occurs in the tumor environment of patients and contributes to NK cell dysfunction (15–19). A disintegrin and metalloproteinase-17 (ADAM17) expressed by NK cells plays a key role in its downregulation by cleaving CD16A in a cis manner at a specific location proximal to the cell membrane upon NK cell activation (13, 14, 20).

CD64 (FcyR1) binds to monomeric IgG with 2–3 orders of magnitude higher affinity than CD16A (24–26). CD64 recognizes the same IgG isotypes as CD16A and is expressed by myeloid cells, including monocytes, macrophages, and activated neutrophils, but not NK cells (24, 26). We generated the novel recombinant receptor CD64/16A that consists of the extracellular region of human CD64 for high affinity antibody binding, and the transmembrane and intracellular regions of human CD16A for mediating NK cell signal transduction. CD64/16A also lacked the membrane proximal ADAM17 cleavage site found in CD16A. In this study, we stably expressed CD64/16A in NK92 cells, a cytotoxic human NK cell line that lacks endogenous FcγRII, and CD16/CD16A transmembrane and cytoplasmic domains. PCR amplification was performed from the generated cDNA. The PCR fragments were purified and mixed together with the forward primer 5′-CGG GAA TTC GGA GAC AAC ATG TGG TTC TTG ACA A-3′, the reverse primer 5′- CGG GAA TTC TCA TTT GTC TTG AGG GTC CTT TCT-3′ (underlined nucleotides are EcoR I sites), and Pfu50 DNA polymerase (Invitrogen) to generate the recombinant CD64/16A receptor. CD64/CD16A and CD16A cDNA (CD16A-176V variant) was inserted into the retroviral expression vector pBMN-IRES-EGFP and virus was generated for NK92 cell transduction, as previously described (14).

MATERIALS AND METHODS

Antibodies

All mAbs to human hematopoietic and leukocyte phenotypic markers are described in Table 1. All isotype-matched negative control mAbs were purchased from BioLegend (San Diego, CA). APC-conjugated F(ab′)2 donkey anti-human or goat anti-mouse IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The human IgG1 mAbs trastuzumab/Herceptin and rituximab/Rituxan, manufactured by Genentech (South San Francisco, CA), and cetuximab/Erbitux, manufactured by Bristol-Myers Squibb (Lawrence, NJ), were purchased through the University of Minnesota Boynton Pharmacy. Recombinant human L-selectin/IgG1 Fc chimera was purchased from R&D Systems (Minneapolis, MN).

Generation of Human CD64/16A

Total RNA was isolated from human peripheral blood leukocytes using TRIzol total RNA isolation reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized with the SuperScript premplantation system (Invitrogen). The recombinant CD64/16A is comprised of human CD64 extracellular domain and CD16A transmembrane and cytoplasmic domains. PCR fragments for CD64 (885 bps) and CD16A (195 bps) were amplified from the generated cDNA. The PCR fragments were purified and mixed together with the forward primer 5′-CGG GAA TTC GGA GAC AAC ATG TGG TTC TTG ACA A-3′, the reverse primer 5′- CGG GAA TTC TCA TTT GTC TTG AGG GTC CTT TCT-3′ (underlined nucleotides are EcoR I sites), and Pfu50 DNA polymerase (Invitrogen) to generate the recombinant CD64/16A receptor. CD64/CD16A and CD16A cDNA (CD16A-176V variant) was inserted into the retroviral expression vector pBMN-IRES-EGFP and virus was generated for NK92 cell transduction, as previously described (14). For this study, the transduced NK92 cells were sorted

Table 1 | Antibodies.

| Antigen | Clone | Fluorophore | Company |
|---------|-------|------------|---------|
| CD56    | HCD56 | PE-CY7     | BioLegend, San Diego, CA |
| CD10   | Hit3a | PE         | BioLegend |
| CD16   | 3G8   | APC        | BioLegend |
| CD16   | 3G8   | none       | Ancell, Bayport, MN |
| CD7    | CD7-6B7 | PE/CY5 | BioLegend |
| CD33B/NKP44 | P44-8 | APC       | BioLegend |
| CD335/NKP48 | 9E2  | APC        | BioLegend |
| CD159a/NKP22A | Z199  | APC        | Beckman Coulter, Brea, CA |
| CD314/NKP2D | 1D11 | PerCP/Cy5.5 | BioLegend |
| CD158a/KIR2DL1 | HP-MA4 | PE | BioLegend |
| CD158c1/KIR2DL2/L3 | DX27 | PE | BioLegend |
| CD158e1/KIR3DL1 | DX9  | PE        | BioLegend |
| CD94   | DX22  | PE         | BioLegend |
| CD64   | 10.1  | APC        | BioLegend |
| CD64   | 10.1  | none       | Ancell |
| CD34   | 5E11z | PE         | BioLegend |
| CD45   | 2D1   | APC        | BioLegend |
| CD43   | CD43-10G7 | APC | BioLegend |
| CD62/L-selectin | LAM1-116 | APC | Ancell |
by flow cytometry to derive populations with homogenous expression of CD16A or CD64/16A, but consisted of a mixed clonal population to avoid the effects of random genomic integration of vector DNA in a single NK92 transductant, as we have done in previous studies (14, 20). Additionally, CD64/CD16A CDNA was inserted into a pKT2 sleeping beauty transposon vector and used along with SB100X transposase for iPSC transduction, as previously described (14). The nucleotide sequences of all constructs were confirmed by direct sequencing from both directions on an ABI 377 sequencer with ABI BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

**Cells**

Fresh human peripheral blood leukocytes from platelethapheresis were purchased from Innovative Blood Resources (St. Paul, MN). Peripheral blood mononuclear cells were further enriched using Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and NK cells were purified by negative depletion using an EasySep human NK cell kit (StemCell Technologies, Cambridge, MA), as per the manufacturer’s instructions, with >95% viability and 90–95% enrichment of CD56+CD3− lymphocytes. Viable cell counting was performed using a Countess II automated cell counter (Life Technologies Corporation, Bothell, WA). The human NK cell line NK92 and the ovarian cancer cell line SKOV-3 were obtained from ATCC (Manassas, VA) and cultured per the manufacturer’s directions. The NK92 cells required IL-2 for growth (500 IU/ml), which was obtained from R&D Systems and the National Cancer Institute, Biological Resources Branch, Preclinical Biologics Repository (Frederick, MD). Heat inactivated Gibco FBS was purchased from Thermo Fisher Scientific (Waltham, MA), and heat inactivated horse serum was purchased from Sigma-Aldrich (St. Louis, MO). For all assays described below, cells were used when in log growth phase.

The iPSCs UCBiPS7, derived from umbilical cord blood CD34+ cells, have been previously characterized and were cultured and differentiated into hematopoietic progenitor cells as described with some modifications (14, 28–31). iPSC culture and hematopoietic differentiation was performed using TeSR-E8 medium and a STEMdiff Hematopoietic Kit (StemCell Technologies), which did not require the use of mouse embryonic fibroblast feeder cells, TrypLE adaption, spin embryoid body formation, or CD34+ cell enrichment. To passage iPSCs, cells were dissociated with Gentle Cell Dissociation Reagent (StemCell Technologies) and aggregates ≥50 µm in diameter were counted with a hemocytometer and diluted to 20–40 aggregates/ml with TeSR-E8 medium. Each well of a 12-well plate was pre-coated with Matrigel Matrix (Corning Inc., Tewksbury, MA) and seeded with 40–80 aggregates in 2 ml of TeSR-E8 medium. Cell aggregates were cultured for 24 h before differentiation with the STEMdiff Hematopoietic Kit, as per the manufacturer’s instructions. At day 12 of hematopoietic progenitor cell differentiation, the percentage of hematopoietic progenitor cells was determined using flow cytometric analysis with anti-CD34, anti-CD45, and anti-CD43 mAbs. NK cell differentiation was performed as previously described (32). The iPSC-derived NK cells, referred to here as iNK cells, were expanded for examination using γ-irradiated K562-mbIL21-41BBL feeder cells at a 1:2 ratio in cell expansion medium [60% DMEM, 30% Ham’s F12, 10% human AB serum (Valley Biomedical, Winchester, VA), 20 µM 2-mercaptoethanol, 50 µM ethanolamine, 20 µg/ml ascorbic Acid, 5 ng/ml sodium selenite, 10 mM HEPES, and 100–250 IU/ml IL-2 (R&D Systems)], as described previously (14, 29–31).

**Cell Staining, IFNγ Quantification, and Flow Cytometric Analysis**

For cell staining, 0.5 × 10⁶–1 × 10⁶ cells were stained with the indicated mAbs (Table 1) using flow buffer (dPBS containing 2.5% FBS and 0.02% sodium azide) and 1 × 10⁴–5 × 10⁴ cells per sample were acquired, as previously described (11, 14, 20). For controls, fluorescence minus one was used as well as appropriate isotype-matched antibodies since the cells of interest expressed FcRs. A 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. A Zombie viability kit was used to assess live vs. dead cells, as per the manufacturer’s instructions (BioLegend).

To assess the capture of soluble trastuzumab, rituximab, cetuximab, or L-selectin/Fc chimera, transduced NK cells were incubated with 5 µg/ml of antibody for 2 h at 37°C in MEM-α basal media (Thermo Fisher Scientific) supplemented with IL-2 (200 IU/ml), HEPE (10 mM), and 2-mercaptopethanol (0.1 mM), washed with MEM-α basal media, and then stained on ice for 30 min with a 1:200 dilution of APC-conjugated F(ab)₂ donkey anti-human IgG (H+L). To detect recombinant human L-selectin/Fc binding, cells were stained with the anti-L-selectin mAb APC-conjugated Lam1-116. To compare CD16A and CD64/16A staining levels on NK92 cells, the respective transductants were stained with a saturating concentration of unconjugated anti-CD16 (3G8) or anti-CD64 (10.1) mAbs (5 µg/ml), washed extensively with dPBS containing 2% goat serum and 2 mM sodium azide, and then stained with APC-conjugated F(ab)₂ goat anti-mouse IgG (H+L). This approach was used since directly conjugated anti-CD16 and anti-CD64 mAbs can vary in their levels of fluorophore labeling. IFNγ quantification was performed by a cytometric bead-based Flex Set assay (BD Biosciences, San Jose, CA), per the manufacturer’s instructions. The minimum and maximum limit of detection for the assay was 1.8 and 2,500 pg/ml, respectively. All flow cytometric analyses were performed on an FACSCelesta instrument (BD Biosciences). Data was analyzed using FACS Diva v8 (BD Biosciences) and FlowJo v10 (Ashland, OR).

**Cell-Cell Conjugation Assay and ADCC**

NK92 cells used in these assays were transduced with pBMN-IRES-EGFP empty vector or vector containing CD64/16A or CD16A CDNA. For all transductants, nearly 100% of the cells expressed GFP. The NK92 transductants were initially serum-starved for 2 h at 37°C in MEM-α basal media (Thermo Fisher Scientific, Waltham, MA) supplemented with IL-2 (200 IU/ml),

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HEPES (10 mM), and 2-mercaptoethanol (0.1 mM). SKOV-3 cells were labeled with CellTrace Violet (Molecular Probes, Eugene, OR) per the manufacturer’s instructions, incubated with 5 μg/ml trastuzumab for 30 min and washed with MEM-α basal media. NK92 cells and SKOV-3 cells were then resuspended in the supplemented MEM-α basal media at 1 × 10^6 and 2 × 10^6/ml, respectively. For a 1:2 Effector:Target (E:T) ratio, 100 μl of each cell type was mixed together, centrifuged for 1 min at 20 × g and incubated at 37°C for the indicated time points. After each time point, the cells were gently vortexed for 3 s and immediately fixed with 1% paraformaldehyde in PBS at 4°C. Cells were immediately analyzed by flow cytometry for which 2 × 10^4 cells per sample were acquired for analyses. The percentage of conjugated NK cells was calculated by gating on GFP and CellTrace Violet double-positive events.

To evaluate ADCC, a DELFIA EuTDA-based cytotoxicity assay was used according to the manufacturer’s instructions (PerkinElmer, Waltham, MA). Briefly, target cells were labeled with Bis(acetoxymethyl)-2-2:6:2 terpyridine 6,6 dicarboxylate (BATDA) for 30 min in their culture medium, washed in culture medium, and pipetted into a 96-well non-tissue culture-treated U-bottom plates at a density of 8 × 10^3 cells/well. A tumor targeting mAb was added at the indicated concentrations and NK cells were added at the indicated E:T ratios. The plates were centrifuged at 400 × g for 1 min and then incubated for 2 h in a humidified 5% CO_2 atmosphere at 37°C. At the end of the incubation, the plates were centrifuged at 500 × g for 5 min and supernatants were transferred to a 96 well DELFIA Yellow Plate (PerkinElmer) and combined with europium. Fluorescence was measured by time-resolved fluorometry using a BMG Labtech (PerkinElmer) and combined with europium. Fluorescence was measured by time-resolved fluorometry using a BMG Labtech (PerkinElmer) and combined with europium. Fluorescence was measured by time-resolved fluorometry using a BMG Labtech. ADCC for each sample is represented as Percent Specific Release and was calculated using the following formula:

\[
\text{Percent Specific Release} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100
\]

For each experiment, measurements were conducted in triplicate using two-three replicate wells.

Statistical Analyses

Statistical analyses were performed by use of GraphPad Prism (GraphPad Software, La Jolla, CA, USA). After assessing for approximate normal distribution, all variables were summarized as mean ± SD. Comparison between 2 groups was done with Student’s t-test, with p < 0.05 taken as statistically significant.

RESULTS

Expression and Function of CD64/16A in NK92 Cells

We engineered a recombinant FcγR that consists of the extracellular region of human CD64 and the transmembrane and cytoplasmic regions of human CD16A, referred to as CD64/16A (Figure 1A). The human NK cell line NK92 stably expressing this recombinant receptor were initially used to examine its function. These cells lack endogenous FcγRs but can mediate ADCC when expressing recombinant CD16A (14, 20, 27). As shown in Figure 1B, NK92 cells expressing CD64/16A were positively stained by an anti-CD64 mAb, whereas parental NK92 cells or NK92 cells expressing CD16A were not. CD16A is known to undergo ectodomain shedding upon NK cell activation resulting in its rapid downregulation in expression (10–13, 20). CD16A as well as its isofrom CD16B on neutrophils is cleaved by ADAM17 (10), and this occurs at an extracellular region proximal to the cell membrane (13, 14). The ADAM17 cleavage region of CD16A is not present in CD64 or CD64/16A (Figure 1A). We found that CD16A underwent a >50% decrease in expression upon NK92 stimulation by ADCC, whereas CD64/16A demonstrated little to no downregulation (Figure 1C).

To establish the functional activity of CD64/16A, we determined its ability to promote E:T conjugation, induce ADCC, and stimulate IFN-γ production. SKOV-3 cells, an ovarian cancer cell line that expresses HER2, were used as the target. A two-color flow cytometric approach was used to quantify the conjugation of NK92-CD64/16A cells and SKOV-3 cells in the absence and presence of the anti-HER2 therapeutic mAb trastuzumab. A bicistronic vector was used for CD64/16A as well as GFP expression, and its fluorescence was used to identify the NK92 cells. SKOV-3 cells were labeled with the fluorescent dye CellTrace Violet. The incubation of NK92-CD64/16A cells with SKOV-3 cells alone resulted in a low level of conjugation after 60 min of exposure (Figure 2A). E:T conjugation was markedly increased in the presence of trastuzumab, and this was effectively disrupted by the presence of the anti-CD64 mAb 10.1 (Figure 2A), which blocks IgG binding (33). The addition of trastuzumab, however, did not enhance E:T conjugation by NK92 cells transduced with an empty vector expressing only GFP (NK92 control cells) (Figure 2A).

An increase in SKOV-3 cell conjugation by NK92-CD64/16A cells corresponded with increased cytolytic activity. We determined direct target cell killing by NK92-CD64/16A cells using an ADCC assay in which various concentrations of trastuzumab and E:T ratios were examined. We show in Figure 2B effective SKOV-3 cytotoxicity by NK92-CD64/16A cells in the presence of trastuzumab that decreased with lower mAb concentrations and lower E:T ratios. To confirm the role of CD64/16A in the induction of target cell killing, we also performed the assay in the presence the anti-CD64 mAb 10.1, which effectively blocked ADCC (Figure 2C).

Cytokine production is also induced during ADCC and NK cells are major producers of IFNγ (4, 34). NK92-CD64/16A cells exposed to SKOV-3 cells and trastuzumab produced considerably higher levels of IFNγ than when exposed to SKOV-3 cells alone (Figure 2D). Taken together, the above findings demonstrate that the CD64 component of the recombinant receptor engages...
tumor-bound antibody, and that the CD16A component promotes intracellular signaling leading to degranulation and cytokine production.

CD64/16A as a Docking Platform For Antibodies

CD64 is distinguished from the other FcγR members by its unique third extracellular domain, which contributes to its high affinity and stable binding to soluble monomeric IgG (26). We compared the ability of NK92 cells expressing CD64/16A or the high affinity variant of CD16A (CD16A-176V) to capture soluble therapeutic mAbs. NK92 cell transductants expressing similar levels of CD64/16A and CD16A (Figure 3A) were incubated with trastuzumab for 2h. After which, excess antibody was washed away and the cells were stained with a fluorophore-conjugated anti-human IgG antibody and then evaluated by flow cytometry. As shown in Figure 3B, NK92-CD64/16A cells captured considerably higher levels of trastuzumab than did the NK92-CD16A cells (8.1-fold increase ± 1.3, mean ± SD of 3 independent experiments). In addition, the NK92-CD64/16A cells efficiently captured the tumor-targeting mAbs Erbitux/cetuximab and Rituxan/rituximab, as well as the fusion protein L-selectin/Fc (Figure 3C). We then tested whether NK92-CD64/16A cells with a captured tumor-targeting mAb mediated ADCC. For this assay, we compared equal numbers of NK92-CD64/16A and NK92-CD16A cells that were incubated with the same concentration of soluble trastuzumab, washed, and exposed to SKOV-3 cells. We observed that target cell killing by NK92-CD64/16A cells with captured trastuzumab was significantly higher than by these same cells in the absence trastuzumab and was far superior to NK92-CD16A cells at all E:T ratios examined (Figure 3D). In contrast, SKOV-3 cytotoxicity by NK92-CD16A and NK92-CD64/16A cells was not significantly different if trastuzumab was present.
FIGURE 2 | CD64/16A promotes target cell conjugation, ADCC, and IFNγ production. (A) NK92 control cells or NK92-CD64/16A cells, both expressing GFP, and SKOV-3 cells, labeled CellTrace Violet, were mixed at an E:T ratio of 1:2 (1 × 10^5 cells to 2 × 10^5 cells) in the presence or absence of trastuzumab (5 µg/ml) and the anti-CD64 mAb 10.1 (10 µg/ml), incubated at 37°C up to 60 min, fixed, and then analyzed by flow cytometry, as described in the Materials and Methods. For the flow cytometric data, representative data are shown. For the bar graphs, mean ± SD of three independent experiments is shown. Statistical significance is indicated as ***p < 0.001. (B) NK92-CD64/16A cells were incubated with SKOV-3 cells (E:T = 20:1; 1.6 × 10^5 cells to 8 × 10^3 cells) and trastuzumab (tras.) at the indicated concentrations (left panel), or with SKOV-3 cells at the indicated E:T ratios in the presence or absence of trastuzumab (5 µg/ml) (right panel) for 2 h at 37°C. Data are represented as % specific release and the mean ± SD of 3 independent experiments is shown. Statistical significance is indicated as *p < 0.05, **p < 0.01. (C) NK92-CD64/16A cells were incubated with SKOV-3 cells (E:T = 20:1; 1.6 × 10^5 cells to 8 × 10^3 cells) in the presence or absence of trastuzumab (5 µg/ml) and the anti-CD64 mAb 10.1 (10 µg/ml), as indicated, for 2 h at 37°C. Data are represented as % specific release and the mean ± SD of 3 independent experiments is shown. Statistical significance is indicated as **p < 0.01. (D) NK92-CD64/16A cells were incubated with SKOV-3 cells (E:T = 1:1; 1 × 10^5 cells to 1 × 10^5 cells) with or without trastuzumab (5 µg/ml) for 2 h at 37°C. Secreted IFNγ levels were quantified by ELISA. Data is shown as mean of 2 independent experiments.

during the assay and not initially docked to the transductants (Figure 3E). This demonstrates that the different transductants had an equivalent cytolytic capacity. Taken together, these findings show that NK92 cells expressing CD64/16A can stably bind soluble anti-tumor mAbs and IgG fusion proteins, and that these can serve as targeting elements to kill cancer cells.

Expression and Function of CD64/16A in iPSC-Derived NK Cells

We also examined the function of CD64/16A in engineered primary NK cells. Genetically modifying peripheral blood NK cells by retroviral or lentiviral transduction at this point has been challenging (35). Embryonic stem cells and iPSCs can be differentiated into cytolytic NK cells in vitro (28–31, 36),
and these cells are highly amendable to genetic engineering (14, 30, 37, 38). Undifferentiated iPSCs were transduced to express CD64/16A using a sleeping beauty transposon plasmid for non-random gene insertion and stable expression. iPSCs were differentiated into hematopoietic cells and then iNK cells by a two-step process that we have previously described (14, 28, 29). For this study, we modified the hematopoietic differentiation method to streamline the procedure by using a commercially available media and hematopoietic differentiation kit, as described in the Materials and Methods. CD34+CD43+CD45+ cells were generated, further differentiated into iNK cells, and these cells were expanded for analysis using recombinant IL-2 and K562-mbIL21-41BBL feeder cells. CD56+CD3− is a hallmark phenotype of human NK cells, and these cells
composed the majority of our differentiated cell population (Figure 4). We also assessed the expression of a number of activating and inhibitory receptors on the iNK cells and compared this to freshly enriched, peripheral blood NK cells. The latter cells have heterogenous phenotypes among individuals and in particular for their inhibitory receptors (39–41). In contrast to the subsets of peripheral blood NK cells expressing the inhibitory KIR receptors KIR2DL2/3, KIR2DL1, and KIR3DL1, the expanded iNK cells lacked expression of these receptors and the activating receptors NKp46 and NKG2D (Figure 4). Of importance is that unlike peripheral blood NK cells, essentially all of the iNK cells were stained with an anti-CD64 mAb (Figure 4), demonstrating the expression of CD64/16A.

To assess the function of CD64/16A, we compared iNK cells derived from iPSCs transduced with either a pKT2 empty vector or pKT2-CD64/16A. The NK cell markers mentioned above were expressed at similar levels and proportions by the two iNK cell populations (data not shown), including CD16A (Figure 5A), but only the iNK-CD64/16A cells were stained by an anti-CD64 mAb (Figure 5A). Both iNK transductants demonstrated increased SKOV-3 cell killing when in the presence of trastuzumab, yet iNK-CD64/16A cells mediated significantly higher levels of ADCC than did the iNK-pKT2 control cells (Figure 5B). The anti-CD16 function blocking mAb 3G8, but not the anti-CD64 mAb 10.1, effectively inhibited ADCC by the iNK-pKT2 cells (Figure 5B). Conversely, 10.1, but not 3G8, blocked ADCC by the iNK-CD64/16A cells (Figure 5B). These findings show that the generated iNK cells were cytolytic effectors responsive to CD16A and CD64/16A engagement of antibody-bound tumor cells. We also treated iNK-CD64/16A and iNK-pKT2 cells with soluble trastuzumab, washed away excess antibody, and exposed them to SKOV-3 cells. Under these conditions, ADCC by the iNK-CD64/16A cells was markedly higher than the iNK-pKT2 cells (Figure 5C), further establishing that CD64/16A can capture soluble anti-tumor mAbs that serve as a targeting element for tumor cell killing.

**DISCUSSION**

CD16A has an exclusive role in inducing ADCC by human NK cells (2–4). The affinity of antibody binding and the expression levels of this IgG Fc receptor modulate NK cell effector functions and affect the efficacy of tumor-targeting therapeutic mAbs (4, 11, 19, 20). To enhance anti-tumor antibody binding by NK cells, we expressed a novel recombinant FcγR consisting of the extracellular region of the high affinity
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**Figure 5** | iNK-CD64/16A cells show enhanced ADCC compared to iNK-pKT2 control cells. (A) iNK cells derived from empty vector (iNK-pKT2) or CD64/16A (iINK-CD64/16A) transduced iPSCs were stained for CD56, CD64, and CD16A, as indicated. The percentage of CD56+ cells positively staining for CD16A or CD64 are indicated in the top right quadrant. (B) iNK-pKT2 or iNK-CD64/16A cells were incubated with SKOV-3 cells (E:T = 10:1, 8 x 10⁴ cells to 8 x 10⁵ cells) in the presence or absence of trastuzumab (5µg/ml), the function blocking anti-CD64 mAb 10.1 (5µg/ml), and the function blocking anti-CD16 mAb 3G8 (5µg/ml), as indicated, for 2 h at 37°C. Data is shown as mean ± SD of three independent experiments. Statistical significance is indicated as ***p < 0.0001. (C) iNK-pKT2 and iNK-CD64/16A cells were incubated in the presence or absence of trastuzumab (5µg/ml), washed, and exposed to SKOV-3 cells (E:T = 10:1, 8 x 10⁴ cells to 8 x 10⁵ cells) for 2 h at 37°C. Data is shown as mean ± SD of three independent experiments. Statistical significance is indicated as ***p < 0.0001.

**Legend:**
- **A** shows the staining of CD56, CD64, and CD16A in iNK-pKT2 and iNK-CD64/16A cells.
- **B** presents a bar graph indicating the percentage of specific release in the presence or absence of trastuzumab, anti-CD64, and anti-CD16 antibodies.
- **C** illustrates the specific release in the presence or absence of trastuzumab and iNK-pKT2.

Interesting observations from the study include:

1. **NK Cell Functionality:** NK cells expressing CD64/16A and the transmembrane and intracellular regions of CD16A. NK cells expressing CD64/16A facilitated cell conjugation with antibody-bound tumor cells, cytotoxicity, and IFNγ production, demonstrating function by both components of the recombinant FcR. CD64/16A lacks the ADAM17 cleavage region found in CD16A and it did not undergo the same level of downregulation in expression during ADCC. Moreover, consistent with the ability of CD64 to stably bind soluble monomeric IgG, NK cells expressing CD64/16A could capture soluble anti-tumor therapeutic mAbs and kill target cells.

2. **Enhanced ADCC:** We demonstrate that CD64/16A was functional in two human NK cell platforms, the NK92 cell line and primary NK cells derived from iPSCs. NK92 cells lack inhibitory KIR receptors and show high levels of natural cytotoxicity compared to other NK cell lines derived from patients (42). NK92 cells have been widely used to express modified genes to direct their cytolytic effector function, have been evaluated in preclinical studies, and are undergoing clinical testing in cancer patients (42, 43). iPSCs are also very amendable to genetic engineering and can be differentiated into NK cells expressing various receptors to direct their effector functions (14, 30, 37, 38). For this study, we streamlined and standardized the hematopoietic differentiation step using a commercial kit. Though the generated iNK cells lacked several inhibitory and activating receptors compared to peripheral blood NK cells and iNK cells in previous studies (29–31), the majority of the cells were CD16A⁺, which is expressed by mature NK cells (44), and mediated ADCC, demonstrating they were cytotoxic effector cells. The particular phenotype of the iNK cells will be important for the desired effector functions. However, to better direct and enhance their anti-tumor activity through the expression of engineered receptors and reduce their off-target effects, it may be advantageous for the iNK cells not to express endogenous inhibitory and activating receptors. Tumor cell ADCC by iNK cells expressing CD64/16A cells was significantly blocked by an anti-CD64 mAb. Interestingly, in contrast to pKT2 vector control iNK cells, ADCC by the iNK-CD64/16A cells was not blocked by an anti-CD16 mAb. Why endogenous CD16A in the iNK-CD64/16A cells did not have a role in the in vitro ADCC assay is unclear at this time. This may be due to a competitive advantage by CD64/16A over endogenous CD16A in binding antibody and/or in utilizing the same pool of downstream signaling factors.

3. **Serial Killing:** An individual NK cell can kill multiple tumor cells in different manners. This includes a process of sequential contacts and degranulations, referred to as serial killing (45, 46), and by the localized dispersion of its granule contents that kills surrounding tumor cells, referred to as bystander killing (47). Further studies are required to determine the effects of CD64/16A expression on these killing processes during ADCC. Inhibiting CD16A shedding has been reported to slow NK cell detachment from target cells and reduce serial killing by NK cells in vitro (48). Due to the CD64 component and its lack of ectodomain shedding, NK cells expressing CD64/16A could be less efficient at serial killing...
and more efficient at bystander killing. An important next step will be to assess the anti-tumor activity of NK cells expressing CD64/16A in vivo, which will include the use of NK92-CD64/16A and iNK-CD64/16A cells in tumor xenograft models.

Therapeutic mAbs have become one of the fastest growing classes of drugs, and tumor-targeting mAbs are the most widely used and characterized immunotherapy for hematologic and solid tumors (49). NK cells expressing CD64/16A have several potential advantages as a combination therapy, as their capture of anti-tumor mAbs, either individually or when mixed, prior to adoptive transfer provides diverse options for switchable targeting elements. Modifying NK cells expressing CD64/16A with an antibody would also reduce the dosage of therapeutic antibodies administered to patients. We showed that fusion proteins containing a human IgG Fc region, such as L-selectin/Fc, can also be captured by CD64/16A, which may provide further options for directing the tissue and tumor antigen targeting of engineered NK cells. Advantages of the NK92 and iNK cell platforms for adoptive cell therapies is that they can be readily gene modified on a clonal level and expanded into clinical-scalable cell numbers to produce engineered NK cells with improved effector activities as an off-the-shelf therapeutic for cancer immunotherapy (36, 37, 42, 43, 50).

AUTHOR CONTRIBUTIONS

BW and JW collected, assembled, analyzed, and interpreted the data, and wrote the manuscript. KS collected, analyzed, and interpreted the data, and revised the manuscript. RH, HM, DM, YL, and AR collected, analyzed, and interpreted the data. DK analyzed the data and revised the manuscript. All authors contributed to manuscript preparation, read, and approved the submitted version.

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