Intrinsic Functional Potential of NK-Cell Subsets Constrains Retargeting Driven by Chimeric Antigen Receptors

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Abstract

Natural killer (NK) cells hold potential as a source of allogeneic cytotoxic effector cells for chimeric antigen receptor (CAR)-mediated therapies. Here, we explored the feasibility of transfecting CAR-encoding mRNA into primary NK cells and investigated how the intrinsic potential of discrete NK-cell subsets affects retargeting efficiency. After screening five second- and third-generation anti-CD19 CAR constructs with different signaling domains and spacer regions, a third-generation CAR with the CH2-domain removed was selected based on its expression and functional profiles. Kinetics experiments revealed that CAR expression was optimal after 3 days of IL15 stimulation prior to transfection, consistently achieving over 80% expression. CAR-engineered NK cells acquired increased degranulation toward CD19+ targets, and maintained their intrinsic degranulation response toward CD19− K562 cells. The response of redirected NK-cell subsets against CD19+ targets was dependent on their intrinsic thresholds for activation determined through both differentiation and education by killer cell immunoglobulin-like receptors (KIR) and/or CD94/NKG2A binding to self HLA class I and HLA-E, respectively. Redirected primary NK cells were insensitive to inhibition through NKG2A/HLA-E interactions but remained sensitive to inhibition through KIR depending on the amount of HLA class I expressed on target cells. Adaptive NK cells, expressing NKG2C, CD57, and self-HLA–specific KIR(s), displayed superior ability to kill CD19−, HLA low, or mismatched tumor cells. These findings support the feasibility of primary allogeneic NK cells for CAR engineering and highlight a need to consider NK-cell diversity when optimizing efficacy of cancer immunotherapies based on CAR-expressing NK cells. Cancer Immunol Res; 6(4); 467–80. ©2018 AACR.

Introduction

Chimeric antigen receptor (CAR)-redirected T cells have seen success in CD19+ hematologic malignancies, leading to cure of acute lymphoid leukemia (ALL) and lymphoma of B-cell origin (1–5). The success of T-cell–based CAR strategies depends on distinct T-cell subsets (6). CAR-modified CD8+ memory stem cells provide superior antitumor responses in xenograft models compared with conventional CD8+ T cells (7). Likewise, central memory T cells can persist and repopulate functional memory niches (8, 9). Selective engineering of specific cell populations has also been tested clinically with promising results (10, 11). Engineering of defined subsets may provide more uniform outcomes and more predictable safety profiles. Other cell types have been studied for redirection (12), including natural killer (NK) cells targeting hematologic (13, 14) and solid tumor cells (15, 16). CAR-transfected NK cells showed cytotoxicity in vitro and in vivo (17). Expanded allogeneic NK cells were used with minimal reported severe adverse side effects attributed to NK cells. Furthermore, expanded NK cells and NK-cell lines transduced with anti-CD19 CAR have reached the clinic and are being tested in several phase II trials (NCT01974479, NCT02892695, and NCT03056339). Still, the potential of NK-CAR immunotherapy remains largely untapped.

Many studies have used feeder–cell–expanded NK cells or the NK-92 cell line as a template for CAR engineering (5, 18). Thus, it remains unknown how repertoire diversity within the NK-cell compartment might influence the functional potential of CAR-redirected NK cells. NK cells are controlled by the integration of...
signals from activating and inhibiting receptors; the inhibiting receptors dominate (19). Inhibitory receptors include the killer cell immunoglobulin-like receptor (KIR) and CD94/NKG2A. KIRs bind to polymorphic residues on groups of HLA class I alleles. NKG2A binds to HLA-E, an HLA class Ib molecule. Together, these receptor families establish the functional capacity of NK cells in a process termed NK-cell education (20, 21). NK cells that lack self-HLA specific (self-specific) inhibitory receptors are not eliminated but are rendered hyporesponsive and therefore remain tolerant to normal tissues. Like T cells, NK cells also undergo continuous differentiation from naïve to terminally differentiated stages (22). NK-cell differentiation is associated with a transcriptionally regulated functional maturation involving increased expression of effector molecules and a shift from cytokine responsiveness to receptor-mediated triggering of effector function (23).

Here, we studied the feasibility of CAR engineering of short-term stimulated primary NK cells. We explored whether CAR engineering is influenced by the intrinsically heterogeneous functional potential in the NK-cell repertoire, which is determined by NK-cell education and the status of NK-cell differentiation.

Materials and Methods

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Materials and Methods

Cells and culture conditions

Human K562, NALM-6, Raji 271.221 wild-type (221 wt), Bjab, ROS-50, and murine P815 cell lines were cultured in complete medium of RPMI 1640 with glutamine (Sigma) supplemented with 10% (20% for Bjab and ROS-50) heat-inactivated FBS (Sigma) and 10 ng of IL15 (Miltenyi Biotec). NK cells were maintained with Geneticin (Thermo Fisher Scientific) and 221 cells transduced to overexpress HLA-E presenting signal peptide of HLA-A (221.AEH) were maintained in Hygromycin B (2C5/F5), anti-CD3 (UHCT1), anti-CD14 (M5E2), anti-CD19 (HB19), anti-CD107a (HA43), and anti-DNAM1 (Drx11) from BD Biosciences; anti-KIR2DL1/S1 (EB6), anti-KIR2DL2/S2/L3 (GL183), anti-NKG2A (z199), and anti-CD56 (N901) from Beckman Coulter; anti-DNAM1(Tx25), anti-KIR3DL1(Dex9), anti-CD57(HCD57), and anti-IFNy(R27) from BioLegend; anti-KIR2DL1(143211), anti-KIR2DL3(180701) from R&D Systems; and anti-KIR2DL1(REA284), anti-KIR2DL1/S1(PB6), anti-NKp30(AF29-4D12), and anti-NKG2C (REA205) from Miltenyi Biotec. Streptavidin Qdot605 (Invitrogen) was used to detect biotinylated mAbs. A Live/Dead Fixable Aqua Dead Cell Stain (Invitrogen) was used to exclude dead cells from the analyses. Anti-C919 CAR was detected using a goat anti-human Immunoglobulin (Ig)G1 Fc antibody (Jackson ImmunoResearch Europe Ltd.). Anti-HLA-ABC (W6/32), from BD Biosciences, was used to detect expression of HLA class I in cell lines and PBMC. Data were acquired by FACSdiva software on BD LSRII equipped with a 488-nm laser, a 633-nm laser, and a 405-nm laser. Ultra-Comp beads (eBiosciences) were stained with each of the fluorochrome-conjugated Abs separately and used as compensation controls. Acquired data were analyzed in FlowJo 10.3 (TreeStar).

Vectors construction and in vitro mRNA synthesis

Both of the anti-C919 CAR constructs—FMC63-IgGFc-CD28-OK40-CDS3 and FMC63-CD8STK-41BB-CD3S (kindly provided by Dr. Martin Pule, University College London)—are comprised of a single-chain fragment variable domain (scFv) derived from the FMC63 mouse hybridoma. The first construct additionally comprises an IgG1-CH2CH3 spacer region and costimulatory domains from CD28 and OX40, and the second consists of CD8 spacer region and 4-1BB costimulatory domain. This CAR was originally described in ref. 24 and cloned into a retroviral SFG vector by M. Pule and colleagues. For the purpose of this study, both constructs were recloned from the retrovirus vector SFG into the mRNA expression vector pCIpA102 as previously described (26). Anti-Reverse Cap Analog (Trilink Biotechnologies Inc.) was used to cap the mRNA. The mRNA was assessed by agarose gel electrophoresis and quantified with Nanodrop (Thermo Fisher Scientific).

CAR mRNA electroporation

Previously described transfection protocol was adapted for the optimized CAR expression in NK cells (26). Cytokine stimulated primary NK cells or B cell–depleted PBMCs were washed and resuspended in serum-free SCGM medium at 10 × 10⁶ cells/mL. mRNA was mixed to a final volume of 400 µL of the cell suspension at 100 µg/mL and electroporated in a 4-mm gap cuvette at 300 V and 2 ms using a BTX 830 Square Wave Electroporator (BTX Technologies Inc.). Transfected cells were immediately transferred to pre-warmed SCGM medium supplemented with 5% human serum and 20 ng/mL of IL15 then cultured overnight (minimum 15 hours).

Antibodies and flow cytometry

The following conjugated antibodies were used: anti-Gran-<ref>yme B (2C5/F5), anti-CD3 (UHCT1), anti-CD14 (M5E2), anti-CD19 (HB19), anti-CD107a (HA43), and anti-DNAM1 (Drx11) from BD Biosciences; anti-KIR2DL1/S1 (EB6), anti-KIR2DL2/S2/L3 (GL183), anti-NKG2A (z199), and anti-CD56 (N901) from Beckman Coulter; anti-DNAM1(Tx25), anti-KIR3DL1(Dex9), anti-CD57(HCD57), and anti-IFNy(R27) from BioLegend; anti-KIR2DL1(143211), anti-KIR2DL3(180701) from R&D Systems; and anti-KIR2DL1(REA284), anti-KIR2DL1/S1(PB6), anti-NKp30(AF29-4D12), and anti-NKG2C (REA205) from Miltenyi Biotec. Streptavidin Qdot605 (Invitrogen) was used to detect biotinylated mAbs. A Live/Dead Fixable Aqua Dead Cell Stain (Invitrogen) was used to exclude dead cells from the analyses. Anti-C919 CAR was detected using a goat anti-human Immunoglobulin (Ig)G1 Fc antibody (Jackson ImmunoResearch Europe Ltd.). Anti-HLA-ABC (W6/32), from BD Biosciences, was used to detect expression of HLA class I in cell lines and PBMC. Data were acquired by FACSdiva software on BD LSRII equipped with a 488-nm laser, a 633-nm laser, and a 405-nm laser. Ultra-Comp beads (eBiosciences) were stained with each of the fluorochrome-conjugated Abs separately and used as compensation controls. Acquired data were analyzed in FlowJo 10.3 (TreeStar).

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NK-cell expansion

NK cells isolated from PBMC were cocultured with 221.AEH cell lines at NK:feeder ratio of 10:1 for 14 days in RPMI with 10% heat-inactivated FBS (Sigma) and 10 ng of IL15 (Miltenyi Biotec).

Human subjects and culture of primary NK cells

Buffco cells (Leukocytes remnants of whole blood after ultra-centrifugation to isolate red blood cells and clotting factors containing plasma) from healthy donors were purchased without identifier from blood bank (Ullevål Hospital, Oslo). Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by density gradient centrifugation (Lymphoprep; Axis-Shield, and SepMate; Stemcell Technologies). CD19–depleted PBMC or NK cells magnetically purified by negative selection (Miltenyi Biotec) were obtained from freshly isolated PBMC and cultured at 37°C in 5% CO2 in CellGro SCGM (CellGenix GmbH) supplemented with 5% human serum (Trina Biorec) and 20 ng/mL of IL15 (Miltenyi Biotec) for at least 72 hours prior to mRNA transfection.

HLA genotyping

Genomic DNA was isolated from 200 µL of buffy coat using the DNeasy blood and tissue kit (Qiagen). KIR ligands were determined using the KIR HLA ligand kit (Olerup SSP) for detecting the HLA-Bw4, -C1, and –C2 motifs.
The medium was replaced every 48 hours and feeder cells were replenished on day 7 of culture. Donors with at least 10% adaptive NK cells (NKG2C\(^+\) and CD57\(^-\)) among NK cells prior to culture were selected for the expansion to ensure retention of the subset at the end of culture. NK cells after 14 days of expansion were transfected with CAR and tested as per short-term cultured NK cells described above.

**Functional flow cytometry assays**

NK cells after expansion and electroporation procedures were seeded with 2:1 E:T ratio in U-bottom 96-well plates in RPMI full medium and incubated for 4 hours at 37\(^\circ\)C and 5% CO\(_2\). The assay was performed within 24 hours after transfection. For functional assays, anti-CD107a was added together with monensin (GolgiStop\({\text{TM}}\)) and brefeldin A (GolgiPlug\({\text{TM}}\)) at the start of the assay. At the end of the assay, cells were stained for surface markers to identify the NK-cell subsets. After surface staining, the cells were fixed and permeabilized with BD Cytofix\({\text{TM}}\) and CytoPerm\({\text{TM}}\) for intracellular staining of IFN\(\gamma\) according to the manufacturer's instructions from BD Biosciences.

**Killing assay**

Purified NK cells were stimulated with IL15 (10 ng/mL) for 3 days, electroporated with HA21 mRNA, and rested overnight in the presence of IL15. The next day, NK cells were stained with CellTrace Far Red dye-CITF (Thermo Fisher), washed, and incubated with NALM-6 in V-bottom 96-well plates at various E:T ratio in the RPMI full medium for 2 hours at 37\(^\circ\)C and 5% CO\(_2\) in the presence of FITC-DEVD-FLMK (Abcam) that allows for detection of activated caspase 3 in living cells. At the end of the assay, cells were washed and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher) for 20 minutes, washed, and resuspended in 200 \(\mu\)L of staining buffer. Cells were analyzed using BD LSR II and BD High Throughput Sampler (BD Biosciences). From every well, 100 \(\mu\)L of cell suspension was aspirated and analyzed. Cytotoxicity of NK cells was calculated based on the number of live target cells (negative for staining of active caspase 3 and dead cells) within the population of CITF\(^+\) cells.

**Microwell assay and imaging**

NK cells were stained with 0.6 \(\mu\)mol/L CellTrace Calcein Red-Orange AM and 221 target cells with 1 \(\mu\)mol/L CellTrace Calcein Green AM and 2.5 \(\mu\)mol/L CellTrace Far Red (Thermo Fisher) at 37\(^\circ\)C for 10 to 20 minutes. The stained cells were seeded onto a silicon-glass microchip divided into two separate compartments containing wells with dimensions of 50 \(\times\) 50 \(\times\) 300 \(\mu\)m\(^3\). Imaging of the microchip was performed using a Zeiss LSM 710 microscope equipped with an environmental chamber kept at 37\(^\circ\)C and 5% CO\(_2\). Images were acquired using a 10\(\times\) objective every 10 minutes for 6 hours. Excitation was provided by a 488 nm Ar-laser (0, 3%), 561 nm DPSS-561-10 laser diode (1, 0%), and 633 nm HeNe-laser (1, 1%), and emissions were collected with maximal pinhole diameter. The Zen 2011 (Zeiss) software package was used for collecting data. Image processing and analysis was conducted using a customized script in MATLAB (Simulink; ref. 27).

**CAR expression time course**

For experiments requiring CAR surface staining on NK cells in the presence of target cells, NK cells were purified, stimulated with IL15 (10 ng/mL) for 3 days, electroporated with HA21 mRNA, and rested overnight in CellGro SCGM (CellGenix GmbH) medium supplemented with 5% FBS and 10 ng/mL IL15. On the next day, CAR NK cells were seeded in the U-bottom 96-well plate either alone or together with CD19\(^+\) cells (Raji and NALM-6) or CD19\(^+\) control mouse cell line P815 at 1:1 E:T ratio in the RPMI full medium and incubated at 37\(^\circ\)C and 5% CO\(_2\). CAR staining was performed at different time points 0 to 24 hours (time 0 hour indicates the initial CAR expression on the NK cells prior to stimulation with target cells). Two additional rounds of target cells were added to NK cells (at time 4 hours and 12 hours). At each time point, NK cells were stained with anti-CAR (goat anti-human Fc antibody), anti-CD3, anti-CD14, anti-CD19 and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher) for 20 minutes, washed and resuspended in 200 \(\mu\)L of staining buffer. Cells were analyzed using BD LSR II and BD High Throughput Sampler (BD Biosciences).

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism software 6.0 and Microsoft Excel with statistical plug-in package from Real Statistics. For comparisons of matched groups, Wilcoxon matched test was used. A Mann-Whitney U test was used for comparisons of unpaired groups. A Pvalue < 0.05 was considered statistically significant. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

**Results**

Optimizing electroporation of CAR mRNA into primary human NK cells

Transfection of resting or short-term stimulated human NK cells has shown varying efficiencies (28). We used time-kinetics experiments to optimize conditions for redirection of primary NK cells (Fig. 1A). Freshly isolated NK cells were either directly transfected or stimulated with IL15 for up to 5 days before electroporation with anti-CD19 CAR mRNA. Transfection efficiency improved both in magnitude and reliability with increased length of stimulation, reaching 80% to 90% CAR\(^+\) NK cells after 3 days of prior stimulation (Fig. 1A). Therefore, in subsequent experiments, NK cells were stimulated with IL15 for 72 hours before transfection to ensure robust CAR expression.

To determine the stability of CAR expression after transfection, we labeled NK cells with cell-trace violet immediately after isolation on day 0 of culture. We transfected these cells on day 3 and followed the expression of CAR over 4 days (until day 7 of culture). CAR transfection efficiency was similar in dividing and non-dividing cells (Fig. 1B and C), reaching 80% to 90% within 24 hours after transfection. Thereafter, CAR expression declined, particularly in dividing cells. CAR expression also declined in non-dividing cells 48 hours after transfection, becoming undetectable 4 days after transfection (Fig. 1B and C). Thus, both cell proliferation rate and receptor turnover contributed to diminished CAR expression in primary NK cells 48 hours after electroporation.

The anti-CD19 CAR construct selected in this study (HA21) consists of an anti-CD19 single-chain fragment variable (scFv) fragment bearing the extracellular CH3 domain, CD28 with its transmembrane domain, and intracellular OX40 and CD3\(\varepsilon\) signaling domains (Fig. 1D; ref. 26). The construct lacked the Fc-binding CH2 domain, such that NK cells could not bind in cis through CD16. Transactivation could occur only through CD19 binding the scFv region. The activating signal is mediated via CD3\(\varepsilon\), an activating adaptor protein also used by various...
NK-activating receptors: CD16, Nkp44, and Nkp30. Almasbak and colleagues established nine variants of anti-CD19 CAR with different lengths of hinge spacers and intracellular signaling motifs for engineering of T cells (26). To investigate the effects of these structural modifications on CAR expression and transfection efficacy, HA21 was compared with a series of alternative constructs in terms of hinge-spacer length and signaling motifs shown in Fig. 1D. As expected, HA22, having only scFv without any hinge spacer (Fig. 1D), was undetectable by anti-human IgG1 Fcγ and its expression could not be evaluated by FACS. The transfection efficiency of the remaining four constructs was similarly high (Fig. 1E and F). Thus, the length of spacer and presence

Figure 1. Optimization of primary NK-cell redirection by CAR. A, Histogram showing CAR (empty) expression against background in mock (filled) transfected NK cells after various durations of cytokine (IL15) stimulation. B, Representative dot-plot overlays illustrating CAR expression (red) against background from mock (blue)-transfected NK cells over the next 4 days after transfection and different generations of division in culture. C, Summary graphs from two experiments with two donors included in each experiment (n = 4) illustrating CAR expression over 4 days after transfection and various generations of division. D, CAR constructs used in the study. E, Representative histograms showing CAR expression of different constructs. F, Summary graph from two experiments with three donors included in each experiment (n = 6), illustrating CAR expression of different constructs in primary NK cells. G, Representative diagram from 1 of 3 donors illustrating NK-cell KIR and NKG2A repertoire 24 hours after CAR transfection.
of OX40 or CD28 did not influence the expression of the CAR. Finally, the transfection itself did not alter the NK-cell repertoire, and no preferential cell losses were observed (Fig. 1G).

**Functionality of NK cells transfected with alternative CAR-encoding constructs**

Next, we evaluated functional responses of the transfected NK cells. We first tested HA21-transfected primary NK cells against various CD19$^+$ and CD19$^-$ cell lines (Fig. 2A and B). K562 and 221 are sensitive to natural cytotoxicity by NK cells due to their low HLA expression but only 221 expresses CD19. NALM-6 and Raji are a CD19$^+$ pre-B cell leukemia cell lines that express HLA-C1 and HLA-C2, which are ligands for KIR2DL2/3 and KIR2DL1, respectively. Compared with mock-transfected cells, CAR (HA21) redirected NK cells showed CD19-specific responses to both NALM-6 and 221 cells with near 2-fold increase of degranulation ($P < 0.01$). CAR-modified NK cells did not show increased degranulation against CD19$^-$ K562 cells nor a response to the murine cell
line P815 (Fig. 2B) as compared with mock-transfected cells. Thus, NK cells transfected with CAR mRNA exhibited target-specific redirection response, retaining natural cytotoxicity against class I-deficient CD19+ target cells without autoactivation.

Functional responses of cytotoxic lymphocytes depend on their ability to form immune synapses and generate an activating signal within the cell. The signaling motifs and length of the spacer on the CAR-encoding construct affect the functionality of redirected T cells (25). Therefore, we asked whether intracellular signaling motifs and length of the spacer could alter the functional response by comparing HA21 with other constructs (Fig. 2C and D). Redirected NK cells showed similar degranulation and cytokine release in response to P815 and K562 (Fig. 2C and D) as compared with mock-transfected cells. This confirmed that redirection neither modified functional response nor caused autoactivation in NK cells even with HA20 that has a full spacer that could potentially bind to CD16. However, NK cells redirected by HA21 and HA22 showed better responses to CD19+ targets 221 and NALM-6 compared with NK cells modified with other constructs, suggesting that removal of the CH2 region may increase CAR potency. There was no difference in degranulation or cytokine release between HA21- and HA22-transfected NK cells (Fig. 2C and D). Thus, removal of the CH3 domain did not confer a functional advantage in vitro. Because HA21 could be detected with anti-lg and provided consistent NK redirection, this construct was selected and validated against a second-generation CAR based on the CD8 hinge region and with 4-1BB and CD3ζ as signaling motifs (Fig. 2E and F and Supplementary Fig. S1). HA21 and 41BB/CD3ζ CAR-engineered primary NK cells showed similar functional activity in all conditions tested (Fig. 2E and F).

CAR expression kinetics experiments revealed stable CAR expression during the first 48 hours after transfection (Fig. 1B and C). However, it remained unclear whether this expression would be lost upon interaction with target cells, compromising the serial killing potential of NK cells. Therefore, we studied the CAR expression after 24 hours of incubation of NK cells with target cells. Following the first exposure to CD19+ targets, CAR expression was reduced within 2 hours to approximately 50% then at 12 hours to 30% of initial levels and remained so beyond 12 hours despite additional rounds of target-cell exposure. The decline in CAR expression within this timeframe was dependent upon presence of the CD19 antigen, because exposure of NK cells to CD19+ P815 cells had no effect on the CAR expression (Fig. 2G). Despite this downregulation of CAR expression, CAR-engineered NK cells displayed higher killing potential in FACS-based killing assays (Supplementary Fig. S2A) and contributed to a greater serial killing in a microwell readout (Supplementary Fig. S2B and Supplementary Video S1; ref. 27).

Directed NK-cell response defined by functional diversity

The functional capacity of NK cells is established through a transcriptionally regulated differentiation program and fine-tuned by inhibitory and activating receptors during NK-cell education (29). We asked whether differentiation states influenced the ability of NK cells to respond to CAR-mediated redirection. We stratified NK-cell responses based on expression of inhibitory receptors (NKG2A and KIRs) and markers of terminal differentiation (CD57 and NKG2C; refs. 29–31; Fig. 3A). Terminally differentiated NK cells express CD57 (22), display epigenetic alterations in signaling components (23, 32, 33), and are more proficient in performing antibody-dependent cellular cytotoxicity (ADCC; ref. 30).

CAR redirection with HA21 or the 41BB/CD3ζ had no effect on NK-cell function against CD19+ HLA class I-deficient K562 cells, establishing the intrinsic functional diversity of the six different NK-cell subsets (Fig. 3B and C, left). Redirected NK cells across all subsets showed increased degranulation and greater IFNγ production against CD19+ targets relative to mock-transfected NK cells (Fig. 3B and C, middle and right). The response hierarchy among the different NK-cell subsets followed a similar trend as mock transfected cells, suggesting that the intrinsic functional capacity established through NK-cell differentiation constrained the response of CAR-transfected NK cells. The least differentiated subset (KIR+ NKG2A+ CD57-) had the weakest response. Adaptive NK cells (NKG2C+ CD57+ KIR+ NKG2A+) showed the greatest response to CAR engagement such that most CAR+ cells degranulated and produced IFNγ toward CD19+ targets. Thus, the state of NK-cell differentiation determines the quality of CAR-redirec
ted NK-cell responses.

Redirected NK cells harness the functional capacity established by education

To evaluate the effect of NK-cell education on the functional response of redirected NK cells, we monitored CAR-induced responses in NK cells expressing various combinations of self- and non-self-specific inhibitory KIR (2DL3+ NK cells in C1/C1 donors and 2DL1 in C2/C2 donors) and/or NKG2A (Fig. 4A). For both mock- and CAR-transfected NK cells, subsets expressing self-specific KIR exhibited stronger degranulation responses than NK cells expressing a non-self-specific KIR against K562, 221 wt cells and NALM-6 cells. Thus, the functional imprints of NK-cell subsets achieved through education were retained despite short-term IL15 stimulation and CAR transfection (Fig. 4B). Similar results were observed when IFNγ production was assessed (Supplementary Fig. S3). Although both educated and uneducated CAR-transfected NK cells exhibited greater response to CD19+ target cells, the response was always higher in the educated self-KIR expressing subsets. Educated subsets showed a greater increase in response upon CAR transfection. Therefore, the intrinsic functional capacity of a given NK-cell subset influenced the effector response in CAR-redirected NK cells.

CAR-redirected NK cells overcome NKG2A inhibition

The net outcome of NK-cell target interactions depends on both intrinsic functional potential of the cell and the input from activating and inhibitory receptors at the immune synapse. Therefore, we next examined whether CAR redirection could overcome NKG2A inhibition and boost NKG2C stimulation in the context of HLA-CD19+ 221 AEH cells transduced to overexpress HLA-E presenting signal peptide of HLA-A (34). We stratified the functional response by gating on NKG2A and NKG2C single-positive NK cells (Fig. 5A). Mock-transfected NKG2C+ NK cells (Fig. 5B, right graph, in blue) showed greater response to targets transduced to express HLA-E compared with 221 wt cells, whereas NKG2A+ NK cells were less responsive to HLA-E+ target cells (Fig. 5B, left graph). However, upon CAR redirection, the NKG2A+ NK cells responded better to both wild-type and HLA-E-transduced CD19+ targets as compared with mock-transfected cells and lost their susceptibility to HLA-E/NKG2A-mediated inhibition (Fig. 5B, left graph, in red). CAR-redirected NKG2C+ NK cells displayed a better response when stimulated by a combination of the CD19
Figure 3.
CAR-redirected NK cells’ response to various cell lines according to their differentiation status. A, Representative gating strategy and contour plots to identify NK cells at various differentiation states and their functional response (CD107a and IFNγ). Summary graphs showing (B) NK-cell degranulation response (data from 14 experiments with two to three donors in each experiment, n = 33) and (C) IFNγ release against CD19+ HLA-I–deficient (K562) CD19+ HLA-I low (221) and CD19+ HLA-I high (NALM-6) according to selected stages of NK-cell differentiation (data from six experiments with two to three donors in each experiment, n = 14). Statistically significant difference between mock- and CAR-transfected cells (all subsets combined) as indicated in the legend.
antigen and HLA-E (Fig. 5B, right graph, 221.AEH in red) compared with either of the ligands alone (Fig. 5B, right graph, 221.wt in red and 221.AEH in blue, respectively). Thus, CAR redirection can overcome NKG2A-mediated inhibition and, to some degree, boost the response of NKG2C⁺ NK cells to HLA-E–expressing target cells. Among the donors studied thus far, only one had a preexisting adaptive NK-cell subset with high levels of NKG2C. Therefore, to test the potential additive effects of CAR-redirected stimulation and NKG2C/HLA-E interactions, we expanded adaptive NKG2C⁺ NK cells from CMV⁺ donors by coculture with 221.AEH for 2 weeks as previously described (35). Mock-transfected adaptive NK cells responded to stimulation by target cells that...
expressed HLA-E (Fig. 5C). This response was boosted by CAR redirection, yielding superior overall responses of this particular subset against HLA-E-expressing targets. The NK cells did not respond to murine P815 cell lines. Both conventional and adaptive NK cells responded similarly to NK-cell targets K562 and 221.wt according to their CD19 expression.

CAR-redirected NK cells remain sensitive to KIR inhibition
Although CAR redirection overcame NKG2A-mediated inhibition, stronger KIR-HLA inhibition might influence the response of CAR-redirected NK cells. Therefore, we tested the degranulation response of CAR-redirected single KIR, NKG2A NK cells from HLA-C heterozygous donors who express both HLA-C1 and C2 against HLA-Cw3(C1) or Cw4(C2)-transduced 221 cells (Fig. 6A and B). Despite a similar increase in degranulation of CAR-redirected single KIR+ NK cells (Figs. 4B and 6B), we found that interactions between the KIR and their cognate HLA ligand dampened the CAR-redirected NK-cell response (Fig. 6B).

These results prompted us to explore the net effects of education and ligand inhibition on CAR-redirected primary NK cells targeted against tumor cell lines expressing HLA-C at physiological levels. We tested the responses of CAR-redirected NK cells from C1/C2 donors against two CD19+ HLA-C2 homozygous cell lines, Bjab and ROS-50. Although ROS-50 cells expressed little HLA class I, expression of HLA class I in Bjab cells was similar to expression in normal B cells derived from PBMCs (Fig. 7A).
difference in HLA class I expression on the targets correlated with the degree of inhibition of mock- and CAR-redirected 2DL3 and 2DL1 single-positive NK cells (Fig. 7B). Degranulation of 2DL3+ and 2DL1+ NK cells was similar against ROS-50 cells with low HLA class I expression (Fig. 7B, right). In contrast, both mock- and CAR-transfected 2DL1+ NK cells were inhibited by Bjab cells, expressing higher levels of HLA class I (Fig. 7B, left). Thus, redirected self-KIR+ NK cells respond to their redirected target but remain sensitive to inhibition by physiological levels of cognate HLA class I.

**Discussion**

NK cells are an alternative source of allogenic effector cells for CAR engineering. Here we show the feasibility of mRNA electroporation for CAR engineering of short-term activated NK cells. By probing the functional responses of NK-cell subsets at the single-cell level, we determined the impact of subset diversity on function and specificity of CAR-engineered primary NK cells. We found that CAR signaling cooperated with differentiation status and education to regulate function of NK-cell subsets through self-MHC interactions. Thus, CAR-triggered functional responses reached a maximum in adaptive NK cells, which are both terminally differentiated and educated through expression of self-specific inhibitory KIR. Although CAR signaling overrode NKG2A-mediated inhibition, CAR-NK cells remained sensitive to inhibition by KIR binding to cognate MHC class I on target cells. Thus, therapeutic strategies based on CAR-engineered NK cells may depend on the functional diversification of human NK cells as well as the HLA genotypes of both donor and recipient.

We focused our analysis on short-term cultured NK cells transfected with mRNA. In a therapeutic setting, such CAR-NK cells will likely only provide antitumor effects for a short time period, limited by transient expression of the construct and rejection by the host immune system (18). Indeed, most CAR-redirected cells lost their CAR expression within 72 hours after transfection or sooner following interaction with target cells.

Thus, the effector response of mRNA-transfected NK cells is of limited durability. However, at the same time, toxicity can be managed due to the kinetics of NK-cell turnover and CAR expression without the need to control their persistence beyond their usual lifespan by means of inserting suicide genes. The mRNA-transduction strategy may offer a safer and faster way for clinical testing of CARs against new targets. Transiently expressed CARs may also be useful in settings where a limited treatment period is desirable, such as for avoiding long-term toxicity or eliminating residual disease before bone marrow transplant. Repeated injection of mRNA CAR electroporated T cells mediated regression of large vascularized flank mesothelioma tumors in a preclinical model based on transfer into NOD/scid/γc−/− mice (36).

Current strategies that connect one donor to one product may be useful for early application of new CAR constructs for new
clinical indications. However, full-scale clinical implementation of CAR therapy will most likely require off-the-shelf cellular products that are transferable across institutions. Our results showing the diversity of outcomes for different subsets and receptor signaling should inform development of master cell banks of NK cells for CAR engineering. Both transient mRNA transfection and stable expression systems such as lentiviruses and transposons will be useful (13, 37–39). Off-the-shelf CAR therapy will require use of third party allogeneic effector cells. However, transposons will be useful (13, 37–39). Off-the-shelf CAR therapy carries the risk of off-target toxicity caused by the endogenous TCR. Although strategies to knock down the TCR in T cells exist, NK cells may be a superior source of allogeneic cells for CAR therapy. In fact, transfer of NK cells across HLA barriers has shown limited toxicity and may even contribute to the antitumor response (40).

Our data suggest that off-the-shelf NK-cell products could be generated by selectively expanding or reprogramming adaptive NK cells from either NK-cell precursors or inducible pluripotent stem cells (iPSC), then further enhancing function with CAR engineering (41).

With education, NK cells achieve functional capacity through interactions between their own inhibitory receptors and conserved residues on MHC class I expressed by cells of both stromal and hematopoietic origin (29). The molecular mechanism behind NK-cell education likely operates by lowering the threshold for activation through activating surface receptors. We set out to explore the impact of education on the potency of CAR-engineered NK cells. Like ADCC, CAR engineering led to responses in uneducated NK cells expressing non-self-specific KIR as their only inhibitory receptor. However, educated NK cells remained superior in all functional readouts tested, suggesting that CAR signaling taps into the intrinsic potential of the cell.

We also noticed that more differentiated NK cells, especially terminally differentiated adaptive NK cells, responded better to CAR stimulation. NKG2C+/CD57+ adaptive NK cells typically express self-specific KIR and are therefore both educated and terminally differentiated (35). Adaptive NK cells are an attractive NK-cell subset for cancer immunotherapy due to robust cytotoxicity, cytokine release, and potential for long-term persistence (42). We found that CAR-engineered adaptive NK cells responded better than all other subsets, demonstrating that differentiation-driven functional potential serves as a good basis for CAR signaling.

Adaptive NK cells undergo an epigenetic reprogramming associated with a specific reconfiguration of adaptor molecules including Syk, Eaat-2, and FcRγ (32). Genetic engineering of CAR signaling motifs may serve to restore these activating molecules’ expression to improve cytotoxicity and cytokine release responses of adaptive NK cells against its redirected target. FcRγ and CD3ζ used in this study are adaptor proteins for NK-cell–activating receptors CD16, NKp46, and Nkp30. Creating a CAR with both activating motifs, DAP12, an adaptor for NKG2C and activating KIRs, may be superior to CD3ζ at inducing NK cells to respond (44) and can be applied to further increase cytotoxicity of NK cells. HA27, without the OX40 motif, did not exhibit a similar functional response as HA21 despite the presence of known activating motifs in NK cells, including CD28 and CD3ζ. Therefore, OX40 may

**Figure 7.**

NK cells exhibit KIR-mediated inhibition to cell line expressing normal amounts of HLA-A, A, Histogram illustrating HLA-ABC expression from Bjab (center) and ROS-50 (right) against normal B cells (left). B, Graphs illustrating mock- or CAR-transfected, single KIR expressing CAR-redirected 2DL3 (C1, left) or 2DL1 (C2, right) NK cells’ response to Bjab and ROS-50 cell lines. Donors were all HLA-C1C2 heterozygous and the target are both HLA-C2 homozygous. Data are aggregated from three experiments with two to three donors in each experiment (n = 7).
facilitate activation of NK cells either as a spacer between the two activating motifs or by interacting with intracellular proteins to induce a signaling cascade. Validating the finding that CAR signaling taps into the intrinsic functional potential of a given NK-cell subset, we observed identical response hierarchies using a second-generation T-cell CAR, based on 4-1BB and CD3ζ.

Next, we determined whether CAR-mediated activation modulates the NK cells’ response to its target and in synergy with other activating receptors. We found that CAR redirection increased the degradation response of NKG2C+ NK cells against HLA-E-expressing targets. Thus, CAR signaling moieties not only override inhibitory signaling through NKG2A, they also boost the response of NKG2C+ adaptive NK cells. The additive response of NKG2C+ NK cells with CAR redirection support their utility against tumors that overexpress HLA-E (45).

Although CAR-redirected adaptive NK cells responded to CAR targets, they also respond to KIR inhibition. Inhibitory KIR induces inhibitory signals through multiple tyrosine-based inhibition motifs (ITIM) to achieve tolerance and prevent cytotoxicity against normal cells (19). Similarly, CD94/NKG2A binding to HLA-E also signals via ITIMs. Although CAR redirection overcame NKG2A inhibition, KIR-mediated inhibition was observed in assays with 221 HLA-C1/2 transfecants expressing supraphysiologic levels of HLA-C. Assays with tumor cell lines confirmed that KIR-mediated inhibition also influenced the recognition of targets with physiologic levels of HLA class I. Therefore, although CAR redirection lowered the threshold for NK-cell responses, the function of CAR-engineered NK cells can be negatively regulated by inhibitory signals. Nevertheless, the fact that the threshold for CAR NK-cell inhibition was dependent on the level of HLA-C, and even overcome in the case of NKG2A/HLA-E interactions, suggests that alternative CAR designs may be less sensitive to self-recognition and may pave the way for autologous NK-CAR strategies.

The finding that KIR inhibition under some circumstances dewregulated the function of CAR-engineered NK cells suggests that KIR signaling may be one tool to dampen CAR-driven off-target toxicity under specific circumstances. One possibility is that CAR-rediret KIR matched NK cells to treat tumors with downregulated HLA class I, where KIR can be used to attenuate CAR-NK cells against normal tissues. Conversely, one can envisage combinations of anti-KIR checkpoint therapy similar to what has been proposed for PD-1–mediated inhibition of CAR-T-cell therapy (46). In fact, a large fraction of mature differentiated T cells express KIRs (47). Thus, KIR inhibition may also be relevant in the context of CAR-T-cell therapy.

CAR redirection enhanced the functionality of hypo-responsive KIR-‘NKGA2A’ NK cells. Although the response was inferior to that of adaptive NK cells, the lack of inhibitory receptors may make this subset superior in autologous settings or in HLA-matched conditions where tumor cells express high levels of HLA class I. Given that NKG2A inhibition was overridden by CAR transfection, another attractive subset for CAR engineering may be NKG2A+KIR- NK cells that have greater intrinsic functional potential compared with KIR+ NKG2A+ NK cells. In fact, there are data suggesting that this NK-cell subset provides clinical benefit on its own in the context of stem cell transplantation (48). However, uneducated NK cells can still be inhibited via their KIR (49). Thus, the use of uneducated NK cells for CAR therapy across HLA barriers may lead to a functional limitation because of lack of education and KIR-mediated inhibition (if the tumor expresses ligands for the non-educating KIR).

Taken together, our study shows that CAR engineering of primary NK cells is feasible. We found that CAR signaling can overcome the hyporesponsiveness of uneducated NK cells and mediate killing of target cells. However, educated NK cells still responded more strongly than uneducated NK cells, revealing that the signaling motifs of the CAR construct tap into the intrinsic potential of the NK-cell subset. Redirected CAR-NK cells remain sensitive to KIR inhibition, suggesting that further optimization for CAR-NK therapy may follow from consideration of donor and recipient HLA genotypes. If our in vitro findings can be verified in vivo, CAR-NK cells may become an alternative to CAR T-cell strategies, particularly in allogeneic settings.

Disclosure of Potential Conflicts of Interest
R. Zagodzdon is an external expert/consultant at Helix Immuno-Oncology. S.A.J. Olweus reports receiving a commercial research grant from Kite Pharma and in a consultant/advisory board member for Oxielia—Institutia. K.-J. Malmberg is a Visiting Professor at Karolinska Institutet, reports receiving a commercial research grant from Fate Therapeutics, and is a consultant/advisory board member for Fate Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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References
Intrinsic Functionality of NK Cells Affects CAR Retargeting


# Intrinsic Functional Potential of NK-Cell Subsets Constrains Retargeting Driven by Chimeric Antigen Receptors

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