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Manufacturing

Differentiation of natural killer cells from induced pluripotent stem cells under defined, serum- and feeder-free conditions

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ABSTRACT

Background aims: Traditionally, natural killer (NK) cells are sourced from the peripheral blood of donors—a laborious and highly donor-specific process. Processes for generating NK cells from induced pluripotent stem cells (iPSCs) have demonstrated that it is possible to successfully generate renewable alloreactive NK cells that are not only functional *in vivo* but can also be genetically engineered for enhanced function. However, poor standardization and cumbersome differentiation procedures suggest that further improvements in the control of the differentiation process are necessary.
Methods: Here the authors evaluated the potential of differentiating NK cells from centrally authenticated iPSCs under entirely chemically defined and serum-free conditions as well as their immunotherapeutic potential, after expansion in feeder-free media, against solid tumors targets. To address limitations of current differentiation approaches, the authors did not utilize feeder or stromal cell layers, TrypLE adaptation or peripheral blood during the differentiation process. The authors also evaluated the feasibility of utilizing centrally authenticated iPSC lines, thus circumventing protocol- and donor-induced variability associated with reprogramming approaches, and characterized these iPSC-NK cells in terms of cytotoxicity, cytokine production and degranulation potential against solid tumor cell lines and patient-derived targets.
Results: Differentiation of iPSCs generated NK cells that were predominantly $CD56^+/CD16^+/CD3^-$ and expressed NK activation markers NKG2D, NKp30, NKp44, NKp46 and DNAM-1. These iPSC-NK cells mediated effector functions, including cytotoxicity, degranulation and IFN-γ production, in response to solid tumor targets, including patient-derived cancer cells, and could be cryopreserved and expanded in culture.
Conclusions: The ability to produce NK cells under defined conditions and the functional responses elicited by these iPSC-NK cells suggest that they could represent promising effectors in clinical adoptive transfer settings as a renewable source of donor-independent NK cells for immunotherapy of solid tumors.

Introduction

Though the use of natural killer (NK) cells in cancer immunotherapy against blood cancers and, to a lesser extent, solid tumors is well documented [1], challenges associated with efficient sourcing of these cells persist [2]. The alloreactivity and graft-versus-tumor effects of NK cells benefit from killer cell immunoglobulin-like receptor (KIR) ligand recipient mismatch [3–6], enabling the allogeneic use of these cells in transplant and non-transplant settings [7]. Despite this and their limited induction of graft-versus-host disease compared with T cells, extensive donor identification and selection is required when sourcing NK cells. Peripheral blood is the most common source of NK cells for clinical use. However, this source has a comparatively smaller proportion of mature NK cells when drawn from pathological settings [8–10]. Such patient-derived cells also display functional impairment, further compromising their therapeutic potential [11–14]. Additionally, obtaining high numbers of pure NK cells from leukapheresis, free from residual T and B cells, is often laborious and requires extensive manufacturing, potentially affecting the resultant immunotherapy product.

Blood-derived NK cells present other challenges for use in adoptive cell-based immunotherapy due to their difficulty in being isolated and growing *ex vivo* as well as their limited transduction efficiency. Not only does peripheral blood contain only

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approximately 10% NK cells, but expansion protocols used to obtain large numbers of NK cells from peripheral blood are also varied, often relying on the use of irradiated feeder cells, and have highly diverse expansion yields. Furthermore, despite advances in the development of techniques to genetically modify these cells [15], NK cells remain highly resistant to exogenous gene uptake, thus limiting the efficiency in generating gene-modified NK cells by transducing, for instance, chimeric antigen receptors (CARs).

NK cells are present in larger amounts (30%) in umbilical cord blood, but these cells are phenotypically different from pNK cells and tend to show impaired maturation. NK cells lines, such as NK-92 and NKG, have also been developed in response to some of the challenges associated with sourcing and manipulating blood-derived NK cells. Although some of these lines represent attractive allogeneic innate effectors, they are also phenotypically and functionally different from blood-derived NK cells. For instance, NK-92 cells lack CD16 and are thus unable to participate in antibody-mediated cellular cytotoxicity. This difference has prompted the development of CD16-expressing variants of this cell line [16].

As an alternative source of highly pure NK cells, induced pluripotent stem cells (iPSCs) have been demonstrated to be able to be differentiated into functionally mature NK cells [17–20]. Because of the relative ease of genetically engineering iPSCs compared with NK cells isolated from peripheral or umbilical cord blood, this approach may represent a promising strategy for generating renewable genetically modified NK cells for use in immunotherapy of solid tumors. NK cells derived from CAR-engineered iPSCs are able to efficiently express CAR genes after differentiation and target and kill more refractory tumors [21]. Despite these remarkable advances, improvements in the manufacturing of NK cells from iPSCs are still needed, as iPSC-NK cells are not yet able to fully replace blood-derived NK cells. For instance, the described protocols established for generating NK cells from pluripotent stem cells utilize feeder or stromal cell layers to adapt iPSCs or culture hematopoietic progenitor cells prior to differentiation and take months to complete and require human or bovine sera for differentiation. Moreover, these approaches largely rely on individualized reprogramming protocols to obtain iPSCs, which inevitably introduce process- and donor-specific variability [18,19,22]. In addition, limited characterization has been carried out to evaluate the potential of these cells in the treatment of solid tumors.

To address these shortcomings and better describe the potential of iPSC-NK cells in immunotherapy, the authors sought to evaluate the feasibility of generating NK cells without feeder or stromal cells or sera and with chemically defined components and test their ability to kill solid tumor targets. Moreover, the authors demonstrated the feasibility of generating NK cells from iPSCs that have been publicly authenticated. These iPSCs represent standardized, highly qualified source materials that enable feeder-independent differentiation into NK cells. NK cells generated with this defined, feeder-independent approach display functional competency, can be differentiated reliably and possess potent anti-tumor effector functions that include degranulation and cytokine production in response to solid tumor targets. Collectively, these features render these cells attractive as a new, renewable source of NK cells for use in adoptive transfer immunotherapy of cancer and represent a step toward standardized and defined manufacturing of off-the-shelf NK cells.

Methods

Reagents and cells

The iPSC lines KOLF2 and hiPSC01 were obtained from the Purdue Institute for Integrative Neuroscience Cell Engineering core and cultured on plates coated with Matrigel (354277; Corning, Corning, NY, USA) in mTesR 1 feeder-free maintenance medium (85850; STEMCELL Technologies, Vancouver, Canada) until passage 20 before being discarded. The iPSC lines used in this experiment showed normal karyotypes and were negative for mycoplasma (WiCell, Madison, WI, USA). A short tandem repeat analysis was used for the authentication of the cell lines (WiCell). Lung-adherent epithelial carcinoma A549 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) (ATCC CCL-185). A549 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (11995065; Gibco, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (35-011-CV; Corning) and 1% penicillin/streptomycin (15140122; Gibco) and maintained until passage 20 before being discarded. Patient-derived recurrent glioblastoma (GBM) cells (GBM10) were obtained from Dr Karen Pollok at the Indiana University School of Medicine. GBM10 cell lines were cultured in DMEM (11965092; Gibco) with 10% FBS and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (15630106; Gibco) and maintained until passage 10 before being discarded. Prostate-adherent epithelial adenocarcinoma PC3 cells were obtained from ATCC (ATCC CRL-1435). PC3 cell lines were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin and maintained until passage 20 before being discarded. Chronic myelogenous leukemia K562 cells were obtained from ATCC (ATCC CLL-243). K562 cells were cultured in Iscove's Modified Dulbecco's Medium (12440053; Gibco) with 10% FBS and 1% penicillin/streptomycin and maintained until passage 20 before being discarded. Recombinant human (rh) IL-15 (1110-15) and rhIL-21 (1110-21) were obtained from GoldBio (St Louis, MO, USA), and rhIL-2 (AK8223) was obtained from Akron Biotech (Boca Raton, FL, USA). In addition, rh stem cell factor (SCF) (300-07), rh bone morphogenetic protein 4 (120-05), rh vascular endothelial growth factor (100-20B), rhIL-3 (200-03), rhIL-7 (200-07) and rhFlt3-L (300-19) were obtained from PeproTech (Rocky Hill, NJ, USA). STEMdiff APEL 2 (05270) differentiation medium was obtained from STEMCELL Technologies. Y-27632 rho-associated coiled-coil kinase (ROCK) inhibitor (1254) was obtained from Tocris Bioscience (Bristol, UK).

Human samples and isolation of NK cells from peripheral blood

Blood samples were obtained from healthy adult donors. Natural killer cells were isolated from peripheral blood by negative selection using the EasySep direct human NK cell isolation kit (STEMCELL Technologies). The cells were expanded at 37°C and 5% carbon dioxide (Co_2) for 3–4 weeks using CTS OpTmizer T-cell expansion medium (A1048501; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% human AB (hAB) serum (35-060-CI; Corning), 1% penicillin/streptomycin, 0.2 mM L-glutamine (25030081; Gibco), 10 ng/mL rhIL-15, 500 IU/mL rhIL-2 and 25 ng/mL rhIL-21. The expanded NK cells were >95% $CD3^-/CD56^+$ when assessed by flow cytometry.

Ethics statement

Written informed consent for the primary NK cells used in this study was obtained from all subjects involved in the study. All procedures performed in studies involving human participants were approved by Purdue University's Institutional Review Board (protocol #1804020540), and all institutional safety and biosecurity procedures were adhered to.

Human iPSC generation, culture and differentiation into hematopoietic cells

The KOLF2 iPSCs were generated by the Sanger Institute's Human Induced Pluripotent Stem Cell Initiative (HipSci) project. Briefly, HipSci used the CytoTune 1 Sendai method (Thermo Fisher Scientific) to reprogram dermal fibroblasts obtained from healthy adult volunteers into iPSCs. The hiPSC01 iPSC line was generated by the authors at the Purdue Institute for Integrative Neuroscience Cell Engineering

core facility. Healthy male dermal fibroblasts (ReproCELL USA, Beltsville, MD, USA) were reprogrammed using the StemRNA third-generation reprogramming kit (ReproCELL USA) according to the manufacturer's protocol. The short tandem repeat analysis for the iPSC lines is shown in supplementary Table 7.

Prior to differentiation, iPSC lines KOLF2 and hiPSC01 were thawed and cultured in mTesR 1 feeder-free maintenance medium for 4–5 days on human embryonic stem cell (hESC)-qualified, Matrigel-coated six-well plates to reach 80% confluency. The iPSC lines were passaged using 0.5 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS) (01-862-1B; Biological Industries, Beit HaEmek, Israel). Prior to passaging, hematopoietic differentiation medium (HPDM) was prepared, consisting of STEMdiff APEL 2, 40 ng/mL SCF, 20 ng/mL bone morphogenetic protein 4 and 20 ng/mL vascular endothelial growth factor, which was supplemented with 10 μM ROCK inhibitor for the first 3 days. The iPSCs were passaged and seeded in 100 μL of HPDM supplemented with ROCK inhibitor in each well of an ultra-low attachment, round-bottom 96-well plate (CLS3474; Corning) at a density of 3000 cells/well. Cells were centrifuged for 5 min at 220 g to facilitate formation of embryoid body (EB) structures and were incubated, undisturbed, for 3 days at 37°C and 5% Co_2. On day 3, day 6 and day 9, media changes were performed by removing 70 uL of medium from each well and adding 100 uL of freshly prepared HPDM without ROCK inhibitor. On day 11, hematopoietic progenitor cells were either collected for flow cytometric analysis or transferred to NK cell differentiation cultures using a wide-bore P200 pipette (14-222-730; Thermo Fisher Scientific).

Hematopoietic cell differentiation into NK cells

In the second phase of NK cell differentiation from iPSCs, hematopoietic progenitor cells were seeded in a standard cell culture-treated six-well plate at a concentration of 32 EBs/well in 4 mL NK cell differentiation medium, consisting of STEMdiff APEL 2, 20 ng/mL SCF, 20 ng/mL IL-7, 10 ng/mL IL-15 and 10 ng/mL Flt3L, which was supplemented with 5 ng/mL IL-3. Half of the medium was replaced twice per week for 4 weeks with freshly prepared NK cell differentiation medium containing IL-3 for the first week only, with no IL-3 for the following 3 weeks. After 4 weeks of NK cell differentiation culture, cells were collected and either analyzed phenotypically via flow cytometry or expanded for 3–4 weeks in CTS OpTmizer T-cell expansion medium supplemented with 5% hAB serum, 1% penicillin/streptomycin, 0.2 mM L-glutamine, 10 ng/mL rhIL-15, 500 IU/mL rhIL-2 and 25 ng/mL rhIL-21 prior to cytotoxicity and functionality assays.

To demonstrate the expansion potential of the authors' iPSC-derived NK cells, hiPSC01-derived NK (hiPSC01-NK) cells and peripheral blood-derived NK (pNK) cells were expanded for 2 weeks in a 24-well G-Rex plate (80192M; Wilson Wolf, Saint Paul, MN, USA) in complete NK MACS medium (130-114-429; Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 5% hAB serum, 1% penicillin/streptomycin and 500 IU/mL rhIL-2.

Cryopreservation of partially or fully differentiated NK cells

During phase two of differentiation, partially or fully differentiated NK cells were cryopreserved in CryoStor CS10 cryopreservation medium (07930; STEMCELL Technologies) at a concentration of 1×10^6 cells/mL at day 25, day 32 or day 39 of differentiation. NK cells were kept frozen in liquid nitrogen for 3 days and then thawed in a water bath at 37°C. Thawed NK cells were seeded in NK differentiation medium at a concentration of approximately 2.5×10^5 cells/mL and allowed to differentiate for the remainder of the 39 days. Following full differentiation, cells were phenotypically analyzed via flow cytometry and compared with unfrozen iPSC-derived NK cells. The pNK cells were freshly isolated as described earlier and not frozen or thawed.

Phenotypic analysis and antibodies

Phenotypic analysis was performed using flow cytometry. After 11 days of hematopoietic progenitor differentiation followed by 4 weeks of NK cell differentiation, cells were collected and stained for expression of NK cell surface receptors. Phenotypic characterization was also carried out on iPSC-NK cells following expansion in CTS OpTmizer expansion medium supplemented with 5% hAB serum, 1% penicillin/streptomycin, 0.2 mM L-glutamine, 10 ng/mL rhIL-15, 500 IU/mL rhIL-2 and 25 ng/mL rhIL-21. To prepare cells for flow cytometric analysis, cells were washed with fluorescence-activated cell sorting buffer (1× PBS, 5% FBS) and then stained for 30 min at 4°C with select antibodies: CD56 (Pe-Cy5.5, clone CMSSB, 35-0567-42 and allophycocyanin [APC], clone CMSSB, 17-0567-42; Thermo Fisher Scientific), CD3 (Pe-Cy7, clone UCHT1, 300419; BioLegend, San Diego, CA, USA), CD16 (APC, clone B73.1, 360705; BioLegend), NKp30 (BV711, clone p30-15, 563383; BD Biosciences, Franklin Lakes, NJ, USA), NKp44 (phycoerythrin [PE], clone p44-8, 325107; BioLegend), NKp46 (APC, clone 9E2, 331917; BioLegend), NKG2D (BV711, clone 1D11, 563688; BD Biosciences), NKG2A (fluorescein isothiocyanate [FITC], clone REA110, 130-113-565; Miltenyi Biotec), DNAM (PE, clone DX11, 559789 and PE, clone DX11, 564796; BD Biosciences), CD19 (APC, clone HIB19, 302211; BioLegend), CD94 (PE, clone DX22, 305506; BioLegend), CD158b (PE, clone DX27, 312605; BioLegend), CD158a/h (FITC, clone HP-MA4, 339503; BioLegend), CD158e1 (FITC, clone DX9, 312705; BioLegend), CD161 (BV510, clone HP-3G10, 339921; BioLegend), CD34 (Pe-Cy5, clone 581, 555823; BD Biosciences), CD43 (FITC, clone MEM-59, 315203; BioLegend), CD45 (APC-Cy7, clone 2D1, 368515; BioLegend), HLA-ABC (PE, clone W6/32, 311405; BioLegend). Sytox Green dead cell stain (S34860; Thermo Fisher Scientific) was used to determine cell viability. In all experiments, analysis was performed by gating NK cells as $CD56^+/CD3^-$populations. Gating was performed using fluorescence minus one controls, and analysis was performed using the BD LSRFortessa (BD Biosciences) (see supplementary Figure 13). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). A representative gating strategy for CD56/CD3 cells is shown in supplementary Figure 13.

Cytotoxicity assays against tumor targets

Primary and iPSC-derived (hiPSC01 and KOLF2) NK cells were collected and expanded for 3–4 weeks using CTS OpTmizer T-cell expansion medium supplemented with 5% hAB serum, 1% penicillin/streptomycin, 0.2 mM L-glutamine, 10 ng/mL rhIL-15, 500 IU/mL rhIL-2 and 25 ng/mL rhIL-21 prior to the addition of A549 or PC3 target cells. A549, PC3, GBM10 and K562 target cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) for 20 min at 37°C as described by the 7-aminoactinomycin D/CFSE cell-mediated cytotoxicity assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) to distinguish target (A549, PC3, GBM10 and K562) and effector (pNK and iPSC-NK) cells. Target cells, labeled with CFSE, were incubated for 4–6 h at 37°C and 5% Co_2prior to the addition of effector cells to allow target cells to adhere to the cell culture plate or 1 h for K562 cells. Effector cells (pNK and iPSC-NK) were incubated with target cells (A549, PC3, GBM10 or K562) at effector:target (E:T) ratios of 10:1, 5:1 and 2.5:1 for 4 h in a 24-well plate. Medium was removed before the addition of effector cells, except for K562 cells, where pNK and iPSC-NK cells were added directly to the culture. The pNK and iPSC-NK cells were kept at a density of 5×10^5 cells/mL, whereas target cells were kept at densities of 5×10^4 cells/mL, 2.5×10^4 cells/mL and 1×10^4 cells/mL, corresponding to each E:T ratio. Control wells containing just A549, PC3, GBM10 or K562 cells were cultured for each test. After co-culture for 4 h at 37°C and 5% Co_2, the supernatant containing effector cells was collected, and the target cells were washed with 1× PBS, trypsinized and added to the effector cell supernatant. Cells were stained with 7-aminoactinomycin D (600120;

Cayman Chemical Company) for 15 min at 4°C, and cells were analyzed on the BD LSRFortessa. Data were analyzed using FlowJo software.

Degranulation and IFN-γ production

PC3 target cancer cells were incubated for 6 h in 24-well plates at 37°C and 5% Co_2prior to the addition of effector cells to allow target cells to adhere to the cell culture plate. Effector cells were incubated with PC3 cells for 4 h at E:T ratios of 10:1, 5:1 and 2.5:1. The pNK and iPSC-NK cells, expanded and activated in culture media as previously described, were kept at a density of 5×10^5 cells/mL, whereas target cells were kept at densities of 5×10^4 cells/mL, 2.5×10^4 cells/mL and 1×10^4 cells/mL, corresponding to each E:T ratio. For IFN-γ assays, at the beginning of the co-culture, GolgiPlug (555029; BD Biosciences) was added to measure IFN-γ production. After co-culture for 4 h at 37°C and 5% Co_2, the supernatant containing effector cells was collected, and the target cells were washed with 1× PBS, trypsinized and added to the effector cell supernatant. Cells were stained for 30 min at 4°C for CD56 (APC, clone CMSSB) and CD3 (Pe-Cy7, clone UCHT1) and subsequently fixed using the Cytofix/Cytoperm kit (554714; BD Biosciences) for 20 min at 4°C. Cells were then stained for IFN-γ antibody (PerCP-Cy5.5, clone B27, 560704; BD Biosciences) for 30 min at 4°C and washed with Perm/Wash buffer (554723; BD Biosciences). For degranulation assays, cells were allowed to incubate at 37°C for 6 h prior to the addition of effector cells. CD107a antibody (PE, clone H4A3, 328607; BioLegend) was added at the beginning of co-culture. After 1 h of incubation, GolgiStop (554724; BD Biosciences) was added to detect NK cell degranulation. After an additional 3 h of incubation, cells were collected and washed. Cells were stained for 30 min at 4°C for CD56 (APC, clone CMSSB) and CD3 (Pe-Cy7, clone UCHT1) and analyzed via flow cytometry. Cells for both assays were collected and analyzed on a BD LSRFortessa. Data were analyzed using FlowJo software. Unstimulated samples (expanded NK cells in the absence of cancer cells) were used as controls and subtracted from stimulated samples (expanded NK cells in the presence of cancer cells). Basal degranulation levels are shown in supplementary Table 6. A representative gating strategy is shown in supplementary Figures 13, 14.

Data analysis

Prism 8 (GraphPad Software, San Diego, CA, USA) was used for all data and statistical analysis, with a $P < 0.05$ considered to be significant. Data are presented as mean ± standard error of the mean and represent experiments carried out in at least triplicate. Unpaired or paired *t*-tests were used for single data comparisons of independent groups.

Results

Differentiation of hematopoietic progenitor cells from iPSCs

In an effort to generate hematopoietic progenitor cells in a defined manner, without the use of mouse embryonic feeder cells, undifferentiated feeder-independent iPSCs were supported throughout differentiation into hematopoietic progenitor cells by the generation of spin EBs (Figure 1A) [23,24]. In brief, iPSCs (hiPSC01 and KOLF2) were spin-aggregated in round-bottom 96-well plates at a defined density of 3000 cells/well. EBs were dissociated into a single cell population in 5 mM ethylenediaminetetraacetic acid for 10 min to perform flow cytometric analysis. $CD34^+$ marker expression was significantly higher for KOLF2 iPSCs seeded at a density of 3000 cells/well compared with 6000 cells/well, but no difference was observed for hiPSC01 iPSCs between the two seeding densities (see supplementary Figure 1A). However, in the case of both cell lines, cultures seeded with either fewer or more than 3000 cells generated EBs with abnormal morphology, resulting in lower yield of hematopoietic progenitor cells following 11 days of differentiation (see supplementary Figure 1B). After 3 days of hematopoietic cell differentiation, these cultures formed EB structures and for the following 8 days demonstrated hematopoietic progenitor cell development and growth (Figure 1B). EB cultures were considered healthy if they maintained a round morphology and exhibited continued growth throughout the 11 days of differentiation and subsequently produced higher yields of NK cells following hematopoietic differentiation and NK cell maturation. For both hiPSC01 and KOLF2 iPSC lines, the majority of cells generated following 11 days of hematopoietic progenitor differentiation expressed CD34 (hiPSC01, 60.40 ± 14.68%, KOLF2, 57.03 ± 13.61%), with many cells also expressing CD43 (hiPSC01, 6.92 ± 1.83%, KOLF2, 4.97 ± 2.57%) and CD45 (hiPSC01, 10.46 ± 2.57%, KOLF2, 8.12 ± 3.50%), both of which are characteristic maturation markers of hematopoietic progenitor cells (Figure 1C; also see supplementary Table 1, Figure 2). Co-expression of these receptors is in line with percentages reported in similar studies [18,25].

Generation of mature NK cells from iPSC-derived hematopoietic progenitor cells

After spin EB formation and 11 days of hematopoietic progenitor differentiation, spin EBs were transferred without dissociation or cell sorting to NK cell differentiation medium containing cytokines (IL-3, IL-7, IL-15, SCF and Flt3L) promoting mature NK cell development (defined as alloreactive $CD56^+/CD3^-$). This differentiation protocol yielded phenotypically mature cells at a purity of 31.68 ± 8.90% $CD56^+/CD3^-$ NK cells from hiPSC01 cells and 17.06 ± 6.70% NK cells from KOLF2 cells (Figure 2A). NK cell purity following differentiation varied between cultures to no larger extent than that reported by other methods and was reproducible between differentiation rounds, particularly for KOLF2 cells, for which the variability was approximately 7% (see supplementary Figure 3). This typically depended most significantly on EB size and morphology following hematopoietic differentiation. More specifically, round, smooth EBs that exhibited continuous growth correlated with higher NK cell purity following differentiation. Although iPSCs seeded at a density of 6000 cells/well were able to yield a high $CD56^+/CD3^-$ population, the abnormal EB morphology and lower CD34 yield favored a seeding density of 3000 cells/well. Furthermore, post-differentiation expansion for 2 weeks with NK cell expansion medium supplemented with IL-2, IL-15 and IL-21 improved NK cell purity for both cell lines to greater than 70% and greatly improved yield for each cell culture (see supplementary Table 5, Figure 4).

Following 4 weeks of NK cell differentiation, human NK cells derived from both KOLF2 and hiPSC01 iPSC lines were generally morphologically indistinguishable from primary human blood-derived NK cells (Figure 2B). Additionally, ultra-low attachment six-well plates were investigated to potentially improve differentiation efficiency. However, these plates were not superior to standard plates in the authors' experiments (see supplementary Figure 5). Although NK cells generated using ultra-low attachment six-well plates for the second stage of NK cell differentiation were comparable in numbers and morphologically identical to those generated using standard cell culture-treated six-well plates, normal attachment plates resulted in higher purity of hiPSC-01 NK cells and higher NKp46 and CD94 expression (Figure 2C; also see supplementary Figure 5).

iPSC-derived NK cells under chemically defined conditions show phenotypic maturity via the expression of NK-specific activation receptors

The authors next measured the expression levels of common inhibitory and activating receptors present on mature NK cells, which NK cells use to mediate their cytotoxicity against tumor targets after

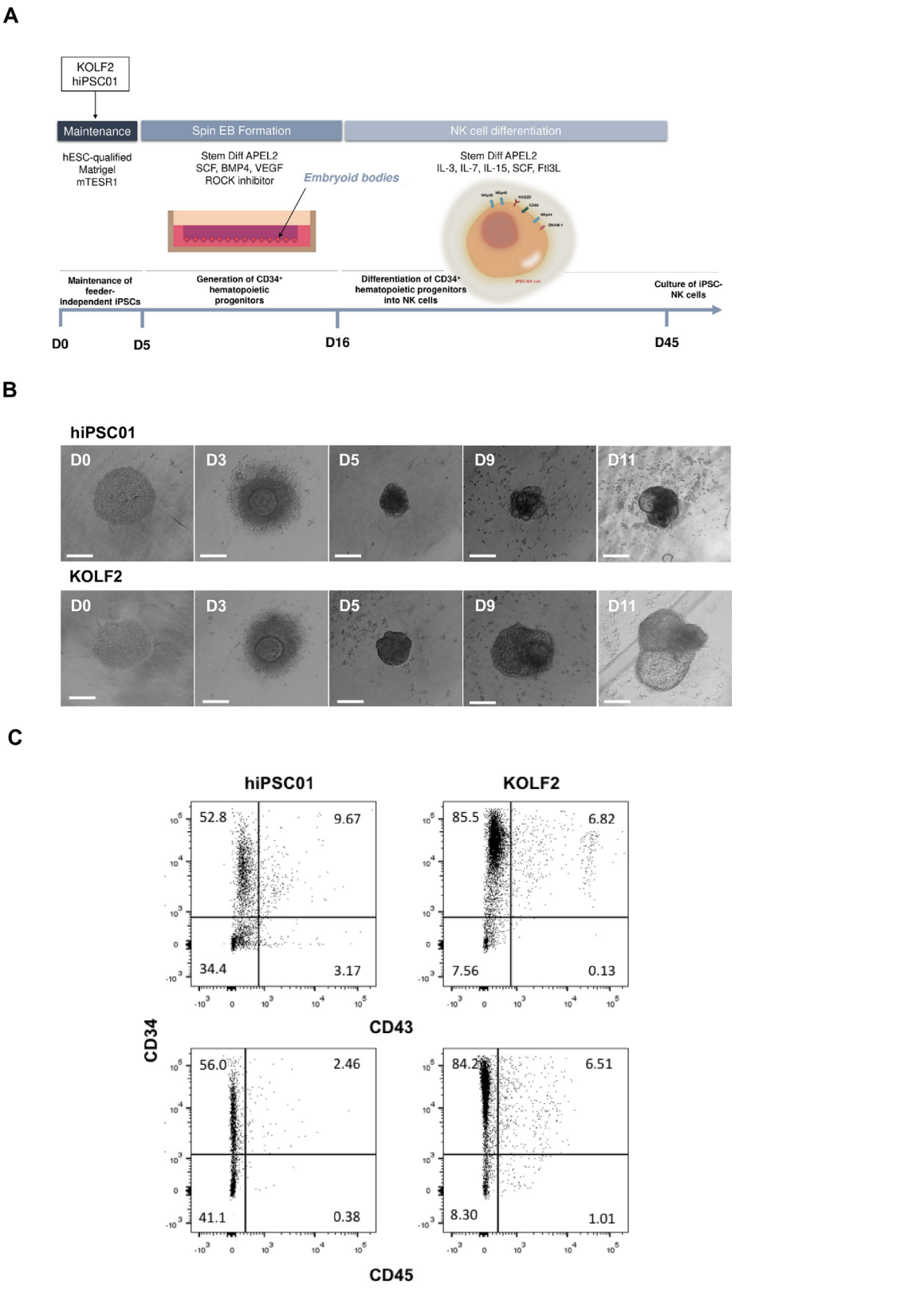

Figure 1. Maintenance of iPSCs and generation of spin EBs and hematopoietic progenitor cells. (A) Diagram showing hematopoietic progenitor and NK differentiation protocol. The authors implemented a two-stage differentiation process to generate NK cells from feeder-independent iPSCs. The iPSC lines KOLF2 and hiPSC01 were maintained in mTesR 1 medium on hESC-qualified Matrigel for 4–5 days, dissociated and transferred into STEMdiff APEL 2 and centrifuged to generate spin EBs. Successfully generated spin EBs were cultured for 11 days to generate $CD34^+$ hematopoietic progenitor cells. This was followed by 4 weeks of differentiation into mature NK cells in the same medium in the presence of cytokines. (B) Phase contrast microscopy images showing morphology of spin EBs and development into hematopoietic progenitor cells over 11 days from KOLF2 and hiPSC01 iPSCs in STEMdiff APEL 2 medium. A representative spin EB is shown for each cell type. (C) Representative dot plot of proportion of $CD34^+/CD45^+$ and $CD34^+/CD43^+$ hematopoietic progenitor cells generated from KOLF2 and hiPSC01 spin EBs as measured by flow cytometry. Scale bar = 500 μm. (Color version of figure is available online).

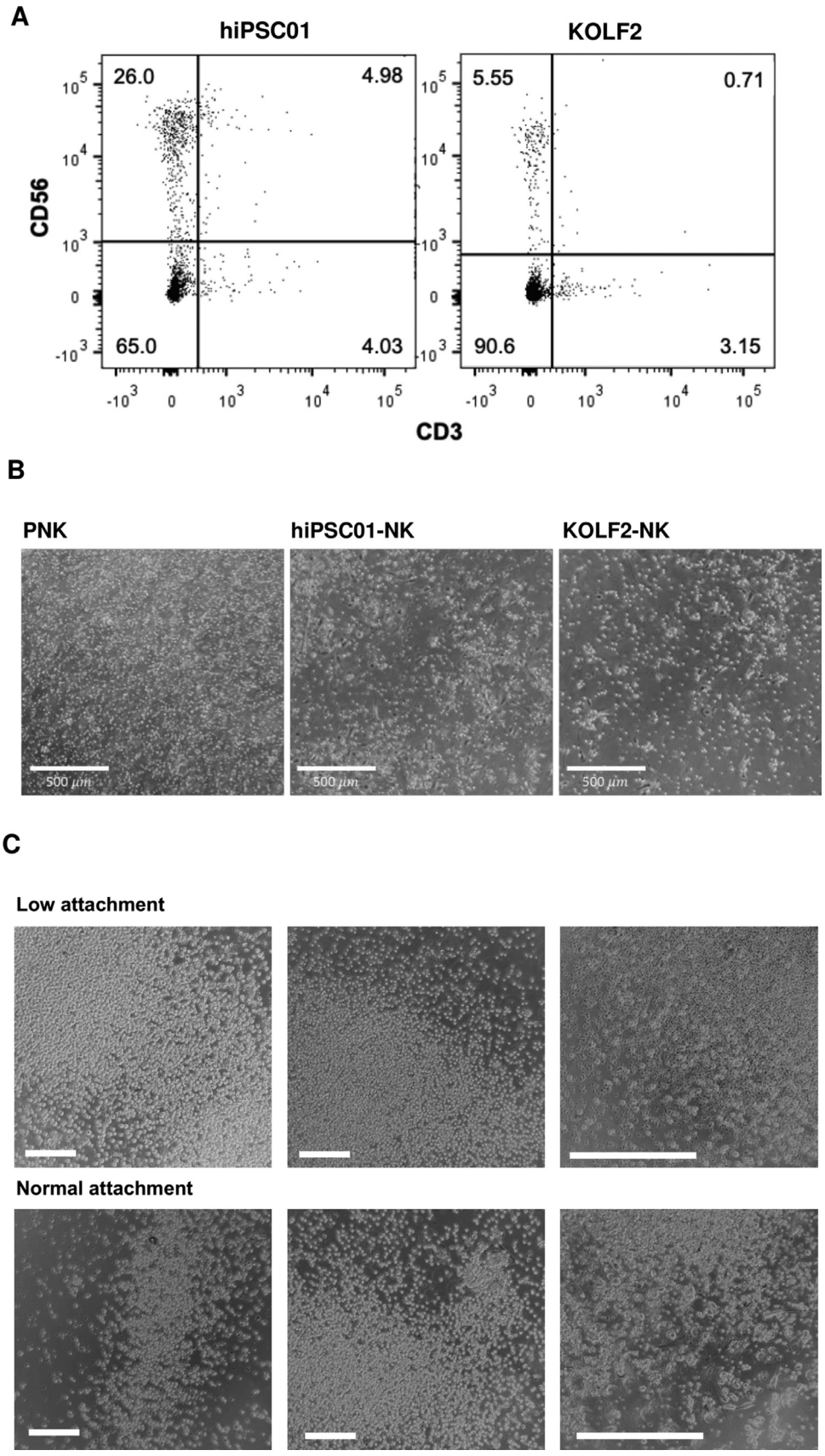

Figure 2. Generation of mature CD56$^+$/CD3$^-$ NK cells from CD34$^+$ hematopoietic progenitor cells. (A) Flow cytometric dot plot showing proportion of CD56$^+$/CD3$^-$ NK cells differentiated from CD34$^+$ hematopoietic progenitor cells from both hiPSC01 (left panel) and KOLF2 (right panel) iPSCs on day 45. Data from a representative differentiation round shown. (B) Morphology of hiPSC01- and KOLF2-derived NK cells as well as pNK cells in culture. The pNK cells were plated at a higher density (day 45). (C) The morphology of differentiated NK cells was similar whether derived from low (top row) or standard (bottom row) attachment cell culture-treated six-well plates on day 45. Shown are representative cultures of KOLF2-derived NK cells. These cells could be maintained in culture while retaining high viability (>70% after expansion) and normal expansion capacity. Scale bar = 500 μm.

differentiation (day 45). Human pNK cells express the major histocompatibility complex class I-dependent inhibitory heterodimer receptor CD94/NKG2A and also activate the receptors NKG2D, NKp30, NKp44, NKp46 and DNAM-1. Mature, fully differentiated iPSC-derived NK cells from both KOLF2 and hiPSC01 cell lines were phenotypically characterized for expression of cell surface markers by flow cytometry on day 45 (Figure 3A,B). Both KOLF2- and hiPSC01-derived $CD56^+$ NK cells expressed CD94 and NKG2A as well as the panel of activating receptors (see supplementary Figure 6). Expression of DNAM-1 was approximately 105-fold higher on KOLF2-derived NK (KOLF-NK) cells and approximately 56-fold higher on hiPSC01-NK cells compared with isotype controls (see supplementary Table 2). Both populations of NK cells expressed the Fc receptor CD16. These cells did not significantly express CD3 or CD19, suggesting that they were not T cells, NK T cells or B cells.

To verify the reproducibility of the differentiation protocol, the authors measured expression of CD56 and CD3 on NK cells derived from KOLF2 and hiPSC01 iPSCs over three separate differentiation rounds via flow cytometry (day 45). The authors found that the proportion of $CD56^+/CD3^-$ NK cells was fairly consistent across multiple rounds of differentiation (Figure 3C) of iPSCs into NK cells, particularly with KOLF2 iPSCs (variability ~7%). Moreover, cryopreservation either during (at day 25 or day 32) or at the end of the differentiation process (day 39) did not significantly change the proportion of $CD56^+/CD3^-$ NK cells (see supplementary Table 3, Figure 7) compared with cells that had not been cryopreserved. Cells plated in NK cell culture media retained their phenotype as primarily $CD56^+/CD3^-$ cells.

The iPSC-derived NK cells were also characterized phenotypically for cell surface marker expression after expansion in culture medium (Figure 4A,B). Both hiPSC01- and KOLF2-NK cells were positive for activating receptors, including NKp44, NKp46, NKp30 and NKG2D. In terms of expression of KIRs, these cells were positive for CD158b (KIR2DL2/DL3) and negative for CD158a/h (KIR2DL1/DS1), with negative CD158e1 (KIR3DL1) expression (see supplementary Table 4, Figure 8).

The hiPSC01- and KOLF2-derived NK cells were able to expand in commercial NK cell expansion medium (Figure 4C; also see supplementary Figure 9) and maintain high viability (>70%) after expansion (see supplementary Figure 12). When compared with rates of expansion of pNK cells in the same medium, KOLF2-NK cell expansion rates were comparable (Figure 4C), whereas hiPSC01-NK cells expanded slightly less. More specifically, with a cell starting number of 300 000 (3000 cells/EB over 100 EBs) 2.93 ± 1.57 million live hiPSC01-NK cells and 3.37 ± 0.19 million KOLF2-NK cells can be obtained following differentiation, which can be expanded by approximately 4- to 5-fold using commercial NK cell medium over a period of 1–2 weeks. When expanded in G-Rex plates, iPSC-NK cells were able to achieve expansion folds of up to 80× per well, which translates to 1920× expansion rates per 24-well plate (see supplementary Figure 9). These expansion rates yield over 10^9 cells/plate, and the expanded iPSC-NK cells displayed cytotoxic ability comparable to that of pNK cells (see supplementary Figure 9C). In summary, this indicates that clinical doses of iPSC-NK cells can be obtained using commercial NK cell media. The use of feeder cells, commonly used to expand NK cells to clinical doses, is another option for expansion of these cells. In addition, expanded iPSC-NK cells exhibited morphologies comparable to those of expanded pNK cells, with somewhat irregular circular to kidney-shaped structures (Figure 4D).

iPSC-derived NK cells are functionally competent and able to degranulate and produce IFN-γ in response to solid tumor targets

To determine the anti-tumor potential of iPSC-derived NK cells using a differentiation process that is chemically defined, feeder- and serum-free, the authors performed killing assays with a variety of tumor cell lines wherein iPSC-NK cells from both KOLF2 and hiPSC01 lines were challenged with the killing of various cancer cell targets after expansion in standard feeder-free NK cell culture medium. Although the differentiation is feeder-free, expansion is a result of choice of medium, and the authors utilized a feeder-free NK cell medium. The authors carried out these killing assays at E:T ratios of 2.5:1, 5:1 and 10:1 against prostate (PC3), lung adenocarcinoma (A549) and recurrent GBM (GBM10) cells, all of which express varying HLA levels (see supplementary Figure 10), and measured killing activity by flow cytometry. KOLF2-NK cells mediated superior killing of prostate cancer cells compared with pNK cells at all E:T ratios (Figure 5A). Similarly, hiPSC01-NK cells mediated superior or comparable killing of prostate cancer PC3 cells (Figure 5B). Superior killing by KOLF2 cells to that of pNK cells was also observed with A549 cells as the targets (Figure 5C), whereas hiPSC01 cells mediated comparable killing of A549 cells at two out of three E:T ratios (Figure 5D), further confirming the functional potency of the iPSC-NK cells derived using the authors' chemically defined protocol. Killing activity comparable to that seen with pNK cells was maintained following expansion of cells in IL-2-, IL-15- and IL-21-containing media [17,26]. No clonal selection was carried out, and killing potential represented population-wide iPSC-NK cell cytotoxicity, meaning selection bias toward specific subpopulations was avoided.

To further establish the cytotoxic potential of the iPSC-derived NK cells, the authors sought to determine their degranulation potential and the production of IFN-γ mediated by these expanded (activated) cells, functions that mature NK cells undergo in response to the presence of cancer cells. Using PC3 cells as targets, the authors set up stimulation assays at three E:T ratios (2.5:1, 5:1 and 10:1). The authors determined the change in percentage expression of IFN-γ from both activated NK cell populations after stimulation with cancer cells by subtracting unstimulated controls (see supplementary Figure 11). KOLF2-NK (Figure 6A) and hiPSC01-NK (Figure 6B) cells were both comparable or superior to pNK cells in producing IFN-γ when stimulated by PC3 cells following activation. Because activated NK cells are able to produce IFN-γ in response to certain stimulation conditions (i.e., specific cytokines), baseline percentage levels likely accounted for the activation-induced cytokine production from these cells. To determine degranulation capacity, the authors measured the change in percentage of NK cell surface expression of CD107a, a marker of cytolytic granule production, via flow cytometry following stimulation with PC3 cells and subtraction of activated NK cells in the absence of cancer cells (see supplementary Figure 11). Both KOLF2-derived (Figure 6C) and hiPSC01-derived (Figure 6D) activated NK cells induced a change in degranulation in response to PC3 cells. These data showed that the authors' iPSC-derived NK cells could be functionally competent in mediating anti-tumor functions against cancer targets, and that their functional competence was comparable among both lines.

The iPSC-NK cells were next challenged to kill GBM10 cells. These cells are derived from a patient with recurrent GBM and are particularly resistant to traditional therapy with temozolomide. Both KOLF2-derived (Figure 7A,C) and hiPSC01-derived (Figure 7B,C) NK cells were able to kill highly treatment-resistant patient-derived recurrent GBM10 cells in a manner comparable to that seen with pNK cells.

Discussion

NK cells have emerged as powerful and promising effectors in adoptive cell-based cancer immunotherapy in large part because of their ability to mediate potent anti-tumor functions within allogeneic transplant settings [7,27]. However, reliance on the peripheral blood of cancer patients for sourcing NK cells risks obtaining cells with severely impaired anti-tumor cytotoxicity [13,28–30]. The challenge in treating tumors with these patient-derived NK cell transplants is further exacerbated by the need for *ex vivo* expansion to sufficient

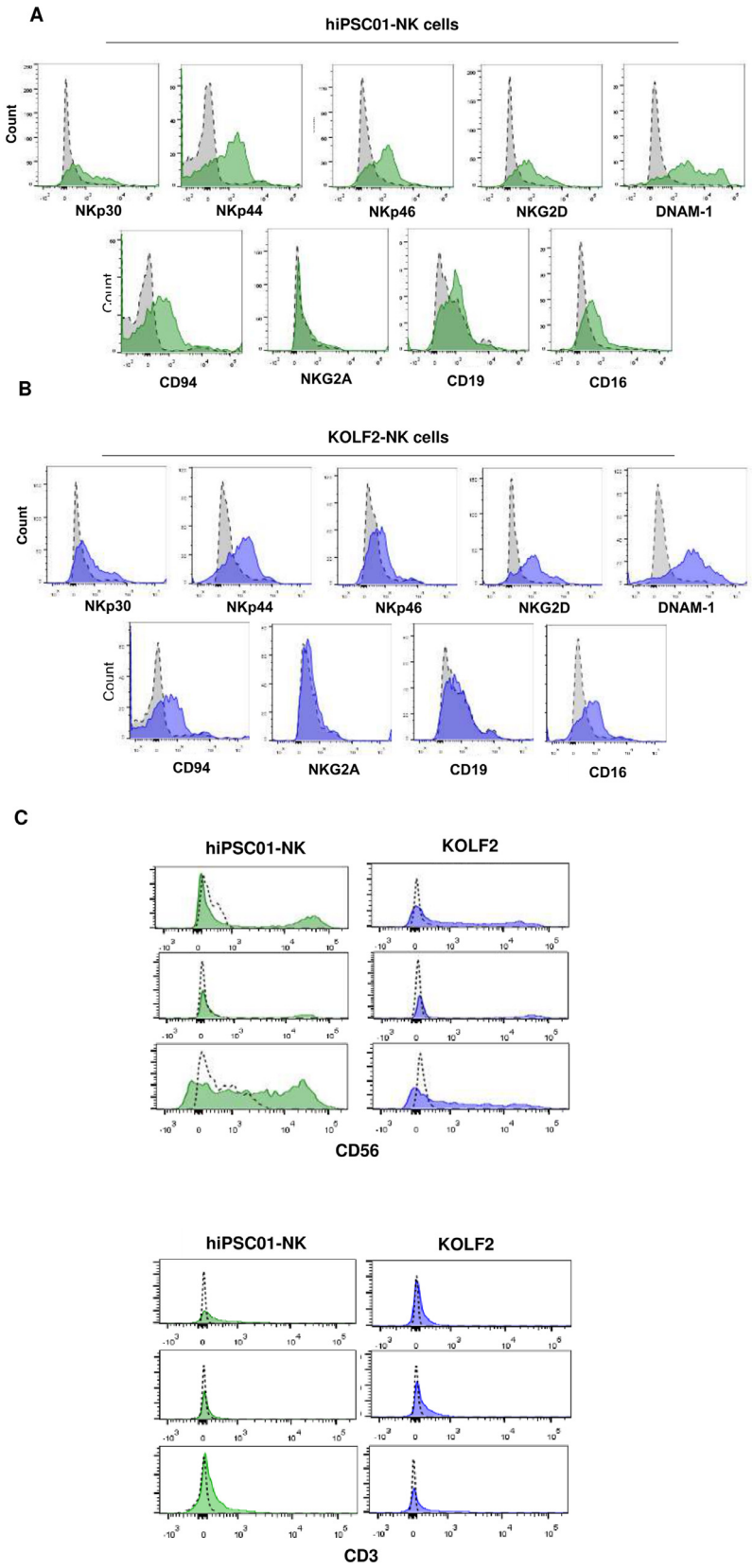

Figure 3. Expression of activating and inhibitory receptors on freshly differentiated iPSC-derived NK cells. Single parameter flow cytometric histograms of expression of activating and inhibitory markers and CD16 measured on $CD56^{+}/CD3^{-}$ NK cells (day 45) differentiated from (A) hiPSC01 iPSCs (green) and (B) KOLF2 iPSCs (blue). Analysis was performed by gating on $CD56^{+}/CD3^{-}$ cells. (C) Reproducibility of the authors' feeder-free differentiation process as indicated by the expression of CD3 and CD56 on NK cells derived from hiPSC01 (left) and KOLF2 (right) iPSCs. Data from three individual and separate differentiation rounds shown. Dashed lines represent control samples. Measurements were normalized by isotype controls and gated on all live cells. (Color version of figure is available online).

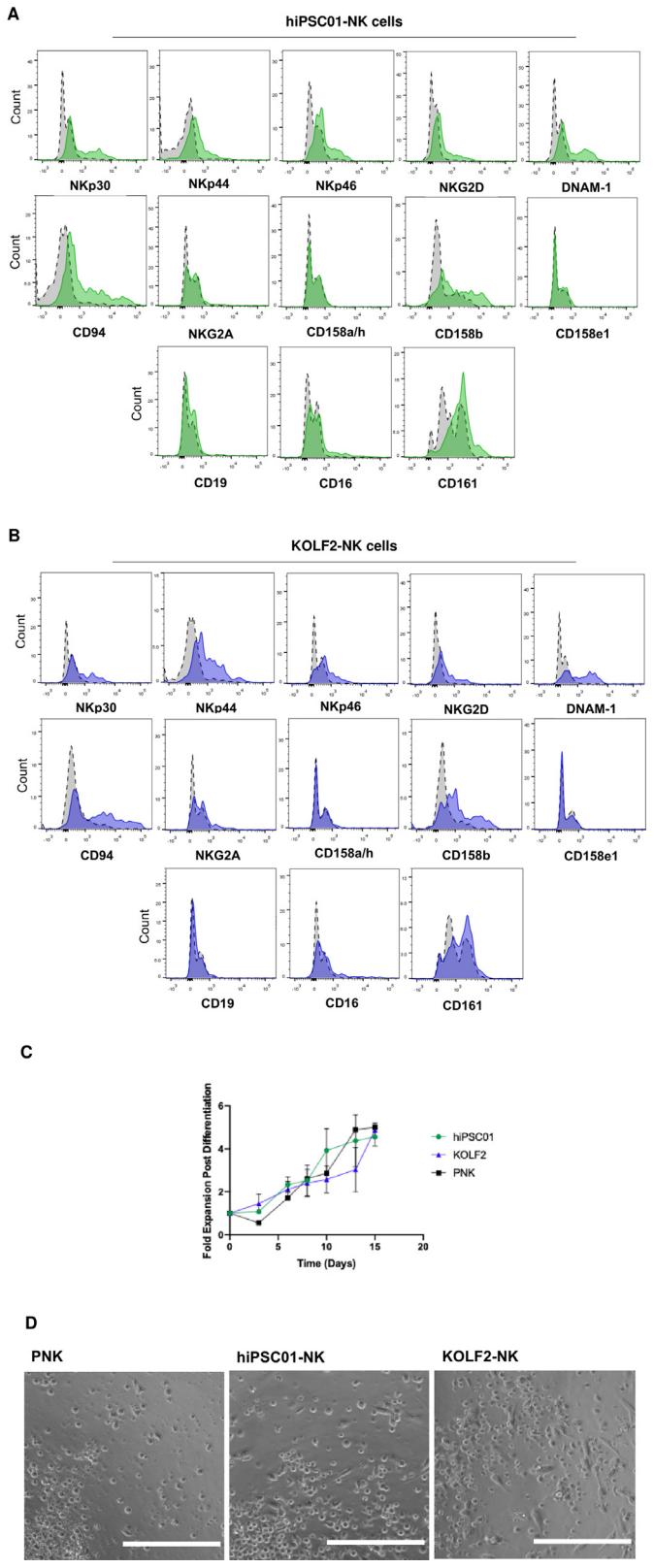

Figure 4. Characterization of expanded iPSC-NK cells. (A) Representative histograms of surface marker expression of hiPSC01-NK cells after expansion. Analysis was performed by gating on $CD56^+/CD3^-$ cells. (B) Representative histograms of surface marker expression of KOLF2-NK cells after expansion. (C) Following differentiation, NK cells derived from both KOLF2 and hiPSC01 iPSCs were cultured in the same NK cell expansion media. Viability was measured by Trypan Blue staining (n = 3). (D) Representative brightfield microscopy images of expanded pNK, hiPSC01-NK and KOLF2-NK cells. Scale bar = 200 μm. (Color version of figure is available online).

numbers for adoptive transfer [31], extending the time patients have to wait to receive treatment. This has fueled the development of protocols for the generation of NK cells from renewable cell sources. Among these, hESCs [22,32] and iPSCs have been shown to generate NK cells with phenotypic and functional profiles similar to those of pNK cells [20,33,34]. To date, protocols developed for the generation of NK cells from iPSCs have relied on the use of feeder cells for the adaptation of iPSCs prior to differentiation [17,35], the culturing of human iPSCs/hESCs on stromal layers (e.g., EL08-1D2) before differentiation [18] or the use of long co-cultures with stromal cells (e.g., M210-B4) for NK cell differentiation following sorting of hematopoietic $CD34^+/CD45^+$ progenitors to improve yields. This translates to laborious and time-consuming procedures that take months to generate functionally mature NK cells. Moreover, these protocols result in either highly heterogeneous $CD56^+$ and $CD56^-$NK populations [22,32] or highly variable yields dependent on the feeder cells used [18].

More recently, a modified Good Manufacturing Practice-friendly protocol was described wherein NK cells were generated from donor peripheral blood-derived cells using integration-free Sendai viral vectors that mediated the reprogramming of peripheral blood-derived cells into iPSCs [19]. The NK cells produced in this process were, moreover, KIR-negative, attractively circumventing the need for KIR-HLA donor matching. Although the use of OP9-DLL1 cells, a modified OP9 cell line expressing the Notch ligand DLL1 as feeders, was able to generate homogeneous $CD56^+/CD45^+$ lymphoid populations, this method nonetheless required feeder-based adaptation of iPSCs as well as the reprogramming of donor-specific peripheral blood-derived cells into iPSCs with specialized protocols. Although a feeder-free protocol for NK cell derivation was recently described [26], there was no indication that the cytotoxicity of these iPSC-derived NK cells was comparable to that of pNK cells with the solid tumor targets tested, including patient-derived cells. The choice of starting cells affects not only the efficiency of reprogramming but also the ability to reproducibly derive functional iPSCs. Moreover, reprogrammed iPSCs show high variability that is at once inter- and intra-line specific as well as donor-dependent [36–39], hampering their efficiency and reproducibility when used in subsequent differentiation protocols. Finally, limited evaluation of these cells' potential in treating solid tumors has been carried out thus far. The authors' approach described here circumvents or improves upon these issues, as it demonstrates the development and characterization of iPSC-NK cells under controlled, feeder-free conditions and functionally and phenotypically characterizes these cells against solid tumor targets in terms of not only cytotoxicity but also degranulation and cytokine production.

Stimulated by the need for improved approaches to generating functional NK cells that are more time-efficient, do not require feeders and are applicable across well-characterized iPSC sources, the authors sought to evaluate the feasibility of a method of generating NK cells from iPSCs that addresses these needs (Figure 1A). The purpose of this study was to generate functional iPSC-NK cells under defined conditions and evaluate their effector activity against solid tumor targets. Studies so far have investigated solid tumor targets in a limited capacity, either only in terms of cytotoxicity or with limited comparison with pNK cells [17,21,25,40]. The authors used the feeder-independent cell line KOLF2 obtained from HipSci. These cells are banked by the European Collection of Authenticated Cell Cultures. KOLF2 is a euploid cell line derived from foreskin fibroblast biopsies of healthy male donors and has been extensively characterized genotypically and phenotypically using validated experimental procedures [41]. The generation of functional NK cells from such cell lines represents a strategy that circumvents the need for reprogramming iPSCs [42], thus avoiding the variability that is associated with the donor fibroblast source and imposed by the reprogramming process. This protocol thus generates functionally competent NK cells that can serve as models

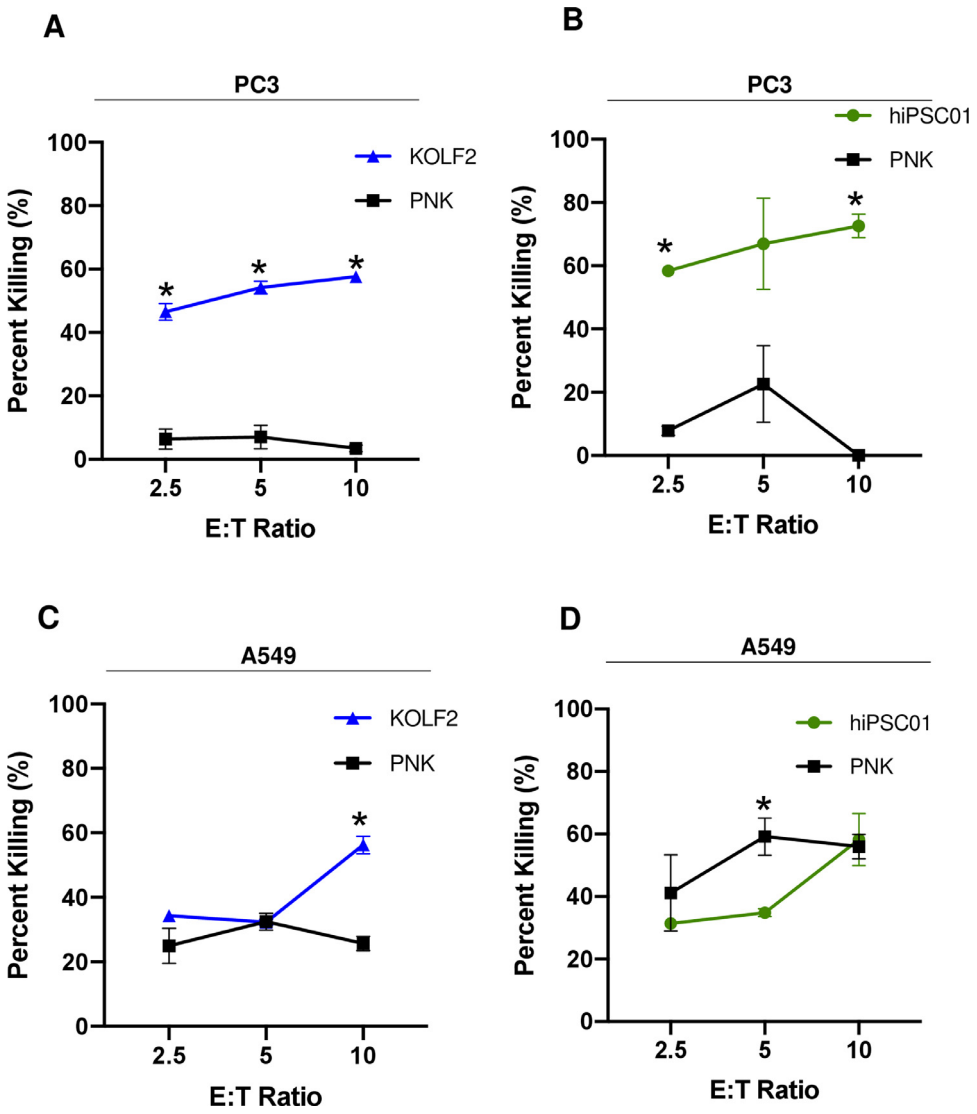

Figure 5. Cytotoxic ability of iPSC-derived NK cells against tumor targets. NK cells derived from either KOLF2 or hiPSC01 cells were challenged with killing various solid tumor cell lines at E:T ratios of 2.5:1, 5:1 and 10:1 after expansion. Measurement of cytolysis of target cells was done by 7-AAD/CFSE staining via flow cytometry. Spontaneous death of cancer cells has been subtracted from all plots. The killing ability of iPSC-derived NK cells compared with that of pNK cells was measured against (A,B) PC3 prostate cancer and (C,D) A549 lung carcinoma cells. The pNK cells were obtained from multiple different donors for technical replicates (n = 3). *$P < 0.05$. 7-AAD, 7-aminoactinomycin D. (Color version of figure is available online).

for the study of NK cell immunobiology and the development of immunotherapies devoid of donor and process-induced variability. In addition, the authors generated NK cells from dermal fibroblast-derived hiPSC01 cells, reprogrammed in the authors' lab using commercial reagents, and compared these NK cells with those derived from the KOLF2 line.

Using a two-stage derivation process that was entirely feeder-free, the authors generated functionally mature NK cells. The high expression of activating receptor DNAM-1 the authors observed could be one mechanism that iPSC-NK cells utilize to eliminate teratoma formation [43]. Expanded and cytokine-activated iPSC-NK cells displayed high levels of expression of activating surface markers (Figure 4A,B) and a repertoire of other receptors, including KIRs, that was comparable among the two iPSC lines.

The authors observed robust anti-tumor functions mediated by these iPSC-derived NK cells. The killing capacity of both KOLF2-NK and hiPSC01-NK cells resulted in killing of solid tumor targets that was at least comparable if not superior to that of pNK cells (Figure 5A–D). The authors also challenged iPSC-NK cells with killing of highly treatment-resistant GBM cells (GBM10). GBM10 cells were derived from a recurrent wild-type p53 GBM from a patient who received prior radiation and salvage chemotherapy. GBM10 cells show high temozolomide and ionizing radiation resistance. Remarkably, iPSC-NK cells showed killing competency that was comparable to that of pNK cells (Figure 7A–C). The equivalence or superiority of the authors' iPSC-NK cells in killing virtually every solid tumor target tested suggests that these cells are able to efficiently mediate cytotoxicity against multiple cancers. The authors have also observed that the iPSC source is a likely factor in determining functional maturity of differentiated NK cells.

The iPSC-derived NK cells are able to display variable KIR expression. Though KIR expression does not appear to, in and of itself, be required for iPSC-NK-mediated cytotoxicity [44], modulation of KIR expression has the potential to nonetheless drive the strength of the response in adoptive transfer settings [6,45]. This does not mean that iPSC-derived NK cell function is driven solely by KIR expression, but the ability to generate KIR-specific NK cells with known and defined KIR haplotypes can facilitate not only their sourcing but also their selection against particular targets that may benefit from targeted mismatch. Alloreactivity of the NK cells generated in this study was evidenced via cytotoxicity, degranulation and cytokine production assays against multiple targets. The observed discrepancy between low degranulation levels

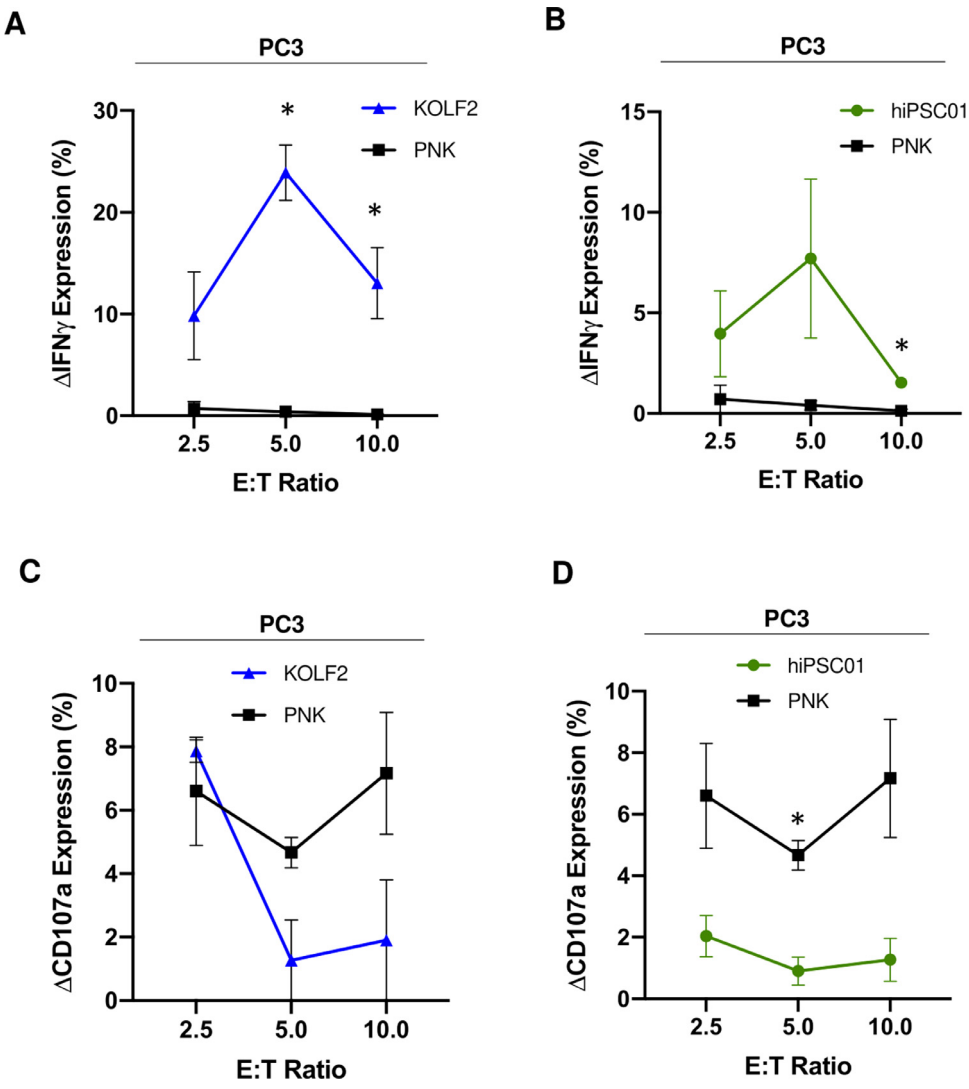

Figure 6. Change in percentage of IFN-γ production and degranulation by iPSC-derived expanded NK cells in response to tumor targets. NK cells derived from (A) KOLF2 iPSCs and (B) hiPSC01 iPSCs produced IFN-γ in a manner comparable to or more efficient than pNK cells when stimulated by prostate cancer PC3 cells and compared with unstimulated cells. IFN-γ was measured by flow cytometry and quantified based on isotype controls. Plotted values represent change obtained after subtracting data for PC3-unstimulated cells. Both (C) KOLF2-derived NK cells and (D) hiPSC01-derived NK cells were able to degranulate in response to PC3 cells. Degranulation was measured by quantifying CD107a via flow cytometry. Measurements were normalized by isotype controls and gated on live cells. Plotted values represent change obtained after subtracting data for expanded NK cells in the absence of cancer cells. The pNK cells were obtained from multiple different donors for technical replicates (n = 3). *P< 0.05. (Color version of figure is available online).

and high cytotoxicity could be explained by the involvement of other mechanisms of killing engaged by iPSC-NK cells, such as via FasL or TRAIL. The authors also measured high levels of IFN-γ produced by iPSC-NK cells, which may sensitize target cells to perforin-dependent killing.

Yield and efficiency, and indeed the phenotypic and functional maturity of differentiated NK cells, are highly dependent on the first stage of the differentiation process; namely, the generation of hematopoietic progenitor cells. In the authors' case, the quality of spin EBs was a significant factor affecting yield of mature NK cells. Intact morphology of spin EBs positively correlated with higher yields of NK cells after differentiation. As three-dimensional cellular aggregates, EBs respond to culture conditions and may carry inherent variability based on media type and source. To reduce such variability and improve the homogeneity and synchronization of EBs, the authors used fully defined conditions for their culture. This improved both their yield and reproducibility and their subsequent response to differentiation triggers. This is different from other published approaches, which relied on largely poorly defined EB culture conditions [18,25]. It bears mentioning that any inherent heterogeneity of individual EBs is related to their biological nature. Scaling up the production of NK cells with the authors' protocol is best achieved by scaling out individual culture wells at the same initial cell concentration rather than plating higher cell densities. For the second stage of differentiation, the authors used previously established cytokines to induce NK differentiation, including IL-3, IL-7, IL-15, SCF and Flt3L [46]. However, modification of this cytokine matrix, such as the elimination of IL-3 to dissuade the generation of myeloid-like cells [26,47], could be one way to improve both purity and yield of the differentiation process. Additionally, the authors' iPSC-NK cells were mostly $CD56^+/CD3^-$ after expansion in culture (see supplementary Figure 4). It is also possible to envisage the straightforward conversion of the authors' current protocol to a fully "xenogeneic-free" process via the replacement of medium used with one that fits such a criterion. Moreover, these iPSC-derived NK cells expanded in culture medium to numbers comparable to the expansion numbers of pNK cells obtained with media alone. Clinical doses of iPSC-NK cells could be obtained by culturing these cells in G-Rex plates, as the authors have shown. Alternatively, they can be grown in the presence of irradiated K562 feeders with IL-15 or IL-21 and 4-1BBL [48], a strategy commonly used to expand NK cells to clinical doses.

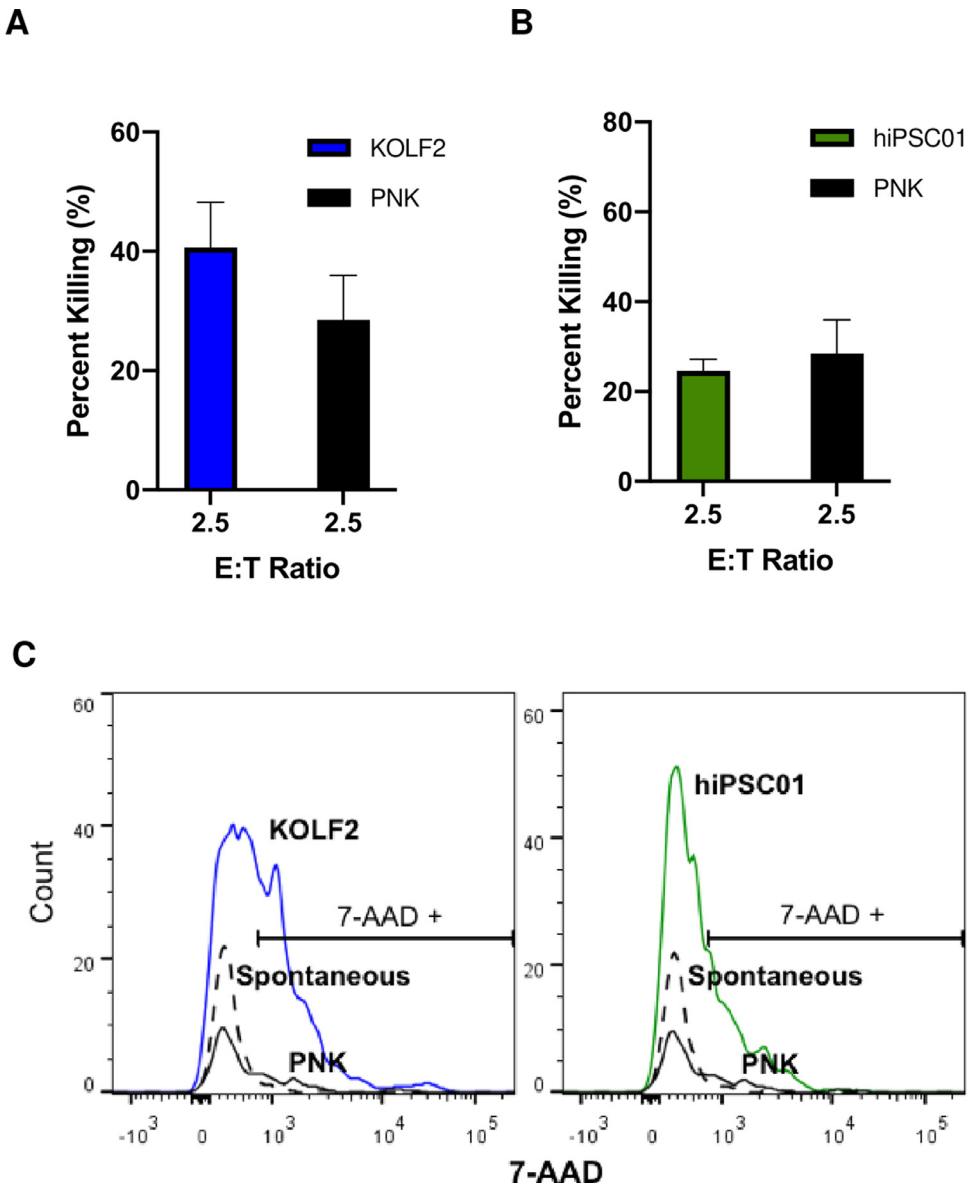

Figure 7. Cytotoxicity of iPSC-NK cells against primary recurrent GBM cells. Cytotoxicity, after NK cell expansion, of (A) KOLF2- and (B) hiPSC01-derived NK cells against patient-derived recurrent GBM cells (GBM10) at an E:T ratio of 2.5:1. NK cells were able to kill recurrent GBM targets with the same efficiency as pNK cells. Cytotoxicity was measured via 7-AAD/CFSE staining by flow cytometry. Spontaneous GBM cell death has been subtracted from plotted killing values. (C) Representative flow cytometric histograms for killing by KOLF2- and hiPSC01-derived NK cells of patient-derived GBM10 cells. Overlay of spontaneous death of GBM10 cells without the presence of NK cells is also shown. The pNK cells were obtained from a single donor for technical replicates (n = 3). 7-AAD, 7-aminoactinomycin D. (Color version of figure is available online).

Collectively, the authors' results demonstrated that centrally derived iPSCs are phenotypically comparable to reprogrammed iPSCs when differentiated into NK cells. The use of centrally authenticated iPSC lines will facilitate the scaling up of culture systems as well as the development of quality-validated protocols to enable the controlled maintenance of iPSCs and differentiation into NK cells, with great value for immunology and immunotherapy applications.

At this stage in the general development pipeline, the authors' claim is not that iPSC-NK cells are superior to pNK cells, but rather that iPSC-NK cells are, as evidenced by the data, a promising and unique effector population for further development in adoptive transfer immunotherapy with potential for targeting solid tumors.

In summary, this is the first study that describes the function of NK cells against solid tumors following derivation from iPSC lines using an entirely feeder-independent, chemically defined protocol. Moreover, this is the first example of the use of iPSC lines that have been centrally authenticated and are readily available for use by other labs. The functional potency of these NK cells against multiple solid tumor targets suggests that they can act as promising allogeneic effectors of adoptive transfer cancer immunotherapy.

Conclusions

The authors have described the function of NK cells differentiated from iPSC lines using a feeder-independent, chemically defined protocol from both reprogrammed and centrally authenticated iPSC lines against solid tumor targets. These authenticated iPSCs represent well-characterized reagents that are able to circumvent protocol-specific reprogramming of iPSCs and drive the development of new immunotherapies for the adoptive transfer of NK cells. The functional performance of NK cells derived in a defined manner against multiple tumor targets makes them promising effectors for further development in adoptive cancer immunotherapy that may be beneficial in scenarios where feeder- or

animal-free conditions are needed or those that can benefit from their ease of genetic manipulation.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: KBL, JM and SM. Acquisition of data: KBL, JM and AMC. Analysis and interpretation of data: KBL, JM and SM. Drafting or revising the manuscript: KBL, JM and SM. All authors have approved the final article.

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Availability of Data and Materials

The data that support the findings of this study are available in the supplementary materials.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2021.05.001.

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