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Basic Research

Artificial feeder cells expressing ligands for killer cell immunoglobulin-like receptors and CD94/NKG2A for expansion of functional primary natural killer cells with tolerance to self

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ABSTRACT

Background aims: Natural killer (NK) cells are promising cells for immunotherapy of cancer, and there are ongoing efforts to improve their *ex vivo* expansion to clinically relevant numbers. This study focused on the development of a C1-, C2-, Bw4 killer cell immunoglobulin-like receptor (KIR) ligand and NKG2A ligand-containing feeder cell line for autonomous expansion of functional NK cells.

Methods: PC3^{PSCA}-derived feeder cells expressing IL-2, 4-1BBL and membrane-bound IL-15-mutDAP12 (mIL-15d) fusion protein in combinations or alone were generated and used for expansion. Expanded NK cells were analyzed with respect to subpopulations, expression of NK cell receptors and immune checkpoint molecules as well as their cytotoxicity against K562 cells, cetuximab-marked tumor cells and autologous B cells.

Results: Only combinatorial expression of IL-2 plus 4-1BBL or IL-2, 4-1BBL plus mIL-15d in feeder cells efficiently expanded NK cells and supported selective outgrowth of NK cells from peripheral blood mononuclear cell samples. Best expansion of NK cells was achieved using PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells. Such expanded NK cells exhibited upregulation of natural cytotoxicity receptors, DNAM-1 and NKG2C and induced expression of high affinity IL-2 receptor, which were paralleled by attenuated KIR and increased expression of NKG2A and ILT2. In addition, elevated TIM-3 levels were noted and PD-1 and T cell immunoreceptor with Ig and ITIM domain (TIGIT) levels remained low. Expanded NK cells were highly cytolytic when encountering K562 cells and cetuximab-marked target cells but remained unresponsive to autologous B cells and target cells with protective levels of human leukocyte antigen.

Conclusions: Collectively, the results demonstrate the feasibility of PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells for robust expansion of NK cells, which remain tolerant to self and could be used in the future for adoptive cell therapy of cancer.

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Introduction

During the last several years, adoptive cell transfer of natural killer (NK) cells for immunotherapy of tumors has received increasing attention [1]. NK cells are characterized by multiple germline-encoded activating and inhibitory receptors that are epigenetically regulated in a stochastic, variegated pattern, resulting in an overlapping diversity of NK cell subsets. By using their array of receptors, NK

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cells remain tolerant to healthy cells but sense and spontaneously kill a wide array of virally infected cells and tumor cells [2]. The current paradigm of NK cell-mediated tolerance against self requires interactions of inhibitory receptors, which are polymorphic killer cell immunoglobulin-like receptors (KIRs) and the CD94/NKG2D heterodimer, with human leukocyte antigen (HLA) class I molecules and non-classical HLA-E molecules on autologous normal cells, respectively [3,4]. Another inhibitory receptor, the immunoglobulin-like transcript 2 (ILT2), also designated leukocyte immunoglobulin-like receptor subfamily B member 1 (LIRLB1) or CD85j, binds to a broad range of HLA class I molecules [5]. Binding to self-HLA molecules by inhibitory NK receptors of developing NK cells is required for achieving the license to kill. Only “licensed,” or “educated,” NK cells have acquired full cytotoxic effector functions enabling them to spontaneously kill target cells (i.e., tumor, foreign or virus-infected cells), whereas non-licensed NK cells remain in a hypo-responsive state [6,7]. Therefore, under homeostatic conditions, NK cells are tolerant against self. Yet this tolerance can be broken by inflammatory cytokines or by an inflammatory environment [8], a finding potentially relevant for setting up *ex vivo* expansion protocols and therapeutic use of NK cells.

In the past, immunotherapy with NK cells has often been limited by the inability to obtain sufficient numbers of pure NK cell populations for cancer treatment. To date, NK cell expansion can be greatly enhanced by feeder cells derived from tumor cell lines or peripheral blood mononuclear cells (PBMCs). Therefore, co-culture systems of irradiated feeder cells and NK cells in media containing IL-2, IL-15 and IL-21 have been developed to generate large numbers of NK cells. Commonly used feeder cell lines are Epstein-Barr virus (EBV)-transformed 721.221, EBV-transformed B cells (EBV-LCLs), EBV/bcr-abl-transformed RPMI8866 and bcr-abl-transformed K562 [9–12].

Stimulation with NK-sensitive K562 cells, which lack protective HLA class I expression and are lethally irradiated, is known to augment NK cell proliferation in combination with IL-2, IL-15 and IL-21 [11,13]. In a further development, K562 cells were genetically modified to express 4-1BBL (CD137L) plus membrane-bound IL-15 or loading of IL-15 receptor alpha (IL15R α) on K562 cells with recombinant human (rh) IL-15 to provide juxtacrine signaling to NK cells; 4-1BBL and IL-15 signaling acts synergistically with exogenously added rhIL-2 to augment K562-specific NK stimulatory capacity, resulting in strong expansion of peripheral blood NK cells without significant proliferation of T lymphocytes [14–16]. The same strong expansion of NK cells is seen when using similar K562 derivatives modified with, for instance, membrane-bound MHC class I chain-related protein A (MICA) plus 4-1BBL [17] or membrane-bound IL-21 plus 4-1BBL [18]. Of note, K562-driven expansion protocols exploit a combined missing self and induced self scenario and have been found to increase expression of natural cytotoxicity receptors (NCRs) and to skew NK cells to KIR-positive populations [15,18]. As reported for K562-IL-15/4-1BBL-driven expansion, neither the expression of cognate HLA ligands on target cells nor increased expression of inhibitory KIRs on K562-IL-15/4-1BBL-expanded NK cells precludes the killing of target cells. Therefore, in such expanded NK cells, inhibitory signaling is apparently negatively affected, resulting in decreased activation threshold of NK cells when encountering activating ligands on target cells [15]. When transplanted following T cell-depleted hematopoietic stem cell transplantation, such expanded NK cells were unexpectedly linked with induction of graft-versus-host disease (GVHD) in a significant fraction (55.6%) of HLA-matched unrelated donor and HLA-matched sibling donor recipients [19]. In a further phase I trial, 54% of recipients treated with mIL-21/4-1BBL-activated haploidentical allogeneic NK cells and receiving post-transplant GVHD prophylaxis after haploidentical T cell-depleted hematopoietic stem cell transplantation also developed GVHD, which, although not statistically significant, was higher than that observed for historic controls (31.8%) [20]. Hence, it appears conceivable that expansion protocols founded on missing self-recognition and that skew NK cells

to a decreased activation threshold could contribute to GVHD if inflammation or other factors upregulate activating ligand expression on nonmalignant tissues.

This work sought to develop and investigate a feeder cell line-based expansion method that avoids “missing self”-driven expansion. In parallel, using genetic modification with the secreted form of IL-2, a simple expansion method that did not require exogenously added cytokines was envisioned.

For the purpose of establishing NK feeder cell lines, the prostate cell line derivative PC3^{PSCA} [21] was chosen, which, in addition to the surface marker prostate stem cell antigen (PSCA), expresses C1 and C2 ligands for dominant inhibitory KIR2DL1 and KIR2DL2/3, respectively, as well as Bw4 ligand for inhibitory KIR3DL1 and inhibitory HLA-E for NKG2A. Moreover, the PC3^{PSCA} cells express Bw6 ligand, which binds to as yet unknown receptors. Artificial NK feeder cell lines were generated by sequential lentiviral gene transfer of co-stimulatory membrane-bound IL-15-mutDAP12 (mIL-15d) fusion protein, 4-1BBL and IL-2, resulting in seven different feeder cell lines. Of note, only feeder cells engineered to secrete IL-2 were capable of inducing NK cell expansion. Best expansion rates of purified NK cells from healthy donors were observed using feeder cells simultaneously expressing IL-2, 4-1BBL and mIL-15d. Such feeder cells also induced a selective and robust outgrowth of NK cells from PBMC samples. Expanded NK cells showed a subpopulation diversity that mainly consisted of CD56bright/CD16+, CD56dim/CD16+ and CD56bright/CD16– cells. In addition, increased expression of NCRs, DNAM-1 and NKG2C was noted. Furthermore, expanded NK cells displayed an attenuated KIR expression and increase in inhibitory NKG2A and ILT2 as well as increase in high-affinity IL-2 receptor and TIM-3. However, surface levels of immune checkpoint molecules PD-1 and T cell immunoreceptor with Ig and ITIM domain (TIGIT) remained low. Robust cytolytic activity against K562 cells lacking protective amounts of HLA class I molecules, a strong cytotoxic response against cetuximab-marked allogeneic tumor cells and no autoreactivity against autologous B cells substantiated a robust missing self-activation, induction of antibody-dependent cell-mediated cytotoxicity (ADCC) and functional tolerance to self. We conclude that artificial feeder cells with expression of KIR ligands for dominant KIRs and the provision of IL-2, 4-1BBL and IL-15 signals enable the outgrowth of educated NK cell populations with tolerance to self and ability to execute lytic activity when properly activated, supporting their clinical use for adoptive immunotherapy.

Methods

Cell lines and clonogenic survival assay

The human prostate cancer cell line PC3^{PSCA}, genetically engineered to express PSCA, has been described previously [21]. All feeder cells derived thereof, as well as the human myelogenous leukemia cell line K562, were maintained in RPMI-1640 (Life Technologies, Carlsbad, CA, USA) with 10% v/v heat-inactivated fetal bovine serum (Life Technologies), 2 mM L-glutamine (Life Technologies), 10 mM HEPES (Life Technologies), 100 U/mL penicillin (Life Technologies) and 0.1 mg/mL streptomycin (Life Technologies). The human embryonic kidney cell line HEK293T was used for production of lentiviral particles and maintained in Dulbecco's Modified Eagle's Medium containing 4.5 g/L glucose (Life Technologies) supplemented with 10% v/v heat-inactivated fetal bovine serum (Life Technologies), 10 mM HEPES (Life Technologies), 100 U/mL penicillin (Life Technologies) and 0.1 mg/mL streptomycin (Life Technologies). The use of primary glioblastoma multiforme (GBM) cells from patients was approved by the local ethical committee (approval reference #EK 323122008) of the Medical Faculty Carl Gustav Carus, Technische Universität Dresden. After obtaining written informed consent from all patients, primary GBM cell lines HT16360-1, HT18199 and

HT18223 were prepared using the Brain Tumor Dissociation Kit (P) (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultivated in Dulbecco's Modified Eagle's Medium as outlined above. All cell lines were cultivated at 37°C and 5% CO₂ in a humidified incubator. All permanent cell lines were authenticated (Multiplexion GmbH, Friedrichshafen, Germany). Adventitious virus polymerase chain reaction (PCR) testing and serological testing of cell culture supernatant from PC3^{PSCA} feeder cells were performed according to GLP standards by the Molecular Diagnostics Laboratory of German Red Cross Blood Donor Centre Frankfurt am Main, Germany. Samples were PCR-negative for hepatitis C virus, hepatitis B virus, hepatitis A virus, ParvoB19 and mycoplasma and PCR-positive for human immunodeficiency virus 1 and 2. Samples were seronegative for human immunodeficiency virus antigen and hepatitis B virus antigen. Long-term survival of PC3^{PSCA} feeder cells after 3 days of co-cultivation with NK cells was tested by plating these cells or 1.000 non-co-cultured PC3^{PSCA} feeder cells as control onto 10-cm dishes. After 2 weeks, cells were stained with crystal violet.

HLA and KIR genotyping

HLA genotyping was performed as described previously, and results are depicted in supplementary Table 1A,B [21]. IPD-KIR Ligand Calculator (EMBL-EBI, Cambridgeshire, UK) was employed to find KIR:KIR ligand mismatches in the graft-versus-host direction when NK cells were confronted with feeder cells (see supplementary Table 1A). Furthermore, KIRs were genotyped to address potential licensing mechanisms in NK cells of donors. In most donors, except donor 3, it was revealed that individually expressed dominant inhibitory KIR2DL1 and KIR2DL2/3 receptors bind to self-HLA, enabling licensing of NK cells. Donor 3 was found to contain one KIR2DL1 allele that was missing its cognate C2 ligand. Furthermore, in all donors expressing KIR3DL1, the cognate HLA ligand (Bw4) was missing (see supplementary Table 1C).

Lentiviral vectors and transduction of cells

For generation of PC3^{PSCA}-derived feeder cells, the self-inactivating lentiviral pHATrick vector [21] containing an internal spleen focus-forming virus (SFFV) U3 promoter followed by a multiple cloning site, a T2A *Thoesa asigna* virus element fused in frame to a puromycin-, zeocin- or hygromycin B-resistance gene, respectively, was used. IL-2 amplified from cDNA reversely transcribed from PBMCs together with an added Kozak sequence and appropriate restriction sites was cloned in frame to T2A-zeocin^R, resulting in pHATrick-IL-2-Zeo^R. A full-length chemically synthesized cDNA of membrane-bound 4-1BBL fused with a vesicular stomatitis virus glycoprotein (VSV-G) epitope tag was obtained from Eurofins Genomics (Ebersberg, Germany) and cloned, adding a Kozak sequence and restriction sites via PCR in frame to T2A-hygromycin B^R, resulting in pHATrick-4-1BBL-Hygro^R. A full-length synthetic cDNA of IL-15 was obtained from Eurofins Genomics. A Kozak sequence, a short (Gly₂Ser₁) linker and AgeI and SphI restriction sites were added to the IL-15 sequence by PCR. To generate mIL-15d, the vector pHATrick-MR1.1-DAP12-mut-Puro^R [22] was used. After excision of the MR1.1 coding region and Ig κ signal sequence with AgeI and SphI, the IL-15 fragment was ligated in frame with the non-functional DAP12 signaling adapter containing an N-terminal c-myc epitope tag, creating the vector pHATrick-mIL-15d-Puro^R. All vectors were verified by DNA sequencing. Lentiviral particle cells were produced by a transient three-vector packaging protocol [21]. Transduction of 5×10^4 PC3^{PSCA} cells in a 24-well with 2 mL of lentiviral supernatant was repeated on two subsequent days. Cells were selected with 20 μ g/mL puromycin (Clontech, Heidelberg, Germany) for mIL-15d, 200 μ g/mL hygromycin B (Life Technologies) for 4-1BBL or 250 μ g/mL zeocin (Life Technologies) for IL-2 for 1 week.

IL-2 enzyme-linked immunosorbent assay

The release of IL-2 by feeder cells was tested in a sandwich enzyme-linked immunosorbent assay (ELISA) (human IL-2 DuoSet ELISA, R&D Systems, Minneapolis, MN, USA). To determine ideal feeder cell densities for future co-cultivation, 1.0 , 2.5 or 5.0×10^4 cells per 24-well were seeded in a final volume of 1 mL complete NK MACS medium (Miltenyi Biotec). After 8, 24, 48, 72 and 96 h, supernatants were diluted and analyzed in triplets according to the manufacturer's protocol using a Synergy 2 multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA).

Western blot analysis

The 4-1BBL-transduced feeder cells were lysed in lysis buffer (10 mM Tris-HCl, pH 8.0; 140 mM NaCl; 1% Triton-X-100). Cell lysates were cleared by centrifugation, separated by SDS-PAGE under reducing conditions and transferred on a Westran polyvinylidene fluoride membrane (Whatman, Dassel, Germany). The protein-loaded and blocked membrane was incubated with a monoclonal anti-VSV-G antibody (1:4000; BD Biosciences, Franklin Lakes, NJ, USA) followed by a horseradish peroxidase-labeled rabbit anti-mouse secondary antibody (1:1000; Dako Denmark, Glostrup, Hovedstaden, Denmark). Equal loading of samples was determined by stripping and re-probing the membrane for total α -tubulin (1:8000; Santa Cruz, Biotechnologies, Dallas, TX, USA). Membranes were visualized and documented using the Luminata Forte Western horseradish peroxidase substrate (Merck Darmstadt, Germany) and the LAS 3000 imager (FujiFilm Europe).

PBMC, NK cell and B cell isolation

The use of human PBMCs, including NK cells from healthy donors, was approved by the local ethical committee (#EK242102007) of the Medical Faculty Carl Gustav Carus, Technische Universität Dresden. After obtaining oral and written consent, PBMCs were isolated by Biocoll gradient centrifugation (Biochrom, Berlin, Germany) and human cytomegalovirus (HCMV) IgG and IgM titers were analyzed using ELISA. Untouched NK cells were prepared using the negative NK Cell Isolation Kit (Miltenyi Biotec). Staining with anti-CD56-APC, anti-CD3-FITC and anti-CD19-PE antibodies (Miltenyi Biotec) routinely confirmed $\geq 90\%$ purity of CD56⁺ NK cells and depletion of CD3⁺ T cells and CD19⁺ B cells. B cells were isolated from PBMCs using the negative Pan B Cell Isolation Kit (Miltenyi Biotec). PBMCs and NK cells were held at 37°C and 5% CO₂ in a humidified incubator.

Expansion of PBMCs and NK cells

NK cells from PBMCs or untouched NK cells were expanded by co-cultivation with feeder cell lines. Briefly, 2.5×10^4 feeder cells were seeded in a 24-well with 1 mL complete RPMI-1640 medium for 24 h. The next day, the medium was substituted with 1 mL NK MACS medium (Miltenyi Biotec) supplemented with 1% NK MACS supplement (Miltenyi Biotec) and 5% human AB serum (c.c. pro, Oberdorla, Germany). After 4–8 h, 5×10^6 PBMCs or 5×10^5 NK cells were seeded to the feeder cells. Every 3–4 days, PBMCs or NK cells were passaged onto new feeder cells (Figure 2A). When using activation beads for expansion of NK cells, an additional 1000 IU/mL rhIL-2 (Proleukin S, Novartis, Nürnberg, Germany) and 20 ng/mL IL-21 (Miltenyi Biotec) were added to the medium. Cell numbers and percentages of NK cell, T cell and NKT cell fractions during PBMC expansion were analyzed by staining 2×10^5 PBMCs with anti-CD56-APC and anti-CD3-PE (Miltenyi Biotec). Proliferation of NK cells was analyzed by carboxyfluorescein succinimidyl ester (CFSE) staining. Briefly, 1×10^7 freshly isolated NK cells were stained with carboxyfluorescein succinimidyl ester (BioLegend, San Diego, CA, USA) according to

the manufacturer's protocol and expanded. The carboxyfluorescein succinimidyl ester intensity of 1×10^5 NK cells was measured daily over the course of 6 days via flow cytometry. Total cell numbers of expanded NK cells were counted by staining cells with anti-CD56-APC (Miltenyi Biotec). IgG isotype controls were included by all measurements. Stained cells were measured using MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec) and analyzed using FlowJo software vX.0.7 (FlowJo, LLC, Ashland, OR, USA).

Flow cytometry analyses of cell surface markers and cytokine production

For analysis of cell surface expression of mIL-15d, at least 3×10^5 feeder cells were stained with anti-c-myc-FITC (Miltenyi Biotec). To determine the amount of contaminating PC3^{PSCA}-derived feeder cells in expanded NK cell samples, cells were simultaneously stained with 1.5 μ g biotinylated scFv(AM1)-P-BAP (anti-PSCA) [6] followed by an anti-Biotin-VioBlue secondary antibody (Miltenyi Biotec) and anti-CD56-APC (Miltenyi Biotec). To assess classical and non-classical HLA as well as LFA-3 expression levels, 1×10^5 PC3^{PSCA} cells were stained with FITC mouse anti-human HLA-ABC (BD Biosciences), anti-HLA-E-PE, anti-HLA-G-APC and anti-CD58(LFA-3)-APC (Miltenyi Biotec). To determine the purity of isolated and expanded NK cells, and to discriminate from T cells and NKT cells, respectively, 2×10^5 cells were simultaneously stained with anti-CD56-APC and anti-CD3-PE (Miltenyi Biotec). For phenotypic analysis of NK cell subpopulations, 2×10^5 NK cells were stained with anti-CD56-APC (Miltenyi Biotec) and anti-CD16-PE (eBioscience, Frankfurt am Main, Germany). For further detailed phenotypic analysis, NK cells were analyzed using anti-CD337(NKp30)-PE, anti-CD336(NKp44)-PE, anti-NKp46-PE, anti-NKp80-PE, anti-CD159c(NKG2C)-APC, anti-CD314(NKG2D)-PE, anti-CD226(DNAM-1)-PE, anti-KIR2DL2/L3/S2-PE (clone DX27), anti-KIR3DL1-PE (clone DX9), anti-CD159a(NKG2A)-APC, anti-CD85j(ILT2)-APC, anti-TIM-3-PE, anti-CD279(PD-1)-PE, anti-TIGIT-Biotin, anti-Biotin-PE, anti-CD25-APC (Miltenyi Biotec) and anti-KIR2DL1/S1/S4-PE (clone HP-3E4, BD Pharmingen). Inflammatory cytokine production was analyzed via stimulation of NK cells with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ g/mL ionomycin (Merck, Germany); 10 μ g/mL brefeldin A (Merck, Germany) was added 1 h later to prevent cytokine secretion. After 5 h, intracellular staining of NK cells was performed using MACS Inside Stain Kit, anti-TNF- α -APC and anti-IFN- γ -APC (Miltenyi Biotec). Epidermal growth factor receptor (EGFR) expression in primary GBM cell lines HT16360-1, HT18199 and HT18223 was investigated using 5 μ g cetuximab (Erbix, Merck) and secondary Cy3 AffiniPure goat anti-human IgG, Fc fragment specific antibody (Jackson ImmunoResearch, Cambridgeshire, UK). To assess classical HLA, GBM cells were stained with FITC mouse anti-human HLA-ABC (BD Biosciences). Appropriate isotype controls were included in all measurements. Stained cells were measured by a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec) and analyzed by FlowJo software vX.0.7 (FlowJo).

Chromium release assay

The cytotoxicity of expanded NK cells was tested using K562 target cells that were devoid of protective HLA class I and HLA-E expression levels [23,24] or primary GBM cell lines HT18199 and HT18223. ADCC of NK cells was analyzed using cetuximab-treated (75 μ g/mL) or untreated primary GBM cell lines HT16360-1, HT18199 and HT18223. Target cell lysis by NK cells was assessed using standard chromium release assay. Briefly, 2×10^6 target cells were labeled with 1.5 MBq sodium ⁵¹chromate (Hartmann Analytic, Braunschweig, Germany) and incubated at 37°C and 5% CO₂. After 1 h, cells were washed several times with PBS and seeded as triplicates in a round bottom 96-well plate (2×10^3 cells per well). The differentially expanded NK cells were starved for 24 h in complete NK MACS medium devoid of cytokines and then added to labeled target cells at

effector to target ratios of 7.5:1 and 15:1 (K562) or 5:1 and 10:1 (GBM cells). After 4 h (K562) or 18 h (GBM cells) of co-cultivation, 25 μ L of cell supernatant was mixed with 150 μ L of scintillation solution Ultima Gold (PerkinElmer, Boston, MA, USA) in a 96-well plate by shaking for 3–5 min at room temperature. The chromium release was measured using a Wallace 1450 Microbeta Trilux Liquid Scintillation and Luminescence Counter (PerkinElmer). Maximal releases were measured by treating target cells with 5% Triton X-100 (Serva, Heidelberg, Germany) and minimum releases by cultivation of target cells with medium alone. Percentage of specific lysis was calculated using the standard formula $100 \times (\text{cpm release target cells} - \text{cpm minimum release}) / (\text{cpm maximum release} - \text{cpm minimum release})$.

CD107a degranulation assay

Tolerance to self of NK cells differentially expanded for 24 days and starved for 24 h in complete NK MACS medium devoid of cytokines was assessed by co-cultivation with freshly isolated autologous B cells and K562 cells in an effector to target ratio of 1:1 and 1:5 in 200 μ L complete NK MACS medium in a V-bottom 96-well plate. After 1 h of co-cultivation, 2 mM monensin (dilution 1:40, Sigma-Aldrich, Taufkirchen, Germany) and anti-CD107a (LAMP-1)-VioBlue (Miltenyi Biotec) were added to the wells and incubated for an additional 3 h. Afterward, CD56 staining was performed and cells were measured by MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec) and analyzed by FlowJo software vX.0.7 (FlowJo). Percentage of CD107a-positive NK cells in medium alone was subtracted from percentage of CD107a-positive NK cells co-cultivated with K562 or B cells.

Statistical analysis

All experiments were performed with NK cells from four or five healthy donors. Parametric *t*-test with Welch's correction was used to compare differences between groups in analysis of cytokine production and ADCC. Differences between groups in other experiments were examined for statistical significance using unpaired nonparametric Mann-Whitney U test. Values of *P* < 0.05 were considered statistically significant.

Results

Generation of PC3^{PSCA} feeder cell lines expressing IL-2, 4-1BBL, and/or mIL-15d

The human prostate cancer cell line PC3 was chosen as the feeder cell line for expansion of NK cells since it expresses HLA-E molecules as well as HLA class I molecules (see supplementary Figure 1A), including dominant C1, C2 and Bw4 ligands for inhibitory KIRs and therefore should avoid missing self-recognition of virtually all donor NK cells (see supplementary Table 1A). Moreover, inhibitory HLA-G and CD58/LFA-3 expression was detected on PC3 cells (see supplementary Figure 1A). In particular, cellular adhesion mediated by CD58/LFA-3 is known to augment activation of CD2-positive NK cells [25,26]. Of note, surface expression of HLA-E in PC3 cells is not only stabilized by cleaved nonamer signal peptides derived from HLA-C but also by HLA-G-derived nonamer peptide displaying a methionine at position –21 of the signal peptides (see supplementary Figure 1B) [27,28].

In particular, it was decided to utilize previously described PC3^{PSCA} cells [21], which were genetically modified to express the surface marker PSCA. PSCA enables specific detection of feeder cells in co-cultures using monoclonal antibodies. PC3^{PSCA} cells were transduced with lentiviral pHATrick vectors encoding IL-2, 4-1BBL fused to a VSV-G epitope tag and mIL-15d containing a c-myc epitope tag, respectively. The vectors contain a *Thosea asigna* virus T2A endoproteolytic cleavage site, allowing simultaneous expression of the gene of interest and the resistance gene (Figure 1A). To generate a

membrane-bound IL-15 (mIL15d), IL-15 was fused to the non-functional signal adapter protein DAP12 (mutDAP12) containing a phosphorylation-defective ITAM, which increases its half-life time on the cell surface (unpublished data). Transduction and antibiotic selection of the human prostate cancer cell line PC3^{PSCA} using vectors encoding IL-2, 4-1BBL and mIL-15d alone or in combination resulted in feeder cell lines depicted in Figure 1B. Generation of all feeder cell lines secreting IL-2 originated from PC3^{PSCA}-IL-2 cells. To determine IL-2 secretion at different cell densities, increasing numbers of PC3^{PSCA}-IL-2 feeder cells were plated and analyzed over time using sandwich ELISA. The results indicated an increased concentration of secreted IL-2 in the medium proportional to the density of seeded cells (Figure 1C). For subsequent experiments with NK cells, 2.5×10^4 feeder cells per 24-well were employed, which were capable of producing 77.7 ng/mL (± 0.13) IL-2 in 72 hours and 98.2 ng/mL (± 0.55) IL-2 in 96 hours and which gave sufficient space for expansion of NK cells. Generation of feeder cell lines genetically engineered with 4-1BBL originated from PC3^{PSCA}-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL cells. Immunoblot analysis using total cell lysates and an anti-VSV-G antibody for detection demonstrated similar expression levels of 4-1BBL protein with a molecular mass close to the calculated value of 29.5 kDa in PC3^{PSCA}-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL feeder cells. (Figure 1D). Generation of feeder cells with ectopic expression of mIL-15d was accomplished in individual transductions of precursor cell lines as depicted in Figure 1B. Robust and similar surface expression levels of mIL-15d in PC3^{PSCA}-mIL-15d, PC3^{PSCA}-IL-2-mIL-15d, PC3^{PSCA}-4-1BBL-mIL-15d and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d cells were confirmed by flow cytometry using anti-c-myc antibody (Figure 1E).

Expansion of purified primary NK cells with PC3^{PSCA}-derived feeder cell lines

CFSE-stained primary NK cells from five healthy donors were cultivated on different feeder cell lines for 6 days and proliferation was monitored daily using flow cytometry analysis (Figure 2B). As expected, NK cell expansion failed when using parental PC3^{PSCA} cells and also failed when using feeder cell lines devoid of ectopic IL-2 secretion but modified with mIL-15d or 4-1BBL alone or in combination (Figure 2B, upper panel). After 9 days of co-culturing NK cells with PC3^{PSCA}-mIL-15d, PC3^{PSCA}-4-1BBL or PC3^{PSCA}-4-1BBL-mIL-15d feeder cells, NK cell numbers remained low, as reflected by the mean expansion factors ranging from 1.4 to 2.9 (Figure 2C), which is in accordance with the results obtained by CFSE analysis. By contrast, NK cells co-cultivated with IL-2-secreting feeder cells showed proliferation starting at day 4 (Figure 2B, lower panel). Of note, NK cells co-cultivated with PC3^{PSCA}-IL-2-4-1BBL had a mean expansion factor of 40.9 ± 8.1 , and those co-cultivated with PC3^{PSCA}-IL-2-4-1BBL-mIL-15d had a mean expansion factor of 47.8 ± 13.0 (Figure 2C). These proliferation factors were significantly better when compared with expansion factors achieved by co-cultivation with PC3^{PSCA}-IL-2 and PC3^{PSCA}-IL-2-mIL-15d feeder cells, which showed only suboptimal cell growth (mean expansion factors 5.1 ± 2.2 and 6.3 ± 2.7 , respectively), and were even better when compared with NK cell expansion using commercially available anti-CD2/anti-NKp46 activation beads plus continuous IL-2/IL-21 treatment (mean expansion factor 16.6 ± 9.9) (Figure 2C). Long-term expansion with IL-2-secreting feeder cell lines over 27 days showed similar differences in expansion rates (Figure 2D). Again, best mean expansion factors in NK cell expansion were achieved when using PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells (86.2 ± 38.8); this was somewhat better when compared with NK cell expansion factors accomplished by co-cultivation of NK cells on PC3^{PSCA}-IL-2-4-1BBL feeder cells (66.1 ± 37.0). Of note, NK cell expansion with PC3^{PSCA} feeder cells was also successful when using RPMI-1640 medium supplemented with L-glutamine and human AB serum (see supplementary Figure 2A) instead of supplemented NK MACS medium.

However, repetitive stimulation with PC3^{PSCA} feeder cells is necessary by passaging NK cell onto new feeder cells every 3–4 days (see supplementary Figure 2B). In addition, expanded and subsequently starved NK cells can be re-stimulated and proliferated when co-cultured again with PC3^{PSCA} feeder cells but fail to proliferate when confronted with K562 target cells (see supplementary Figure 2C). Of note, after 3–4 days, PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells completely disappeared when co-cultured with primary NK cells, as revealed by flow cytometry analysis of PSCA-positive and CD56-positive cell fractions and clonogenic survival assays (see supplementary Figure 3).

Expansion of primary NK cells from PBMCs with IL-2-secreting PC3^{PSCA}-derived feeder cell lines

So far, genetically modified K562 feeder cell lines, which are devoid of protective classical HLA class I expression levels, have been described as selectively expanding NK cells from PBMC samples that still contain fractions of allogeneic T cells [14,18]. It was of special interest whether PC3^{PSCA}-derived feeder cells could enhance outgrowth of NK cells from allogeneic PBMC samples while also limiting proliferation of T cells. To this end, different IL-2-secreting feeder cell lines were used for expansion of PBMCs, and the percentage of NK cells, T cells and NKT cells was determined over 18 days by flow cytometry. Among NK cell expansions using PC3^{PSCA}-derived feeder cells starting from an unfractionated PBMC product, we revealed that only PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cell lines facilitated a gradual outgrowth of NK cells that was associated with a significant decrease in T cell fractions (Figure 3A). More specifically, when using PC3^{PSCA}-IL-2-4-1BBL or PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells, the median percentage of CD56⁺CD3[−] NK cells in the PBMC samples rapidly increased starting at day 4 and reached a plateau at day 11. At day 18 of expansion using PC3^{PSCA}-IL-2-4-1BBL or PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells, mean percentages of NK cells of 55.2% and 64.3% in the expanded cell population were revealed, which were higher than the relative amounts of CD56[−]CD3⁺ T cells (29.1% and 19.3%). In contrast to feeder cells providing simultaneous IL-2 and 4-1BBL stimulation, IL-2- and IL-2-mIL-15d-modified feeder cells did not sufficiently promote selective outgrowth of NK cells from PBMC samples. More specifically, induction of NK cell growth was noted, yet the mean percentages of NK cells in PBMC samples remained lower than the relative amount of T cells. The frequency of CD56⁺CD3⁺ NKT cells barely increased over time using the different feeder cell lines and remained below 10% at day 18. The relative amount of CD56⁺CD3[−] NK cells at the end of the expansion phases directly correlated to the corresponding maximum expansion factors (Figure 3B). Co-cultivation of PBMCs with PC3^{PSCA}-IL-2-4-1BBL-mIL-15d resulted in the highest expansion of NK cells, with a mean expansion factor of $38.5 (\pm 14.3)$. PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-mIL-15d feeder cells induced intermediate proliferation rates, resulting in expansion factors of $22.0 (\pm 6.3)$ and $15.9 (\pm 7.6)$, respectively. Yet the mean maximum expansion factor of NK cells obtained with PC3^{PSCA}-IL-2 feeder cells was only $9.3 (\pm 5.9)$. A significant T cell outgrowth was hardly seen when utilizing any of the IL-2-producing feeder cells and is reflected by low expansion factors for T cells ranging from 2.7 to 4.4 (Figure 3C). In summary, feeder cells induced a selective outgrowth of NK cells, whereas significant expansion of T cells was not observed. As already revealed regarding expansion of purified NK cells, best expansion rates were achieved using PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells followed by PC3^{PSCA}-IL-2-4-1BBL feeder cells. Therefore, PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells were chosen for further experiments.

Characterization of feeder cell-expanded primary NK cells

To assess the effect of expansion protocols on the outgrowth of different NK cell subsets, CD56 and CD16 expression on purified NK

Fig. 1. Generation of PC3^{PSCA} feeder cell lines. (A) Schematic representation of IL-2, 4-1BBL and mIL-15d coding sequences in the lentiviral vector backbone pHATrick. The internal SFFV U3 promoter of the provirus regulates the expression of the transgenes. Furthermore, the vectors contain a *Thosea asigna* T2A endoproteolytic cleavage site, allowing simultaneous expression of the gene of interest and resistance gene; 4-1BBL was fused to a VSV-G epitope tag for detection. The mIL-15d consists of IL-15, a c-myc epitope tag and the non-functional signal adapter protein DAP12 (mutDAP12), which harbors T91S and T102S mutations in its ITAM. (B) Overview of the stepwise generation of the different PC3^{PSCA} feeder cell lines by lentiviral transduction. (C) Long-term measurement of IL-2 secretion of PC3^{PSCA}-IL-2 cells up to 4 days by sandwich ELISA. Cells seeded at densities of 1.0, 2.5 or 5.0 × 10⁴ cells per 24-well in 1 mL complete NK MACS medium. (D) Immunoblot analysis of total protein lysates of wildtype (control) and transduced PC3^{PSCA} feeder cells demonstrating expression of 4-1BBL. The detection of 4-1BBL was accomplished using a VSV-G-specific antibody and HRP-labeled anti-mouse secondary antibody. Staining using α-tubulin antibodies demonstrated equal loading of protein lysates. (E) The surface expression of mIL-15d was determined by flow cytometry analysis using anti-c-myc antibody (filled areas). Cells stained with IgG isotype antibody (open areas) served as control. HRP, horseradish peroxidase; L, leader; pCMV, promoter of cytomegalovirus; R-U5 and ΔU3-R-U5, long terminal repeats for integration of the provirus; SFFV, spleen focus-forming virus.

Fig. 2. Expansion of isolated primary NK cells from five healthy donors with PC3^{PSCA} feeder cell lines. (A) Schematic representation of the expansion schema. (B) Representative CFSE signals of stained primary NK cells from one donor on six consecutive days using parental PC3^{PSCA} and seven different PC3^{PSCA} feeder cell lines. For comparison, expansion of NK cells using activation beads and continuous rhIL-2/IL-21 cytokine support is included. The upper row represents NK cells co-cultured with unmodified PC3^{PSCA} feeder cells as well as with transgenic lines without IL-2 secretion. PC3^{PSCA} feeder cells transduced with the transgene for IL-2 secretion, represented in the lower row, showed a decrease of the CFSE signal from day 4, comparable with the effects of activation beads. (C) Overview of the grand means and expansion factors of primary NK cells cultured with the eight different feeder cell lines or activation beads as control. Expansion factor is calculated via the cell count at day 9, relative to the cell count at the start of expansion at day 1. (D) Overview of the grand means and maximal expansion factors of primary NK cells cultured with IL-2 secreting feeder cell lines or activation beads as control. Maximal expansion factor is calculated via the maximum cell count within the cultivation of 27 days, relative to the cell count on day 1. (Color version of figure is available online).

Fig. 3. Expansion of NK cells from PBMCs with four different IL-2 secreting PC3^{PSCA} feeder cell lines. (A) Percentage of NK cells (CD56⁺/CD3⁺), T cells (CD56⁺/CD3⁺) and NKT cells (CD56⁺/CD3⁺) relative to the total number of PBMCs (gates on living and single-cell PBMCs) was measured by flow cytometry for 18 days using anti-CD56 and anti-CD3 antibodies. Results were shown as mean $\pm$ SD of PBMCs from five different donors. Growth of primary NK cells (B) and T cells (C) from PBMCs of five healthy donors co-cultured with IL-2 secreting feeder cell lines is summarized by maximal expansion factors and the grand means. Maximal expansion factor is calculated via the maximum cell count within the cultivation of 18 days, relative to the cell count of seeded cells at day 0. SD, standard deviation.

cells from five donors expanded with PC3^{PSCA}-IL-2-4-1BBL or PC3^{PSCA}-IL-2-4-1BBL-mIL-15d was analyzed by flow cytometry and compared with data obtained from freshly isolated untouched NK cells or NK cells expanded by activation beads (Figure 4A). Freshly isolated NK cells showed mainly a CD56dim/CD16⁺ phenotype (89% $\pm$ 8.1%), which is typical for the major NK cell population in peripheral blood and indicates mature NK cells [29]. Expansion of NK cells using feeder cells resulted in heterogeneous NK cell populations with three dominant subsets, mainly CD56bright/CD16⁺, accounting for approximately 50%, and CD56bright/CD16[−] and CD56dim/CD16⁺, each accounting for 20–25% of NK cells, respectively (Figure 4B). In contrast to the results obtained with feeder cells, the expansion of NK cells using the activation bead protocol led to the appearance of a major subset of CD56bright/CD16⁺ cells accounting for 64% ($\pm$ 15.7%) and an intermediate subset of CD56bright/CD16[−] cells accounting for 33% ($\pm$ 14.5%) of expanded cells. However, the CD56dim/CD16⁺ subset, representing mature NK cells [29], was vastly reduced (1% $\pm$ 0.7%), which is in contrast to the results obtained in freshly isolated NK cells and feeder cell-expanded NK cells. In addition, the ability to produce cytokines was investigated after 6 hours of stimulation with PMA and ionomycin by intracellular staining. Both CD56bright and CD56dim NK cell subsets expanded with PC3^{PSCA}-IL-2-4-1BBL or

PC3^{PSCA}-IL-2-4-1BBL-mIL-15d produced TNF- α and IFN- γ (see supplementary Figure 4). However, CD56bright NK cells showed a significantly larger expression of TNF- α (average 23.4% versus 48.8%) and IFN- γ (average 26.7% versus 53.0%) compared with CD56dim NK cells (see supplementary Figure 4A,B).

Next, the relative amounts of cells with expression of various activating NK cell receptors, inhibitory NK cell receptors, activation markers and immune checkpoint receptors were investigated. Differentially expanded NK cells from five healthy donors were each compared with corresponding freshly isolated NK cells (Figure 5 and supplementary Figure 5). Irrespective of the used expansion method, activating and co-activating receptors, including Nkp30, Nkp44, Nkp46, Nkp80, NKG2C and DNAM-1, were upregulated in expanded NK cells. However, no dissimilarities between the differentially expanded NK cells, with the exception of Nkp44, which had a slightly broader expression after expansion with activation beads, were detected. NK cells from all expansion groups contained NKG2D NK cell fractions >90%, which did not differ significantly from those observed in freshly isolated NK cells (Figure 5A). The fraction of NK cells expressing KIR2DL2/L3/S2 decreased in all expansion groups. In accordance with KIR genotyping, no signals for KIR3DL1 were observed for NK cells from donor 4. The frequency of KIR3DL1-

Fig. 4. CD56/CD16 NK cell subset compositions after differential expansion of NK cells. Freshly isolated NK cells as well as activation beads, PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cell-expanded NK cells 10 days after isolation from five healthy donors were analyzed concerning their CD56 and CD16 expression levels by flow cytometry. (A) Representative CD56/CD16 staining of NK cells (gray dots) from one donor is shown. NK cells stained with IgG isotype antibodies (black dots) served as control. (B) Overview of the four different NK cell subpopulations from all five donors before and 10 days after expansion is depicted. Displayed are mean values and SD of two independent experiments. SD, standard deviation.

positive NK cells from all other donors was slightly reduced in all expansion groups. The frequency of KIR2DL1/S1/S4-positive NK cells in all groups of differentially expanded NK cells remained, on average, below 25%. Yet expansion using feeder cells revealed slight donor-dependent increases in relative amounts of KIR2DL1/S1/S4-positive NK cells, which might be related to differential compositions of KIR2DL1/S1/S4 alleles (see supplementary Table 1C). In particular, the percentage of KIR2DL1/S1/S4-positive NK cells from donor 1, which lack inhibitory KIR2DL1 alleles and instead contain KIR2DS1, moderately increased during expansion with feeder cells. Yet the fraction of CD94/NGK2A-positive cells as well as of NK cells expressing the co-inhibitory ILT2 receptor was strongly increased in all groups of expanded NK cells when compared with corresponding freshly isolated NK cells. Activation bead-expanded NK cells showed a significantly higher frequency of CD94/NGK2A and, partially, ILT2-positive cells when compared with NK cells expanded by feeder cells (Figure 5B). Additional analysis of the fraction of cells expressing TIGIT and PD-1 associated with exhaustion of lymphocytes revealed low frequencies of PD-1- and TIGIT-positive cells in freshly isolated NK cells as well as in all groups of expanded NK cells (Figure 5C). By contrast, the relative number of TIM-3-positive cells, which is associated with cytokine-induced activation and maturation of NK cells [30], was strongly amplified after expansion of NK cells using feeder cells or activation beads. Yet, in feeder cell-expanded NK cells from different donors, 35–64% of NK cells were found to express the alpha chain (CD25) for high-affinity IL-2 receptor (CD25). Of note, no such strong increase in CD25-positive cells was observed in NK cells that were expanded using activation beads (Figure 5C).

Feeder cell-expanded NK cells exhibit robust cytotoxicity against K562 target cells

It was of special interest whether cytotoxic effector functions of expanded NK cells remained stable, and therefore NK cells expanded with PC3^{PSCA}-IL-2-4-1BBL, PC3^{PSCA}-IL-2-4-1BBL-mIL-15d and activation beads, respectively, were investigated after 3 and 4 weeks of expansion. Cytotoxic activity of expanded NK cells against K562 leukemia cells was investigated by chromium release assay. Of note, the cytotoxicity of PC3^{PSCA}-IL-2-4-1BBL or PC3^{PSCA}-IL-2-4-1BBL-mIL-15d-expanded NK cells at effector to target ratio of 7.5:1 increased in both groups, from approximately 50% at week 3 to 80% at week 4. Similar results were seen when using a higher effector to target ratio of 15:1 (Figure 6A). After 3 weeks of expansion, activation bead-expanded NK cells showed a high average cell lysis, ranging from 73% at effector to target ratio of 7.5:1 to 88% at effector to target ratio of 15:1. In contrast to NK cells expanded using feeder cells, the viability of NK cells expanded using activation beads, including continuous exogenous cytokine support, decreased after 4 weeks (data not shown). Therefore, only expanded NK cells from one donor could be included for testing cytotoxicity against K562 targets. However, such NK cells were still effective in killing K562 targets (Figure 6A). In summary, viability of feeder cell-expanded NK cells remained stable, and their cytotoxicity toward K562 targets increased during prolonged cultivation. By contrast, although activation bead-expanded NK cells preserved their lytic cytotoxicity, their viability gradually decreased during cultivation. Since PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells induced similar phenotypes and cytotoxicity toward K562 targets, subsequent experiments utilized PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells, which produced the best NK cell numbers.

Fig. 5. Expression levels of NK cell surface markers. Freshly isolated NK cells were analyzed for selected surface markers in comparison to PC3^{PSCA}-IL-2-4-1BBL (blue lines), PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cell- (green lines) or activation bead-expanded NK cells (red lines) from five healthy donors 10 days after isolation. Expression levels of activating and co-activating receptors (A) and inhibitory and co-inhibitory receptors (B) as well as exhaustion and activation markers (C) were measured. Percentage of KIR2DL1/S1/S4-positive NK cells from donor 1 are marked by bold lines and squares. Displayed are mean values of three independent experiments (* $P < 0.05$, ** $P < 0.01$). (Color version of figure is available online).

Tolerance of polyclonal feeder cell-expanded NK cells to autologous cells and to allogeneic tumor cells with protective levels of inhibitory NK cell ligands

To evaluate whether expanded NK cells remained tolerant to self, NK cells expanded for 24 days using PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells were confronted with autologous B cells and K562 cells at different effector to target ratios in a CD107a degranulation assay. When given to autologous B cells, the feeder cell-expanded NK cells

remained unresponsive, as indicated by low frequencies of CD107a signals ranging from 1.2% (0.0–2.9%) at an effector to target ratio of 1:1 to 2.8% (0.6–5.6%) at an effector to target ratio of 1:5. Yet NK cells showed significantly increased degranulation when encountering K562 cells devoid of protective HLA class I and HLA-E expression levels, as demonstrated by the increased frequencies of CD107a-positive NK cells ranging from, on average, 12.7% (7.9–16.9%) at an effector to target ratio of 1:1 to 9.5% (4.9–14.1%) at an effector to target ratio of 1:5 (Figure 6B). Next, cytotoxicity of feeder cell-expanded NK cells

Fig. 6. Cytotoxicity induced by missing self-recognition and tolerance to self of expanded NK cells. (A) PC3^{PSCA}-IL-2-4-1BBL, PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cell- or activation bead-expanded NK cells from four healthy donors 3 weeks and 4 weeks after isolation were co-cultured with ⁵¹chromium-loaded K562 cells at different E:T ratios for 4 h. The mean of specific cell lysis of triplets of one representative chrome release assay is shown. (B) Autoreactivity of expanded NK cells was analyzed by CD107a degranulation assay. Therefore, PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cell-expanded NK cells from five healthy donors were co-cultivated with autologous B cells or K562 cells (positive control) at different E:T ratios for 4 h. NK cells in medium alone served as an internal control. CD107a-positive NK cells were measured by flow cytometry. Percentage of CD107a-positive NK cells in medium alone was subtracted from percentage of CD107a-positive NK cells co-cultivated with B cells or K562 (**P* < 0.05, ***P* < 0.01). E:T, effector to target.

toward allogeneic HT18223 and HT18199 primary glioblastoma cells was assessed using chromium release assay (Figure 7A). Both GBM cell lines expressed robust amounts of classical HLA on their cell surface, which can induce tolerance in NK cells (Figure 7C). Conversely, mismatches of single KIR-positive NK cells to C1 and C2 ligands as well as increased levels of activating ligands for NCRs or NKG2D on target cells are considered to decrease the activation threshold of NK cells. Calculated KIR:KIR ligand mismatches to genotyped HT18223 and HT18199 GBM cells (see supplementary Table 1) and licensing status are depicted as inlets in Figure 7A. When expanded NK cells from four donors with a KIR:KIR ligand match based on analysis of HLA alleles, yet with non-licensed NK cells missing Bw4 on target cells, were confronted to HT18223 GBM cells, no substantial cytotoxic response was observed. By contrast, NK cells that were expanded by activation beads, yet displayed similar expression of activating and inhibitory NK cell receptors as feeder cell-expanded NK cells (Figure 5), demonstrated a robust cytotoxic response toward HT18223 cells. A similar outcome was obtained when using HT18199 target cells (Figure 7A). In this experiment, feeder cell-expanded NK cells from donor 1, which had a non-licensed KIR3DL1 that was missing its cognate ligand on the target cells, as well as NK cells from donor 3, which also contained a non-licensed KIR3DL1

and, additionally, a non-licensed KIR2DL1 that was missing its cognate ligand on target cells, remained unresponsive. Interestingly, feeder cell-expanded NK cells from donors 2 and 4, which both contained a non-licensed Bw4 that was missing its cognate ligand in the target cells and additionally exhibited a C2 mismatch in the target cell direction, also remained non-responsive to HT1899 GBM cells. That an insufficient level of activating signals on GBM target cells accounted for the results was rendered unlikely since activation bead-expanded NK cells readily killed the HT18223 and HT18199 GBM cells. Furthermore, decreased cytotoxic function of feeder cell-expanded NK cells was also unlikely since they efficiently eradicated K562 cells (Figure 6A). Interestingly, cytotoxicity toward HT18223 and HT18199 GBM cells was unleashed by pre-treatment of feeder cell-expanded NK cells with 50 IU/mL rhIL-2 (Figure 7B). We therefore concluded that feeder cell-expanded NK cells that expressed high-affinity IL-2 receptor (see CD25 expression in Figure 5C) became hyper-activated after treatment with 50 IU rhIL-2. In contrast, we suggest that inhibitory NK cell receptor signaling in NK cells, which were expanded using PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells and without subsequent rhIL-2 stimulation, is mostly functional when encountering target cells with protective levels of HLA molecules. Moreover, since the inhibitory receptors

Fig. 7. NK cells expanded by co-cultivation with PC3^{PSCA}-IL-2-4-1BBL-mIL-15d remain tolerant for allogeneic tumor cells with expression of HLA class I molecules. (A) Expanded NK cells from four different donors were confronted with allogeneic primary GBM cells from patient HT18223 or patient HT18199. Cytotoxicity of NK cells was analyzed using chromium release assay at an E:T ratio of 5:1. All donors contained non-licensed NK cells for Bw4 (gray boxes). Additionally, donors 2 and 5 contained KIR2DL1 that was missing its C2 ligand in HT18199 cells (black boxes). Donor 3 contained non-licensed KIR2DL1 NK cells missing the C2 ligand in HT18199 cells (gray box). Note the increased lytic cytotoxicity of activation bead-expanded NK cells, whereas NK cells expanded by PC3^{PSCA}-IL-2-4-1BBL-mIL-15d remained unresponsive. * $P < 0.05$. (B) Cytotoxicity toward HT18223 and HT18199 GBM cells was induced by pre-treatment of PC3^{PSCA}-IL-2-4-1BBL-mIL-15d-expanded NK cells with 50 IU/mL rhIL-2 (* $P < 0.05$). (C) Expression of HLA-ABC and EGFR on GBM target cells was measured by flow cytometry (filled areas). EGFR surface expression was assessed using cetuximab and secondary APC-conjugated antibody. Cells stained with isotype antibody (open areas) served as control. (D) Induction of ADCC of NK cells toward cetuximab-marked primary GBM cells was analyzed using chromium release assay at an E:T ratio of 5:1. Note that the strength of the ADCC is directly correlated to amount of EGFR surface expression on target cells (mean $\pm$ SD; * $P < 0.05$). APC, allophycocyanin; E:T, effector to target; SD, standard deviation.

NKG2A and ILT2 were expressed in 82.5% (77.8–86.2%) and 61.2% (55.4–66.6%) of polyclonal feeder cell-expanded NK cells, respectively, we assume that cell numbers of supposed alloreactive CD94/NKG2A- and ILT2-negative single KIR2DL1-positive NK cells from donors 2 and 4 were too low to induce significant cytotoxic responses toward HT1899 target cells. In addition, the 18-h protocol used for the cytotoxicity assays should not lead to the outgrowth of alloreactive NK cells in numbers sufficient for the induction of potent cytotoxic effects, which have been reported to require a longer period of time [31,32].

ADCC of feeder cell-expanded NK cells toward cetuximab-marked allogeneic tumor cells

Thus far, the characterization of feeder cell-expanded polyclonal NK cells has revealed functional tolerance to autologous cells and tumor cells expressing protective levels of inhibitory NK cell receptors. To address whether such NK cells can be redirected to kill GBM cells, a therapeutic anti-EGFR monoclonal antibody (cetuximab) was employed to induce ADCC. For the experiments, HLA class I-positive primary GBM cells with high (HT16360-1), intermediate (HT18199) and no (HT18233) surface expression levels of EGFR were used as targets (Figure 7C). When confronted with GBM targets again, NK cells did not unleash substantial lytic cytotoxicity (Figure 7D). Yet we observed a robust killing of EGFR-positive target cells when employing cetuximab. Moreover, the observed killing efficiencies directly correlated with EGFR expression levels on target cells (Figure 7C), indicating that feeder cell-expanded NK cells can execute lytic cytotoxicity when properly activated.

Discussion

Recent studies suggest that the activation status of NK cells differentially modulates recognition of self-inhibitory ligands and activating ligands on target cells. In particular, an inflammatory environment or hyper-activation of NK cells using high doses of IL-2 or IL-15 can yield NK cells in which inhibitory receptor binding cannot preclude killing of HLA-positive target cells [15]. This finding is potentially relevant when employing *ex vivo* expanded hyper-activated NK cells since they could contribute to GVHD if a disturbed homeostasis of non-malignant tissue induces expression of activating NK cell ligands [19].

In this study, we sought to establish feeder cell lines for autonomous expansion of educated human peripheral NK cells. In particular, by omitting high cytokine doses as well as missing self-recognition of feeder cells, hyper-activation of NK cells ought to be avoided. For this purpose, we chose PC3^{PSCA} cells, derivatives of the prostate cell line that endogenously express C1, C2 and Bw4 ligands for inhibitory KIRs and ILT2 as well as non-classical HLA-E for CD94/NKG2A and therefore provide main inhibitory self-ligands for NK cells from virtually any donor.

In this study, we demonstrate the expansion of educated NK cells by using PC3^{PSCA} cells genetically modified to secrete IL-2 and additionally gene-engineered to express a membrane-bound form of IL-15 and co-stimulatory 4-1BBL. Surprisingly, only feeder cell lines modified with the secreted form of IL-2 were capable of inducing proliferation of NK cells, whereas feeder cells genetically modified with membrane-bound IL-15, 4-1BBL or both failed to induce substantial proliferation. In particular, juxtacrine activation of IL-2/IL-15 receptor of NK cells by membrane-bound IL-15 on K562 feeder cells [14] or by membrane-bound IL-15 of genetically engineered NK cells [33] has been reported to robustly activate and expand NK cells. Thus far, it is tempting to speculate that the mIL-15d molecule used in this study might have decreased activity since its mut-DAP12-proportion might have a higher probability of forming homodimers, which might affect binding to $\beta\gamma$ chains of the IL-2/IL-

15 receptor when compared with the recently described IL-15-CD8alpha fusion protein [14].

Best expansion rates of NK cells ranging from, on average, 66- to 86-fold after 27 days were observed when using feeder cells genetically engineered to secrete IL-2 and to co-express 4-1BBL and membrane bound IL-15, followed by those modified to co-express 4-1BBL. Recent studies have concluded that co-stimulatory signaling of 4-1BBL on 4-1BB-positive NK cells is essential for activation and expansion of peripheral NK cells [14,15,34]. Our results are consistent with this conclusion. Of note, in our study, NK cell expansion was performed at an initial feeder cell to NK cell ratio of 1:20, which gave similar results when compared with recently described expansion protocols using HLA-positive irradiated PBMC-derived feeder cells or irradiated EBV-transformed B cells at a feeder cell to NK cell ratio of 20:1 plus high doses of IL-2 (500–1,000 IU/mL rhIL-2) [35–37]. Furthermore, expansion of NK cells using PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d also needs significantly less feeder cells when compared with the feeder cell to NK cell ratios of 2:1 and 10:1 used for HLA-negative K562 derivatives modified to activate NK cells via combinatorial expression of 4-1BBL with IL-15 or IL-21 [14,15,18]. Yet, when compared with our expansion method for NK cells, K562 feeder cells modified with membrane-bound IL-15 and 4-1BBL led to a higher mean NK cell expansion of 277-fold in 21 days [14]. The same accounts for K562 feeder cells modified with soluble IL-15, MHC class I chain-related protein A (MICA) and 4-1BBL, which enabled 550-fold expansion of NK cells after 24 days [17], whereas K562 derivatives modified with membrane-bound IL-21 and 4-1BBL promoted a lower mean NK cell expansion of 48-fold in 21 days [18]. However, our NK cell expansion protocol does not rely on exogenously given cytokines, which might limit hyper-activation of NK cells as discussed below. Furthermore, when extrapolated for 500 mL peripheral blood, our lab-scale expansion might yield, on average, 4.45×10^9 (range: 2.4×10^9 to 6.3×10^9) NK cells, which might be sufficient for clinical applications. Yet whether a modified ratio of PC3-derived feeder cells to NK cells and the use of bioreactors can further improve the expansion of NK cells to obtain higher cell numbers for repetitive rounds of adoptive cell therapy remains to be investigated. Furthermore, from a regulatory point of view, it might be advantageous to use feeder cell systems that allow NK cell expansion without supplementing exogenous cytokines. In this regard, we also observed a complete eradication of feeder cells when co-cultured with NK cells. Moreover, in case of a potential transfer of PC3-derived feeder cells during adoptive cell therapy, calculated HLA mismatches in the host-versus-graft direction and concomitant immune stimulatory cytokine and co-stimulatory receptor expression of feeder cells should result in enhanced rejection of potentially contaminating feeder cells in the NK cell product by the immune system of the host.

Of note, expansion of NK cells using PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells was accompanied by increased fractions of CD25-positive NK cells and resulted in the appearance of immature-like CD56bright/CD16- and CD56bright/CD16+ populations but also of significant fractions of mature CD56dim/CD16+ NK cells. By contrast, NK cells expanded by expansion beads and IL-2/IL-21 treatment showed no upregulation of CD25 and contained only immature-like CD56bright/CD16- and CD56bright/CD16+ NK cells. We therefore concluded that, in our study, only immature CD56bright NK cells accounting for 9% ($\pm 4.6\%$) of peripheral NK cells and not mature CD56dim/CD16+ NK cells were capable of expanding in response to stimulation by feeder cells or activation beads. Interestingly, CD56bright and CD56dim NK cells robustly secreted IFN- γ and TNF- α upon stimulation with PMA and ionomycin, indicating functional NK cells. Yet CD56bright cells, as commonly known, showed significantly increased secretion of cytokines when compared with CD56dim NK cells. Interestingly, when resting NK cells after expansion were re-stimulated with feeder cells, they were again capable of expanding. By contrast, confrontation of

resting expanded NK cells with K562 target cells did not result in induction of secondary expansion, indicating that, for proliferation, such NK cells are still dependent on cytokine support and co-stimulatory signals. From a clinical point of view, this can be advantageous since an indefinite proliferation in the host might be limited and induction of proliferation likely depends on exogenously given IL-2. In addition, immune checkpoint markers TIGIT and PD-1, which are also associated with exhaustion of innate lymphocytic cells [38], were not upregulated in feeder cell-expanded NK cells. Furthermore, the concomitant upregulation of TIM-3 on NK cells, a marker linked to activation and maturation of NK cells [30], in conjunction with robust cytotoxicity against K562 target cells, demonstrates that expansion by PC3^{PSCA}-IL-2-4-1BBL and PC3^{PSCA}-IL-2-4-1BBL-mIL-15d feeder cells produces functional NK cells. Likewise, both feeder cell lines induced increased expression of activating NK cell receptors, in particular natural cytotoxicity receptors Nkp30, Nkp44 and Nkp46. Together with a robust expression of co-stimulatory DNAM-1 and activating NKG2D receptors, which also has been reported for NK cell expansion protocols using K562 derivatives [14,15], this is indicative of functional NK cells. Interestingly, a moderate increase in NKG2C-positive NK cell subsets was also observed in NK cell populations when using PC3-derived feeder cells. This is an intriguing observation since all donors used in this study were tested HCMV-seronegative. The activating NKG2C receptor is involved in NK cell responses against HCMV-infected host cells presenting HCMV UL40-derived nonamer peptides on HLA-E [39]. Such UL40 peptides are identical to host peptides derived from classical HLA-C and HLA-G expressed by the PC3-derived feeder cells (listed in supplementary Figure 1B) and together with CD58/LFA-3 on feeder cells likely contribute to the activation and outgrowth of NKG2C-positive NK subsets via stimulation of NKG2C and CD2, respectively [25,39,40].

To this end, our study demonstrates efficient expansion of non-exhausted functional CD25-positive NK cells with tolerance to self and to tumor cells with protective amounts of inhibitory NK cell ligands in an autologous and allogeneic setting, respectively, but with high lytic activity when encountering tumor cells with loss of protective HLA expression or facing tumor cells marked with a therapeutic anti-tumor antibody. Of note, the dominance of inhibitory versus activating signals in such expanded NK cells can be mitigated by low doses of rhIL-2, resulting in the killing of tumor cells irrespective of their expression of protective levels of classical HLA I molecules and HLA-E. This study raises the prospect that using NK cells generated in this manner for adoptive cell transfer could prove less damaging to stressed normal tissues and efficacious when properly activated.

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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: AT, SM JF and GS. Acquisition of data: SM, JF and MF. Analysis and interpretation of data: SM, JF, MF, FM and AT. Drafting or revising the manuscript: AT, SM, JF, MF, GS and FM. All authors have approved the final article.

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Supplementary materials

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