# CELLULAR THERAPIES IN CANCER

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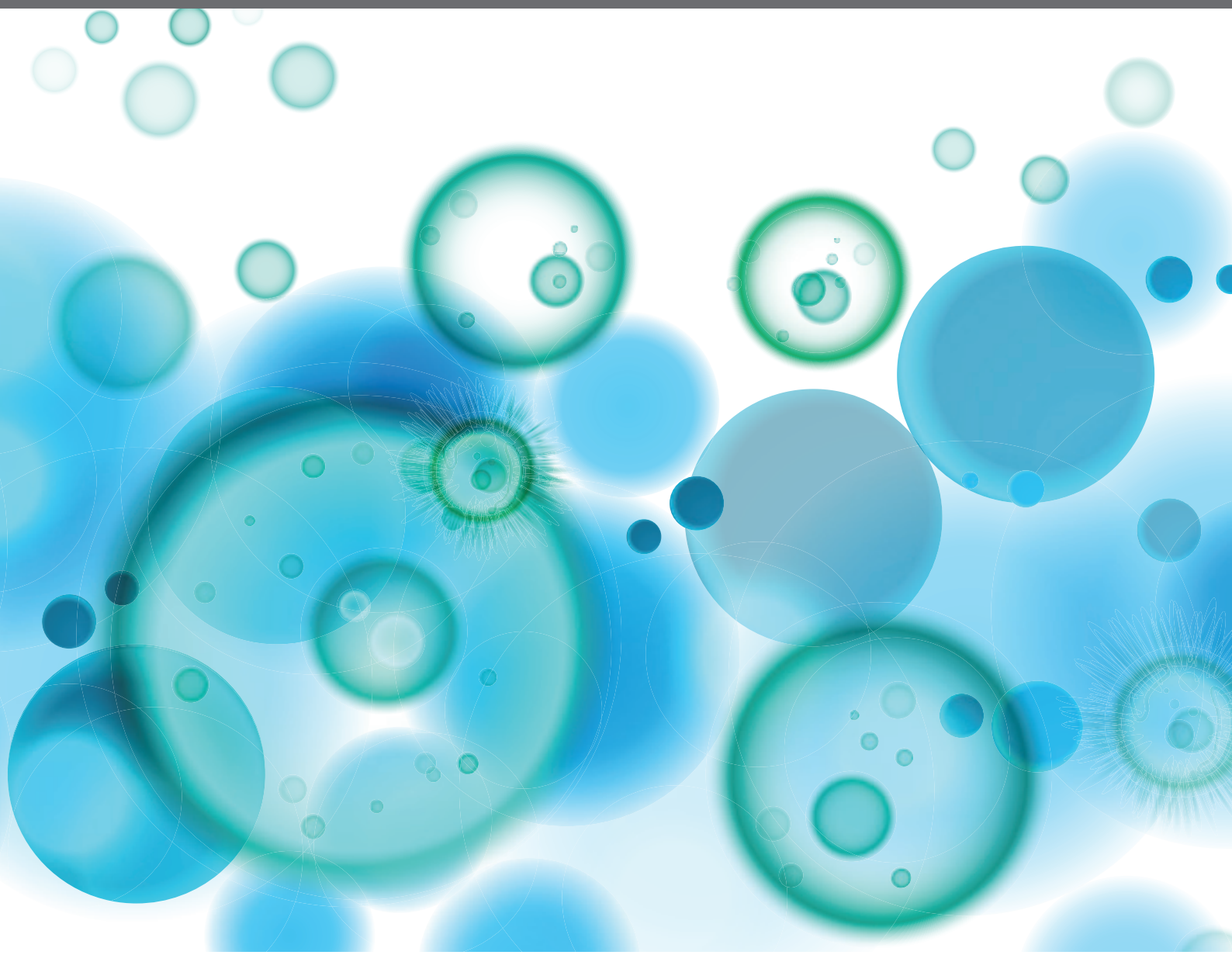

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# CELLULAR THERAPIES IN CANCER

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EDITORIAL
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# Editorial: Cellular Therapies in Cancer

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## Editorial on the Research Topic

## Cellular Therapies in Cancer

The field of cellular therapy is evolving rapidly with novel therapeutic modalities that include a wide spectrum of products. These include tumor-infiltrating lymphocytes (TILs), engineered T-cell receptor (TCR), chimeric antigen receptor (CAR)-T cells, cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and others. Among these, CAR T-cell therapy was the first to be approved by the US Food and Drug Administration (FDA) for the treatment of patients with B lymphoid malignancies. Specifically, Tisagenlecleucel (Kymriah) was approved in August 2017 for the treatment of patients with refractory B-cell acute lymphoblastic leukemia (ALL) up to the age of 26, and in May 2018, for adults with refractory B-cell lymphoma and Axicabtagene Ciloleucel (Yescarta) was approved for adults with refractory B-cell lymphoma in October 2017. However, a number of challenges have hindered the widespread use of engineered immune cells beyond B-cell malignancies. Some of these challenges include the lack of ideal targets for solid tumors, antigen loss mediating cancer relapse, the complexity of generating a patient-specific cell product, toxicity owing to the "on-target off-tumor" reactivity and cytokine release syndrome/neurotoxicity, inefficient trafficking to tumor sites, and tumor-mediated immunosuppression, to name a few. Advances in synthetic biology and genetic engineering, and the investigation of new platforms for cellular therapies have paved the road for the development of strategies to bypass some of these challenges, in order to advance the field forward and make a wider clinical impact. This collection of published articles is comprised of a series of original research and reviews highlighting recent advances in different forms of cellular therapies employing both T and NK cells against hematologic malignancies as well as solid tumors.

- Hoffmann et al. addressed a major question regarding the phenotype of CAR-T cells generated from untreated chronic lymphocytic leukemia (CLL) patients and specifically the ratio of naïve to effector T cells ($T_N/T_E$) in the final product and their expansion and persistence following adoptive transfer. The authors found that compared to healthy donors, the $T_N/T_E$ ratio was low in CAR-T products generated from untreated CLL patients, constituting a challenge for long-lasting CAR-T effects. As the high proportion of malignant B cells in CLL patients may hinder expansion of CAR-$T_N$ cells, the authors suggested that depletion of CLL cells prior to CAR-T production may improve the quality of the product (Hoffmann et al.).
- Qin et al. also addressed the survival and functionality of the transferred T cells, but from another angle through exploring different culture conditions to generate more effective CTLs for adoptive therapy. They proposed a new protocol to generate potent CTL clones using mouse embryonic fibroblast-conditioned medium (MEF-CM) and showed that these cells have higher potential to persist long-term in tumor-bearing and non-tumor bearing mice. Characterization

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of these cells suggests that MEF-CM enhances the effector functions of $CD8^+$ T cells and thus augmenting antitumor immunity (Qin et al.).

- Khan et al. investigated another important problem concerning the paucity of tumor antigens in the development of cellular therapies. The authors report overexpression of cathepsin G (CG) in cell lines and in primary ALL blasts and investigated CG as a tumor target for immunotherapy of ALL. Specifically, they showed that CG1-specific CTLs efficiently kill primary ALL blasts *in vitro* (Khan et al.).
- Houghtelin and Bollard reviewed the role of virus-specific T cells (VSTs) as a minimally toxic therapy in immunocompromised patients with viral infections. They also discuss future directions, including the use of naïve donor sources and third-party banks (Houghtelin and Bollard).
- Parisi et al. reviewed the role of alloreactive NK cells against acute myeloid leukemia (AML), and assessed biomarkers predictive of response, dosing, and other aspects which could maximize their efficacy.
- Herrera et al. explored a method to generate a large number of mature NK cells that could be used in NK cell-based immunotherapy. Using OP9 feeder cells, they were able to generate large numbers of mature and functional NK cells from umbilical cord blood (Herrera et al.).
- Lieberman et al. similarly aimed to explore an optimal approach to expand NK cells. They used a clinically validated expansion protocol using an engineered K562 cell line expressing membrane-bound interleukin (IL)-15 and 4-1BB ligand (K562-mbIL-15-4-1BBL) as feeder cells together with exogenous high dose IL-2 in the culture medium. They showed that NK cells expanded *ex vivo* in the presence of K562-mbIL-15-4-1BBL are not terminally mature but instead have high cytokine production capacity and antitumor efficacy. They concluded that this method could be an ideal source of long-lived and highly potent NK cells for cellular therapy (Lieberman et al.).
- Mehta and Rezvani reviewed the approach of genetically engineering NK cells to express a CAR (CAR-NK cells). As NK cells do not cause graft-vs.-host disease (GVHD), they can be used as a readily available "off-the-shelf" cellular therapy that could increase accessibility of this treatment for many more patients (Mehta and Rezvani).

Despite impressive successes in the management of CD19+ B-cell hematologic malignancies, progress in the solid tumor field has been met with a number of challenges. This is due to several factors, such as limited trafficking of adoptively transferred immune cells into the solid tumor sites, and the immunosuppressive tumor microenvironment. Importantly, identifying specific tumor antigens that are highly and uniformly expressed among solid tumors with low expression on normal tissues has been challenging. Adoptive transfer of TILs as an anti-cancer therapy pioneered by Steven Rosenberg at the National Institutes of Health (NIH) over two decades (1) has resulted in significant objective response rates in patients with metastatic melanoma and is being explored in other cancer settings (2). TCR-T cell therapy has also been commonly explored in solid tumors and is showing promise in some clinical trials (3). In addition, growing efforts are being made to expand the use of CAR-based therapies for solid tumors.

- Seliktar-Ofir et al. investigated a method to select tumor-specific TILs instead of the classic randomly isolated TILs. As CD137 is a co-stimulatory marker induced upon the interaction of T cells with their target cells, they explored a method to select CD137-expressing T cells *ex vivo*. CD137-selected TILs had superior antitumor activity and were enriched for T cells recognizing neoantigens and shared tumor antigens (Seliktar-Ofir et al.).
- Lo Presti et al. reviewed the use of a minor, yet clinically significant, population of T lymphocytes- the γδ T-cells. These cells have distinctive features justifying their use in anti-cancer immunotherapy; namely, they are not subject to major histocompatibility complex restriction, they are less dependent on co-stimulatory molecules than αβ T-cells, and they have known cytotoxicity against cancer cells (Lo Presti et al.).
- Jiang et al. presented their research in CAR T-cell therapy for hepatocellular carcinoma (HCC). They established patient-derived xenografts (PDX) mouse models of HCC and evaluated the cytotoxicity of anti-glypican 3 (GPC3)-CAR-T cells *in vivo*. GPC3 CAR-T cells efficiently suppressed tumor growth in one model, and eradicated the tumor in two other models where GPC3 was highly expressed (Jiang et al.).
- Mirzaei et al. reviewed the advances made in CAR T-cell therapy for solid tumors. They outlined the barriers to the effectiveness of this approach in solid tumors and some of the strategies to overcome these (Mirzaei et al.).
- Le and Thai reviewed the application of T- and NK-cell based immunotherapies in neuroblastoma and other childhood solid tumors such as other central nervous system tumors and sarcomas. They also delineated key differences between adult and fetal/neonatal immune systems (Le and Thai).
- Uppendahl et al. focused on the role of NK cell-based immunotherapy in gynecologic malignancies. They reviewed the advances made in harnessing these cells for clinical application against ovarian, cervical and uterine cancer (Uppendahl et al.).
- Tian et al. sought to enhance the activity of NK cells against $HER2^+$ breast cancer. Based on previous research showing that Herceptin could increase the cytotoxicity of lymphocytes against HER2 expressing tumors (4), they investigated the effect of engaging NK cells with Herceptin on their antitumor activity. They found that Herceptin increased NK cell proliferation, migration and cytotoxicity against $HER2^+$ breast cancer cells. In a pilot study, Herceptin-treated NK cells were administered to a $HER2^+$ breast cancer patient who could not tolerate the cardiotoxic side effects of Herceptin, leading to shrinkage in lung nodular metastases (Tian et al.).

In summary, we anticipate that this collection of original articles and reviews will increase our understanding of the progress made in the field of cellular immunotherapies for cancer and will serve as an inspiration for future research efforts. Despite the numerous challenges encountered in moving the field of cellular therapy forward, the success achieved thus far is

exciting and promising for a paradigm shift in the years to come. With the exploding advances in genome editing and synthetic biology, efforts from around the world will continue to advance the field toward curative approaches for highly resistant cancers.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest:** MD Anderson Cancer Center and Takeda have entered a license agreement and research agreement to develop CB-CAR NK cells for the treatment of B-cell malignancies and other cancers, which creates an institutional conflict of interest under MD Anderson policy. KR has received an education grant from Affirmed and Pharmacyclics; is a consultant for EMD serono and Virogin; and is a member of the Kiadis data and safety monitoring board.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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# Anti-GPC3-CAR T Cells Suppress the Growth of Tumor Cells in Patient-Derived Xenografts of Hepatocellular Carcinoma

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**Background:** The lack of a general clinic-relevant model for human cancer is a major impediment to the acceleration of novel therapeutic approaches for clinical use. We propose to establish and characterize primary human hepatocellular carcinoma (HCC) xenografts that can be used to evaluate the cytotoxicity of adoptive chimeric antigen receptor (CAR) T cells and accelerate the clinical translation of CAR T cells used in HCC.

**Methods:** Primary HCCs were used to establish the xenografts. The morphology, immunological markers, and gene expression characteristics of xenografts were detected and compared to those of the corresponding primary tumors. CAR T cells were adoptively transplanted into patient-derived xenograft (PDX) models of HCC. The cytotoxicity of CAR T cells *in vivo* was evaluated.

**Results:** PDX1, PDX2, and PDX3 were established using primary tumors from three individual HCC patients. All three PDXs maintained original tumor characteristics in their morphology, immunological markers, and gene expression. Tumors in PDX1 grew relatively slower than that in PDX2 and PDX3. Glypican 3 (GPC3)-CAR T cells efficiently suppressed tumor growth in PDX3 and impressively eradicated tumor cells from PDX1 and PDX2, in which GPC3 proteins were highly expressed.

**Conclusion:** GPC3-CAR T cells were capable of effectively eliminating tumors in PDX model of HCC. Therefore, GPC3-CAR T cell therapy is a promising candidate for HCC treatment.

**Keywords: cell therapy, T cells, CAR, hepatocellular carcinoma, PDX**

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## INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers and is one of the deadliest cancers in Asia (1–3). Current curative approaches for liver cancer mainly involve partial liver resection, liver transplantation, chemotherapy, and transarterial chemoembolization (4, 5). Despite enormous advances in the diagnosis and treatment of liver cancer in the recent decades, the 5-year survival rate has remained at about 10% (6, 7). Thus, more novel potential strategies, such as immunotherapy with genetic engineering of T cells to express chimeric antigen receptor (CAR), are now being tested in clinical trials (http://www.clinicaltrials.gov). For accelerating the steps of clinical trials, careful preclinical evaluations in models that closely mirror the clinical situation are urgently required.

Patient-derived xenografts (PDXs) refer to a procedure in which cancerous tissue from a patient's primary tumor is implanted directly into an immunodeficient mouse (8). This technique offers several advantages over standard cell line xenograft models. Unlike cancer cell lines, primary tumor cells are directly derived from human tissues and are not subjected to frequent high-serum environments and passages. Thus, PDX models are more biologically stable when passaged in mice in terms of mutational status, gene expression patterns, drug responsiveness, and tumor heterogeneity (9). Despite these benefits, only two studies report the use of PDX models of HCCs in drug testing (10, 11). No study has yet examined the use of CAR T cells in PDX models of HCC. Thus, it is necessary to carry out preclinical evaluation of novel CAR T cells against HCCs in PDX models.

It has been shown that glypican-3 (GPC3), a 580-AA heparan sulfate proteoglycan, expresses in 75% of HCC samples but not in healthy liver or other normal tissue (12). GPC3 is, therefore, a suitable target for CAR T cell therapy. Two previous studies showed the promising activity of GPC3-CAR T cells against HCC cell lines *in vivo* (13, 14). However, the capacity of GPC3-CAR T cells to eliminate HCC has not been evaluated in PDX models yet. In this study, we established and characterized primary human HCC xenografts to assess the cytotoxicity of adoptive GPC3-CAR T cells.

## MATERIALS AND METHODS

### Establishment of HCC Xenografts

Written informed consent was obtained from 12 patients, and the study received ethics approval from the Research Ethics Board of GIBH and the Second Affiliated Hospital of Guangzhou Medical University. All experimental protocols were performed in accordance with guidelines set by the China Council on Animal Care and the Ethics Committee of Animal Experiments at GIBH. The mice were provided with sterilized food and water *ad libitum* and housed in negative pressure isolators with 12-hour light/dark cycles. The isolation was performed following a previously described method with some modifications. The diagnosis of HCC was confirmed by histologic analysis in all cases. HCC tissues were transplanted into NOD/SCID/IL2rg$^{-/-}$ (NSI) mice that were sourced from Li's lab (15–17). Primary HCC tumors were placed in RPMI 1640 in an ice bath. Thin slices of tumor were diced into ~25 mm$^3$ pieces. The tissue was transplanted subcutaneously in the right flank of 8-week-old male NSI mice. Growth of the established tumor xenografts was monitored at least twice weekly through measurement of the length (a) and width (b) of the tumor. The tumor volume was calculated as $(a \times b^2)/2$. For serial transplantation, tumor-bearing animals were anesthetized with diethyl ether and sacrificed *via* cervical dislocation. Tumors were minced under sterile conditions and transplanted in successive NSI mice as described earlier.

For the Huh-7 and HepG2 xenograft model, mice were inoculated subcutaneously with $2 \times 10^6$ Huh-7 cells on the right flank. When the tumor volume was approximately 50–100 mm$^3$, the xenografts were randomly allocated into two groups, and the mice were given intravenous injection of human GPC3-CAR T or Control-CAR T cells in 200-μL phosphate-buffered saline solution as indicated. The tumor volume was calculated as $(a \times b^2)/2$.

### Genes and Lentiviral Vectors

To generate CARs-targeting GPC3, the genes of anti-GPC3 scFv, based on GC33 antibodies (18) and anti-CD19 scFv as Control ScFv, were first synthesized and subcloned in frame into lentiviral vectors containing expression cassettes encoding an IgM signal peptide and CD3ζ, CD28ζ, and 4-1BBζ signaling domains under the control of an EF-1α promoter. The sequence of each cloned CAR was verified *via* sequencing.

### Cell Lines and Reagents

A total of 293 T cells were used for lentivirus production and were cultured with DMEM (Gibco, Life Technologies), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 IU/mL of penicillin, and 100 IU/mL of streptomycin. HepG2 (HB-8065, purchased from ATCC), Huh-7 (gifted from Dr. Xiaoping Chen, GIBH), and A549 (CCL-185, purchased from ATCC) were transduced with a lentiviral vector co-expressing GFP and luciferase. HepG2-GL (HCC line, stably transfected with GFP and luciferase), Huh7-GL (HCC line, stably transfected with GFP and luciferase), and A549-GL (lung adenocarcinoma line, stably transfected with GFP and luciferase) cells were cultured with DMEM (Gibco, Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 IU/mL of penicillin, and 100 IU/mL of streptomycin. Human recombinant interleukin (IL)-2 was obtained from Peprotech. Polyethylenimine, an efficient transfection agent, was purchased from Life Technologies. Anti-GPC3 and anti-AFP were purchased from Santa Cruz Biotechnology, anti-CD3 (BV421) from Biolegend, and the remainder from eBioscience: CD45RO (Clone UCHL1), CD38 (clone HIT2), CD45 (clone HI30), CD19 (clone HIB19), CD5 (clone UCHT2), CD137 (clone 4B4-1), CD62L (clone DREG-56), CCR7 (clone 3D12), CD3 (clone OKT3), CD86 (clone IT2.2), PD-1 (clone eBioJ105), CD44 (clone IM7), TIM3 (clone F38-2E2), CD25 (clone BC96), CD49d (clone 9F10), CD18 (clone 6.7), CD27 (clone O323), CD163 (clone eBioGHI/61), CD326 (clone 1B7), CD66b (clone G10F5), CD3 (clone WM-59), CD206 (clone 19.2), CD80 (clone16-10A1), CD24 (clone eBioSN3), CD42b (clone

HIP1), CD36 (clone eBioNL07), CD127 (clone eBioRDR5), LAG3 (clone 3DS223H), CD107a (clone eBioH4A3), CTLA4 (clone 14D3), CD28 (clone CD28.2), CD56 (clone TULY56), CD49f (clone eBioGoH3), HLA-DR (clone L243), CD4 (clone OKT4), and CD8 (clone OKT8).

## Isolation, Transduction, and Expansion of Primary Human T Lymphocytes

Peripheral mononuclear cells (PBMCs) were separated *via* density gradient centrifugation (Lymphoprep, Stem Cell Technologies, Vancouver, BC, Canada). Primary human T cells were isolated from PBMCs *via* negative selection using the pan T Isolation Kit (Miltenyi Biotec, Germany). T cells were cultured in RPMI 1640 supplemented with 10% FCS (Gibco, Life Technologies), 100-U/mL penicillin, and 100 g/mL streptomycin sulfate (R10) and were stimulated with particles coated with anti-CD3/anti-CD28 antibodies (Miltenyi Biotec, Germany) at a cell-to-bead ratio of 1:2. Approximately 72 h after activation, T cells were transfected with supernatant containing lentiviral vectors expressing Control or GPC3-CARs. After transduction for 12 h, T cells were cultured with R10 medium supplemented with IL-2 (300 IU/mL). T cells were fed with fresh media every 2 days and were used within 21 days of expansion in all experiments.

## Cytotoxicity Assays

The target cells HepG2-GL, Huh-7-GL, and A549-GL were incubated with Control-CAR T or GPC3-CAR T cells at the indicated ratios in triplicate wells in U-bottomed, 96-well plates. Target cell viability was monitored 24 h later by adding 100 µL/well substrate D-Luciferin (potassium salt; Cayman Chemical, USA) resolved at 150 µg/mL. The background luminescence was negligible (<1% the signal from the wells with only target cells). The viability percentage (%) was, therefore, equal to the experimental signal/maximal signal, and the killing percentage was equal to 100 − viability percentage.

## Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay kits for IL-2 and interferon-γ were purchased from eBioscience, San Diego, CA, USA, and all ELISAs were conducted in accordance with the manuals provided. Control-CAR T and GPC3-CAR T cells were co-cultured at a 1:1 *E*/*T* ratio for 24 h in duplicate wells, from which the supernatant was collected and measured for the concentrations of IL-2 and IFN-γ.

## Quantitative Real-Time Polymerase Chain Reaction (PCR)

mRNA was extracted from cells with TRIzol reagent (Qiagen, Stockach, Germany) and reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (Takara, Japan). All reactions were performed with TransStart Tip Green qPCR SuperMix (TransGene, Beijing, China) on a Bio-Rad CFX96 real-time PCR machine (Bio-Rad, Hercules, CA, USA), using the primers shown in Table S1 in Supplementary Material. Delta CT calculations were relative to β-actin and corrected for PCR efficiencies.

## Flow Cytometry

Flow cytometry for the GFP% of transduced T cells and for GPC3 and PD-L1 expression on HCC cells was performed on a C6 cytometer and analyzed using the FlowJo software. The PBMCs, spleens, and bone marrow (BM) from xenograft mice were treated with a red blood cell lysis buffer (Biolegend), and the cells were stained with anti-hCD3, hCD4, and hCD8 analyzed on a Fortessa cytometer (BD Biosciences). All FACS staining was performed on ice for 30 min and washed with PBS containing 2% FBS before cell cytometry. Mouse tissues were weighed and harvested into ice-cold RPMI 1640. The tissues were manually morselized with a scalpel and then mechanically disaggregated through 40- to 100-mm filters.

## Histological Analysis

Organ or tissue samples were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 4-µm thickness, and stained with hematoxylin and eosin or antibodies (GPC3 and AFP). Images were obtained on a microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Germany).

## Statistics

The data are presented as the mean ± SEM. The results were analyzed *via* an unpaired Student's *t*-test (two-tailed). Statistical significance was defined by a *P* value of less than 0.05. All statistical analyses were performed using the Prism software version 6.0 (GraphPad).

# RESULTS

## Establishment and Characterization of HCCs from PDXs

Of the 12 models, 6 did not grow in the first generation (P1). PDXs of HCC were successfully engrafted from six PDXs in immunodeficient mice (NSI, NOD/SCID-IL2rg$^{-/-}$). Of these, three xenografts were propagated beyond the third generation (P3) (**Figure 1A**), whereas three tumors were still growing in the first generation (P1). Taken together, a success rate of 25% was reached when the third generation was considered successfully engrafted. An overview of the successfully growing PDX models and their clinical characteristics of the original patients are shown in **Table 1**.

To validate the established PDX models, we compared their morphology, immunological markers (GPC3 and AFP), and gene expressions with those of the corresponding primary tumor. Histologic evaluation of the xenografts revealed tumor tissue with morphologic characteristics like those of the original primary human tumor (**Figure 1B**). Immunologic markers of liver tumors such as GPC3 and APF were detected in both primary patient tumors and xenografts (**Figure 1C**). Quantitative reverse transcription PCR was performed to characterize the mRNA expression level of some tumor-related genes in xenografts and primary tumor (**Figure 1D**). These genes are associated with carcinogenesis, aggression, and characterization of HCCs (19, 20). Unsupervised hierarchical clustering of selected transcriptional profiles confirmed that all patients and xenograft pairs cluster

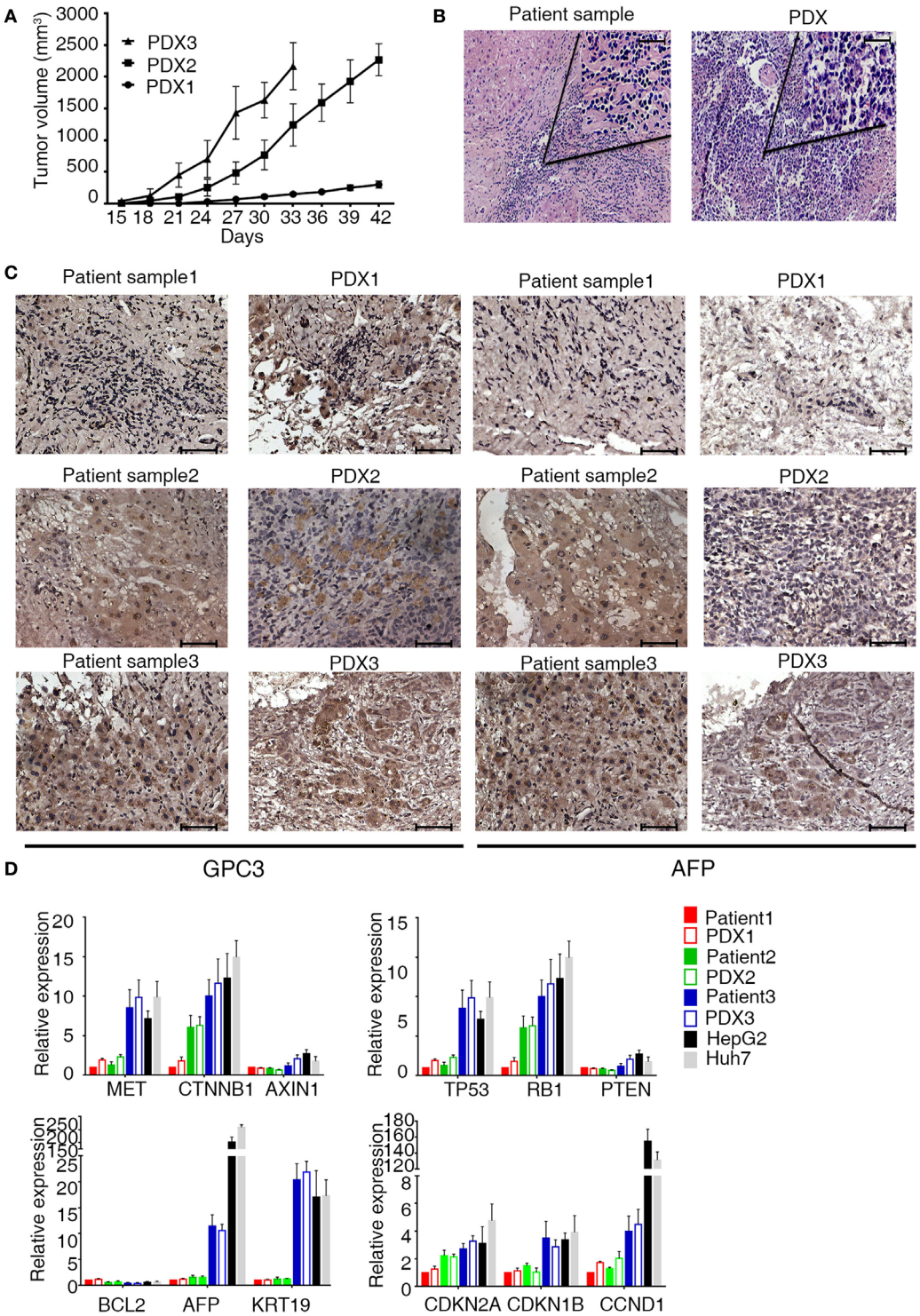


**FIGURE 1** | **Characterization of patient-derived xenograft (PDX) model of hepatocellular carcinoma**. **(A)** Patient-derived xenografts (PDXs) were established, and tumor growth was measured at a given time for each PDX xenograft. Tumor volume was calculated as ($a \times b^2$)/2, the length (a), and width (b) of tumor. **(B,C)** H&E and IHC staining tissue of primary tumor and PDXs. The xenograft demonstrates morphology, glypican 3, and AFP staining consistent with the original human tumor. Brown color indicates positive staining. IgG was used as a negative control. Scale bar is 50 μm. **(D)** mRNA expression level of tumor-related genes [MET (MET proto-oncogene, receptor tyrosine kinase), CTNNB1 (catenin beta 1), AXIN1 (axin 1), TP53 (tumor protein p53), RB1 (RB transcriptional corepressor 1), PTEN (phosphatase and tensin homolog), BCL2 (BCL2, apoptosis regulator), AFP (alpha fetoprotein), KRT19 (keratin 19), CDKN2A (cyclin-dependent kinase inhibitor 2A), CDKN1B (cyclin-dependent kinase inhibitor 1B), and CCND1 (cyclin D1)] in primary tumor and PDXs. Results represent mean ± SD of three individual experiments.

**TABLE 1 | Clinical information of patients.**

| HCC | Sex | Age (years) | Site | HBsAg | Metastasis | Histologic grade | PD-L1 | AFP (UI/mL) | Xenografts |
|---|---|---|---|---|---|---|---|---|---|
| Patient 1 | Male | 47 | Left lobe | – | No | III | – | 4 | 3rd |
| Patient 2 | Female | 47 | Right lobe | + | No | IV | – | 2.15 | 3rd |
| Patient 3 | Male | 53 | Right lobe | + | Intrahepatic | IV | + | 384.99 | 3rd |
| Patient 4 | Male | 50 | Right lobe | + | No | III | – | 3.85 | 1st |
| Patient 5 | Male | 60 | Right lobe | + | No | III | + | 1.68 | 1st |
| Patient 6 | Male | 67 | Left lobe | – | No | III | – | 62.54 | 1st |

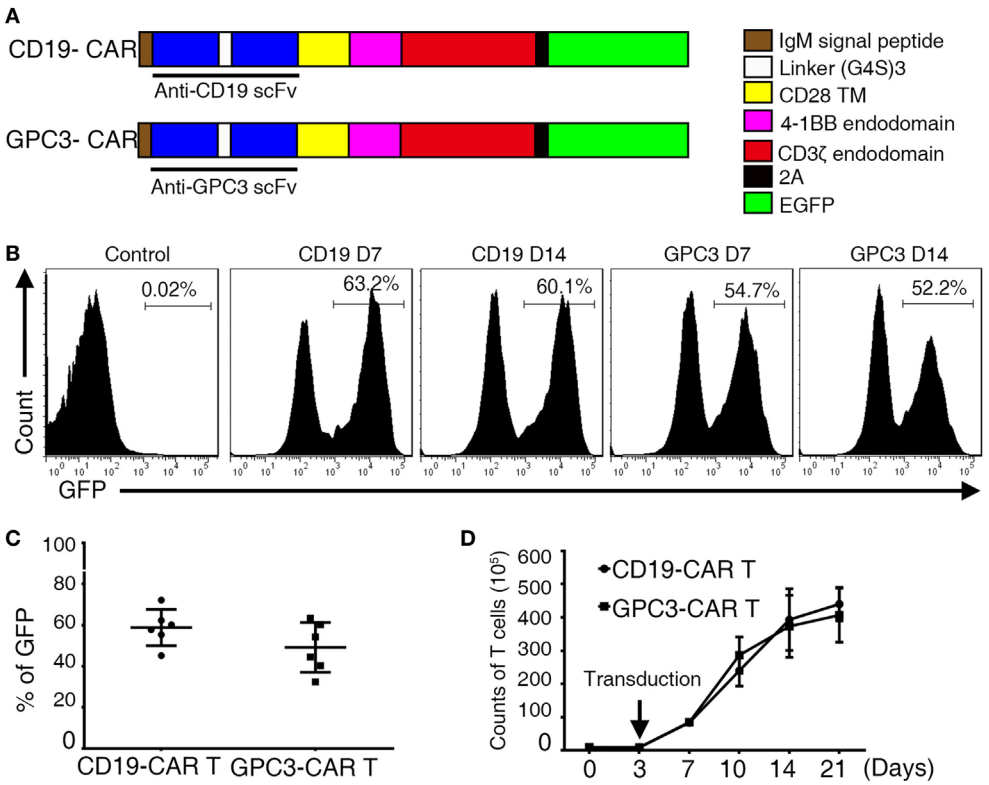


**FIGURE 2 | Construction of chimeric anti-glypican 3 (GPC3) vectors and generation of GPC3-chimeric antigen receptor (CAR) T cells**. **(A)** Schematic representation of a lentiviral vector encoding the signal peptide, anti-GPC3 scFv, CD28 transmembrane domain, 4-1BB costimulatory endodomain, and CD3ζ signaling domain along with eGFP using 2A. **(B)** A representative of GPC3-CARs expression on human T cells transduced with lentivirus was analyzed using flow cytometry, which detected eGFP at days 7 and 14. **(C)** Transduction efficiency. Results represent mean ± SD of six individual experiments. No difference was detected between the percentages of GPC3-CAR T and Control CAR T. **(D)** The expansion of transduced T cells *in vitro* from day 0 to 21. Results represent mean ± SD of three individual experiments.

together (Figure S1 in Supplementary Material). Collectively, our results indicate that PDX of HCCs in the mice recapitulate the original disease and remain stable through three serial transplantations.

## T Cells Engineered to Express GPC3-CARs

The sequencing encoding the anti-GPC3 scFv (Figure S2A in Supplementary Material) was cloned in frame into lentivirus vectors containing CAR expression cassettes with CD28, 4-1 BB, and CD3ζ endodomains (**Figure 2A**). For the generation of T cell populations that expressed the anti-hGPC3-CAR, a two-step optimal expansion protocol was developed. CD3- and CD28-activated T cells after 72 h were transduced with the GPC3-CAR construct to generate GPC3-CAR T cells. The expression of CARs was measured *via* flow cytometry through eGFP expression. CARs were stably expressed from day 7 to day 14 with no significant difference (**Figure 2B**). The frequency of CAR expression was 58.6% for CD19-CAR and 49.2% for GPC3-CAR (**Figure 2C**). Flow cytometric analysis using a goat anti-mouse F(ab)2 confirms that the expression of CAR molecules was consistent with eGFP (Figure S2B in Supplementary Material). The generated CAR T cells were >97% CD3-positive T cells, which consisted of CD4- and CD8-positive T cell subsets with the same ratio as the non-transduced T cells (Figures S3A–D in

Supplementary Material). In our optimal expansion protocol, T cells begin to expand at day 3 and continued to expand until day 21. Reproducible expansion of 20- to 50-folds of T cell can be achieved at day 14 (**Figure 2D**). Collectively, these experiments established a robust two-step method to transduce and expand (up to 50-fold) CAR-transduced T cells from the peripheral blood of healthy donors.

## Phenotypic and Functional Characterization of GPC3-CAR T Cells

To better define the phenotyping of CAR-transduced T lymphocytes after infection, we next performed 35 different cell surface markers. CAR T cells were compared at the beginning (day 0) and the middle (day 14) of the T cell culture process. We observed upregulation of the activation markers CD25 and CD27, the migration maker CCR7 (**Figure 3A**), and the costimulatory receptors CD86 (21) and CD137 (**Figure 3A**), which are indicators of enhanced proliferative potential of T cells. After *in vitro* culture, T cells acquired an intermediate effector memory phenotype with the progressive downregulation of CD28 and CD62L. Moreover, we observed upregulation or downregulation of multiple molecules involved in cell adhesion. CD18, CD44, and CD49d were upregulated, and CD49f, CD107a, and CD56 were downregulated (**Figure 3A**). Notably, key inhibitory and exhaustion-associated molecules such as PD-1, CTLA-4, and TIM3 were upregulated (**Figure 3A**). Importantly, we found strikingly similar CAR T cell phenotypes across all six tested donors, as illustrated in the heat map in Figure S3E in Supplementary Material. A hallmark function of activated T lymphocytes is the production of cytokines. To evaluate this production, we co-cultured CAR T cells with GPC3-positive HCC cell lines as target cells (Figure S4A in Supplementary Material). GPC3-CAR T cells secreted high levels of INF-γ and IL-2 after coincubation with only GPC3-positive targets (**Figures 3B,C**). These data collectively characterize CAR T cells as a highly reproducible cellular product of activated lymphocytes, endowed with migratory potential and natural cytotoxic machinery.

## Effective Serial Killing of GPC3-Positive Human HCC Cells by GPC3-CAR T Cells

To test whether GPC3-CAR T cells could specifically recognize and kill GPC3-positive targets, cytotoxicity assays were

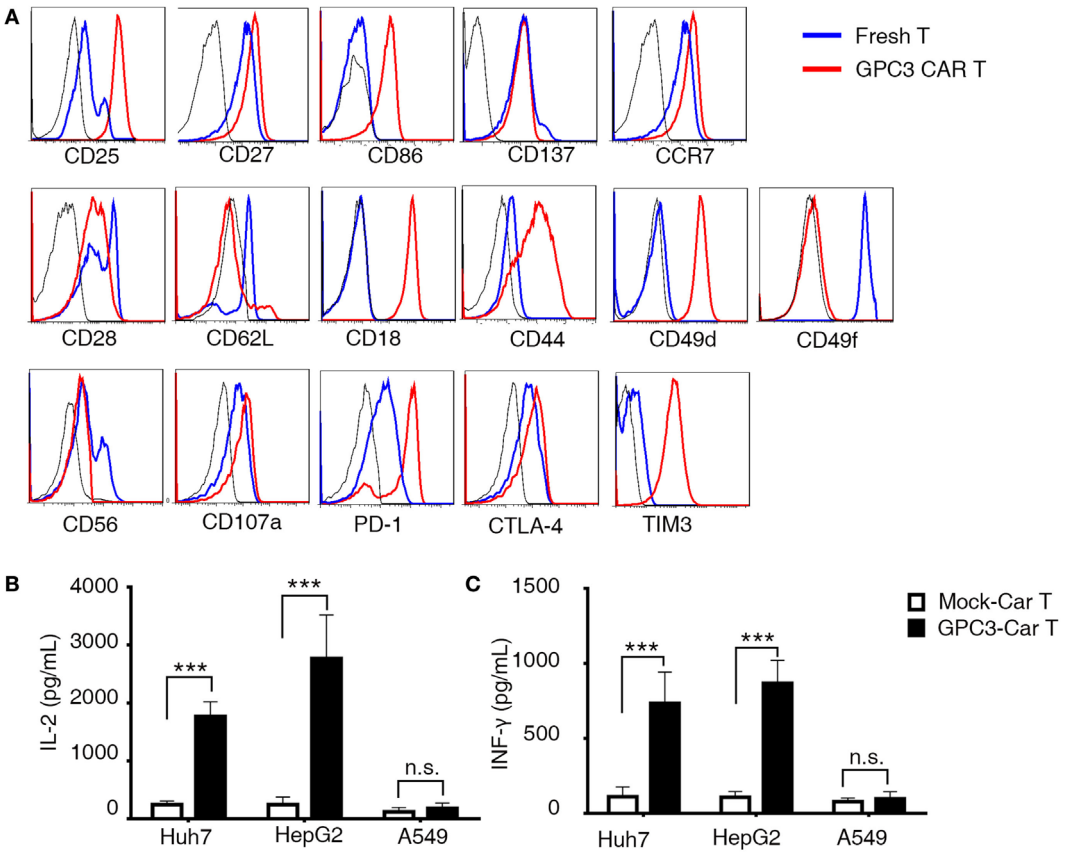


**FIGURE 3 | Phenotypic analysis and cytokines produced in glypican 3 (GPC3)-chimeric antigen receptor (CAR) T cells.** **(A)** Flow cytometry comparison of the common surface phenotype of GPC3-CAR T cells (red line) at day 14 of culture with freshly isolated T cells (blue line). Black line represents the isotype control. Histogram overlays show 16 markers related to lymphocyte activation, differentiation, migration, adhesion, and exhaustion. **(B)** Interferon-γ and **(C)** interleukin-2 were secreted by the indicated modified CAR T cells co-cultured with hepatocellular carcinoma cell lines and A549 for 24 h. Results represent triplicates. ***$P < 0.001$ with *T*-test.

performed by incubating the CAR T cells with GPC3-positive HCC cells (Huh-7and HepG2) and GPC3-negative cells (A549) (Figure S4A in Supplementary Material). GPC3-CAR T cells were highly cytotoxic against the GPC3-positive HCC cells, Huh-7, and HepG2. By contrast, GPC3-CAR T cells did not target GPC3-negtive cells (**Figure 4A**). These data demonstrate that GPC3-CAR T cells selectively target GPC3-positive tumor cells. It had been demonstrated that 4-1BB endodomains ameliorate exhaustion of CAR T cells (22). To further explore cytotoxic potency of GPC3-CAR T cells incorporating 4-1BB costimulatory domains, we performed a co-culture in which CAR T cells were restimulated with GPC3-positive HCC cells every 24 h for three consecutive days at E:T ratios of 1:1 (23). Killing of Huh-7 and HepG2 hepatoma cells was only observed when GPC3-CAR was reconstituted (**Figure 4B**). Taken together, these results indicate that GPC3-CART cells displayed significantly specific and efficient cytotoxicity against GPC3-positive target cells.

## Adoptive Transfer of GPC3-CAR T Cells Suppresses HCC Cell Lines Growth *In Vivo*

To explore the killing of GPC3-positive tumors by GPC3-CAR T cells *in vivo*, we used a subcutaneous xenograft model in which transplant tumors were established in immunodeficient mice using HepG2 and Huh-7 cell lines. Tumors were established for 7 days, and a tumor volume of approximately 50–100 $mm^3$ was obtained. The mice were then treated *via* adoptive transfer of GPC3-CAR T or Control-CAR T cells. Tumor growth was efficiently suppressed by intravenous injection of $5 \times 10^6$ GPC3-CAR T cells ($n = 5$), as compared to a control group that received Control transduced T cells ($n = 5$) (**Figures 5A–D**). We also detected human T cells in the PBMC and tumor tissues of mice with subcutaneous Huh-7 or HepG2 xenografts after T cell infusion (**Figures 5E,F**). The results show that GPC3-CAR T cells can efficiently suppress the growth of HCC cell lines in mice.

## Patient-Derived HCC Xenograft Is Controlled by GPC3-CAR T Cells

Patient-derived xenograft models preserve the heterogeneous pathological and genetic characteristics of the original patient tumors and may provide a precision preclinical model for immunotherapy evaluation. Our results show that GPC3 protein was highly expressed in xenografts of HCCs, so we tested the effect of GPC3-CAR T cells in these PDX models. In all three individual PDX models, $2.5 \times 10^6$ CAR T cells were given by intravenous injection twice after the tumor volume reached 50–100 $mm^3$. The efficient antitumor effect was observed in the xenografts treated with GPC3-CAR T cells compared to the Control-CAR T cells (**Figures 6A–F**). We observed that GPC3–CAR T cells have better cytotoxicity in PDX1 and PDX2 than PDX3. We propose that the heterogenous nature of tumors can affect GPC3-CAR T cells cytotoxicity to tumors *in vivo*. Studies have shown that the highly expressed MET, CTNNB1, and CCND1 are associated with aggression of HCCs (24). MET, CTNNB1, and CCND1 are highly expressed in PDX3 than in PDX1 and PDX2 (**Figure 1D**). This result implies that PDX3 tumor cells are more aggressive. Programed cell death 1 (PD-1), an immunoinhibitory receptor belonging to the CD28 family, has been shown as a frequently used physiologic immunosuppressive mechanism by tumors to invade host immunity (25). Our results show that PD-L1 was highly expressed in PDX3 but not in PDX1 and PDX2 (**Table 1**). These results suggest that tumor aggression and immunosuppressive molecules should be considered in CAR T cell therapy. T cell analysis also shows that GFP-positive T cells are higher in GPC3-CAR T groups than in Control-CAR T groups (**Figures 6G–I**). Taken together, our results demonstrate that GPC3-CAR T cells

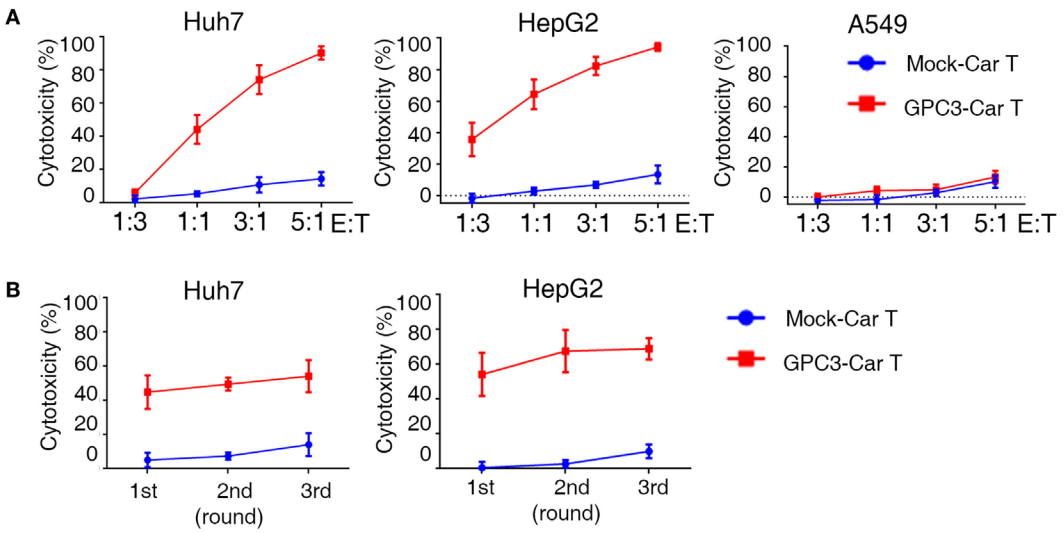


**FIGURE 4 | Cytotoxicity of glypican 3 (GPC3)-chimeric antigen receptor (CAR) T cells against targeted cells *in vitro*. (A)** Cytotoxicity of GPC3-CAR T cells was assayed by coincubation with Huh-7-GL ($GPC3^+$), HepG2-GL ($GPC3^+$), and A549-GL ($GPC3^-$) cells at E:T ratios ranging from 1:3 to 5:1. **(B)** Serial killing of GPC3-CAR cells against Huh-7-GL and HepG2-GL cells at E: T ratios of 1:1 in indicated durations. Results represent mean ± SEM.

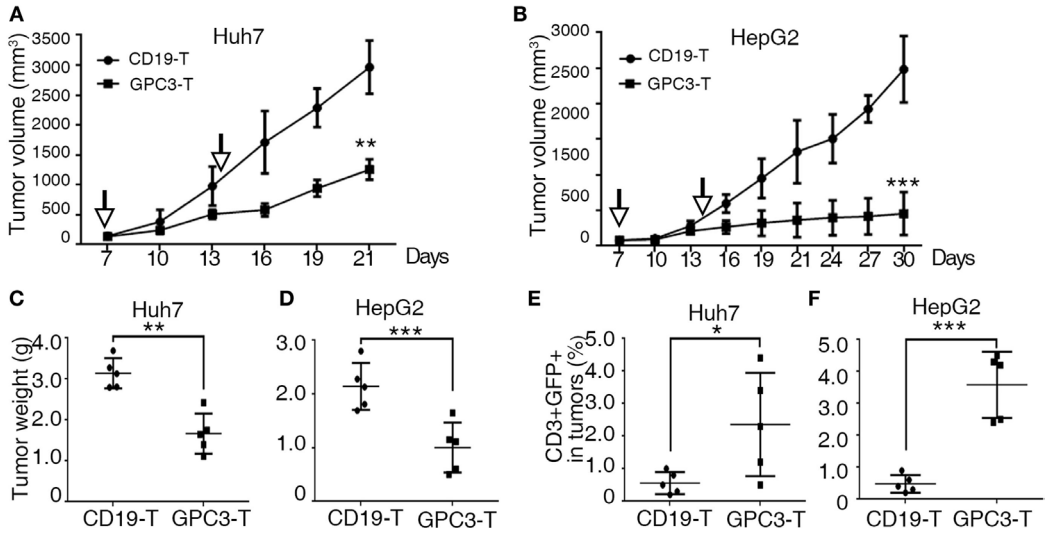


**FIGURE 5 | Cytotoxicity of glypican 3 (GPC3)-chimeric antigen receptor (CAR) T cells against hepatocellular carcinoma cell lines in xenografts**. **(A,B)** Growth curve of Huh-7 and HepG2 xenografts ($n = 5$) treated with the Control- or GPC3-CAR T cells at indicated time point (arrow). At the end of the experiment, the tumors treated with GPC3-CAR T cells were significantly smaller than those in the Control group. **(C,D)** Huh-7 and HepG2 tumor weights from the mice treated with CAR T cells at the end of the experiment, respectively. **(E,F)** GPC3-CAR T cells in tumors were significantly higher than Control-CAR T groups. Results represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ with *T*-test.

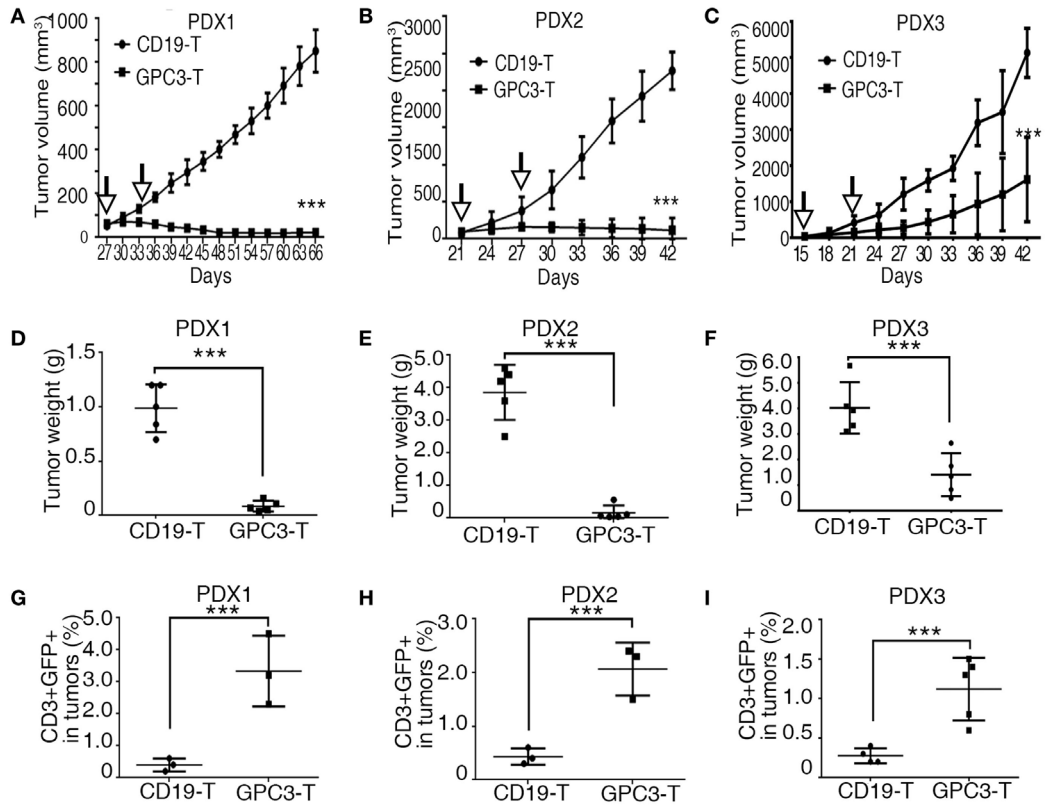


**FIGURE 6 | Glypican 3 (GPC3)-chimeric antigen receptor (CAR) T cells efficiently abolish growth of patient-derived xenografts (PDXs) of hepatocellular carcinoma**. **(A–C)** Growth curve of PDX1, PDX2, and PDX3 ($n = 5$) treated with the Control- or GPC3-CAR T cells at indicated time point (arrow). At the end of the experiment, the tumors treated with GPC3-CAR T cells were significantly smaller than those in the Control group. **(D–F)** PDX1, PDX2, and PDX3 tumor weights from the mice treated with CAR T cells at the end of the experiment. **(G–I)** GPC3-CAR T cells in tumors were significantly higher than Control-CAR T groups. Results represent mean ± SD. $^{***}P < 0.001$ with *T*-test.

were able to efficiently suppress the growth of primary GPC3-positive HCC *in vivo*.

## DISCUSSION

In this study, we report the establishment of three PDX models from primary HCC in NSI mice. The xenografts were successfully serially transplanted while preserving the characteristics of the original patient tumors. We observed that tumors in PDX2 and PDX3 xenografts grow faster than that in PDX1. Our data suggest that their growth behavior is positively correlated with the expression levels of MET, CTNNB1, and CCND1. Previous studies show that MET and CTNNB1 act as oncogenes in HCCs and that CCND1 is the hallmark of cell cycle progression (19, 26, 27).

Previous studies have shown that GPC3-CAR T cells efficiently eradicate liver cancer cells lines that possess a high level of GPC3 expression *in vivo* (13, 14). Here, we observed that GPC3-CAR T cells were less effective to kill HCC cell lines in xenografts, compared to a previous report (13). A possible reason for this difference is that we used NOD/SCID/IL2$^{-/-}$ mice for xenotransplantation, whereas the previous report used NOD/SCID mice, in which natural killer (NK) cells are active against tumor cells. By comparing the subcutaneous growth of Huh-7, we observed that Huh-7 cells grew faster in NOD/SCID/IL2$^{-/-}$ mice than in NOD/SCID mice. In addition, tumor-experienced T cells (28) can promote NK cell activity against tumors cells. It is likely that GPC3-CAR T cells may kill tumor cells in synergy with mouse NK cells.

Patient-derived xenograft models have been commonly used to test drug efficacies and identify biomarkers in a number of cancers, including liver, ovarian, pancreatic, breast, and prostate cancers (9). Previous studies have shown that tumors in PDX models are biologically stable and accurately reflect the histopathology, gene expression, genetic mutations, and therapeutic response of the patient tumor (9). Several recent preclinical studies and clinical trials have demonstrated the efficient activity of CD19-CAR T cells against acute B lymphoblastic leukemia (29). However, CAR T cells that target solid tumors have so far demonstrated limited efficacy. To date, the most positive trials reported have used GD2 CARs to target neuroblastoma (3 of 11 patients with complete remissions), HER2 CARs for sarcoma (4 of 17 patients showing stable disease), and HER1 CARs for lung cancer (2 of 11 patients with partial responses) (30). We report that GPC3-CAR T cells impressively eradicated tumors from PDX1 and PDX2, which were less aggressive and were PD-L1 negative. In contrast, GPC3-CAR T cells were less cytotoxicity to tumors in PDX3 that were more aggressive and highly expressed PD-L1, suggesting that we need to combine CAR T cell therapy and immune checkpoint inhibitors to achieve higher efficacy of eliminating PD-L1-positive HCC. Similarly, two recent reports showed that combining CAR therapy and PD-1 blockade was efficacious in breast cancer and mesothelioma models (31, 32). Downregulated expression of GPC3 in HCC cells may affect GPC3-CAR T-specific cytotoxicity to tumor cells. While our data show that the percentage of GPC3-positive cells was not changed in control and GPC3-CAR T treatment *in vivo* (Figures S4B,C in Supplementary Material).

In summary, we established and characterized three GPC3-positive PDXs of HCC. We also show that GPC3-CAR T cells suppressed tumor growth but with different efficacies in the PDX models of the three individual patients. Therefore, PDX models can potentially be used to evaluate the efficacy of GPC3-CAR T cell therapy for treating HCC in individual patients.

## AUTHOR CONTRIBUTIONS

ZJ and XJ contributed to the conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing. SC, XW, YL, SL, and QLiang contributed to the provision of study material or patients and collection and/or assembly of data. BL, SW, and QW provided administrative support. YY and DP contributed to the conception and design and provided financial support. YY, QLiu, and PLiu contributed to the conception and design. PX and PLi contributed to the conception and design, data analysis, and interpretation, manuscript writing, and final approval of manuscript and provided financial support. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00690/full#supplementary-material.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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# OP9 Feeder Cells Are Superior to M2-10B4 Cells for the Generation of Mature and Functional Natural Killer Cells from Umbilical Cord Hematopoietic Progenitors

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Adoptive natural killer (NK) cell therapy relies on the acquisition of large numbers of mature and functional NK cells. An option for future immunotherapy treatments is to use large amounts of NK cells derived and differentiated from umbilical cord blood (UCB) $CD34^+$ hematopoietic stem cells (HSCs), mainly because UCB is one of the most accessible HSC sources. In our study, we compared the potential of two stromal cell lines, OP9 and M2-10B4, for *in vitro* generation of mature and functional $CD56^+$ NK cells from UCB $CD34^+$ HSC. We generated higher number of $CD56^+$ NK cells in the presence of the OP9 cell line than when they were generated in the presence of M2-10B4 cells. Furthermore, higher frequency of $CD56^+$ NK cells was achieved earlier when cultures were performed with the OP9 cells than with the M2-10B4 cells. Additionally, we studied in detail the maturation stages of $CD56^+$ NK cells during the *in vitro* differentiation process. Our data show that by using both stromal cell lines, $CD34^+$ HSC *in vitro* differentiated into the terminal stages 4–5 of maturation resembled the *in vivo* differentiation pattern of human NK cells. Higher frequencies of more mature NK cells were reached earlier by using OP9 cell line than M2-10B4 cells. Alternatively, we observed that our *in vitro* NK cells expressed similar levels of granzyme B and perforin, and there were no significant differences between cultures performed in the presence of OP9 cell line or M2-10B4 cell line. Likewise, degranulation and cytotoxic activity against K562 target cells were very similar in both culture conditions. The results presented here provide an optimal strategy to generate high numbers of mature and functional NK cells *in vitro*, and point toward the use of the OP9 stromal cell line to accelerate the culture procedure to obtain them. Furthermore, this method could establish the basis for the generation of mature NK cells ready for cancer immunotherapy.

**Keywords: hematopoietic stem cells, umbilical cord blood, natural killer cells, *in vitro* cell differentiation, immunotherapy**

## INTRODUCTION

Natural killer (NK) cells constitute 10–15% of peripheral blood (PB) lymphocytes and display a half-life of approximately 7–10 days in circulation (1). They can also be found in cord blood (CB) in a similar frequency to PB (2), but the small volume in CB units represents the difficulty in obtaining suitable numbers of NK cells needed for clinical use (3). Human NK cells are phenotypically described as $CD3^{-}CD56^{+}$ cells within the lymphocyte population (4), and they are classified as a subset within the group 1 of innate lymphocyte cells, capable of producing IFN-γ, and exert cytotoxicity (5). According to the intensity of the expression of the CD56 receptor, *in vivo* differentiated mature NK cells are divided into $CD56^{bright}$ and $CD56^{dim}$ subpopulations (6). $CD56^{bright}$ cells constitute less than 10% of circulating NK cells, produce high levels of inflammatory cytokines, and have none or low expression of CD16. $CD56^{dim}$ NK cells express CD16 and contain an abundance of granules that arm them for cytolytic activity against viral-infected and cancer cells (7). NK cells are originated from $CD34^{+}$ hematopoietic progenitors (4). Before reaching a mature stage, they acquire progressively and orderly different surface markers, being classified into stage 1 ($CD34^{+}$, $CD45RA^{+}$, $CD117^{-}$, $CD94^{-}$, $CD56^{-}$, $CD16^{-}$), stage 2 ($CD34^{+}$, $CD45RA^{+}$, $CD117^{+}$, $CD94^{-}$, $CD56^{-}$, $CD16^{-}$), and stage 3 ($CD34^{-}$ $CD117^{+}$, $CD94^{-}$, $CD56^{-}$, $CD16^{-}$). Once they reach a mature stage, NK cells are phenotypically described by their surface markers as stage 4 ($CD34^{-}$, $CD94^{+}$, $CD117^{+/-}$, $CD56^{bright}$, $CD16^{+/-}$) and stage 5 ($CD34^{-}$, $CD94^{+/-}$, $CD117^{-}$, $CD56^{dim}$, $CD16^{+}$) (8).

Current NK cell-based cancer immunotherapy aims to reverse the tumor-induced NK cell dysfunction that is observed in patients with cancer and to increase and sustain NK cell effector functions (9, 10). The low numbers of these cells in PB and, even lower numbers in CB, have led to several approaches to expand and/or activate freshly isolated autologous or allogeneic NK cells by culturing with different interleukins, such as IL-2, IL-15, and IL-21 (11–14). $CD34^{+}$ hematopoietic progenitors from umbilical cord blood (UCB) are being considered a source for the production of a large number of NK cells (15, 16). Obtaining NK cells from UCB $CD34^{+}$ hematopoietic progenitors has been extensively described (17). However, further research is needed to obtain even larger numbers of mature and functional NK cells ready to use in cancer immunotherapy.

In this study, we aimed to evaluate the production of functional and mature NK cells from UCB $CD34^{+}$ hematopoietic progenitors with two different culture conditions, where OP9 and M2-10B4 cell lines are used as feeder layers. OP9 is typically used as a support for the differentiation of $CD34^{+}$ cells from embryonic stem cells (ESCs) or pluripotent stem cells (18–21). Instead, M2-10B4 is a good support to maintain $CD34^{+}$ cells in a long-term culture, acting like a hematopoietic niche (22). Our data show that these two culture conditions generated a large number of mature and functional NK cells. Furthermore, the presence of OP9 feeder cells in the culture generated a higher amount of mature NK cells in a faster manner when compared with culture conditions with M2-10B4 feeder cells.

## MATERIALS AND METHODS

### Umbilical Cord and PB Samples and Cell Lines

Umbilical cord blood and PB samples were obtained with prior signed informed consent and ethical committee approval from the Basque Ethics Committee for Clinical Research [Comité Etico de Investigación Clinica de Euskadi-CEIC-E (PI2014138)]. Fully signed written informed consent was obtained from the pregnant mothers. UCB units that contain between $1.5 \times 10^{9}$ and $8 \times 10^{8}$ mononuclear cells were used for investigation purposes. One fresh UCB unit (less than 30 h between the extraction and the processing) was used to perform a set of experiments (Table S1 in Supplementary Material). OP9, M2-10B4, and K562 cell lines were purchased from ATCC (CRL-2749, CRL-1972, and CCL-243, respectively). OP9 cells were cultured with α-MEM (Gibco), 20% fetal bovine serum (FBS) (Hyclone), 1% penicillin/streptomycin, and 1% Glutamax. M2-10B4 cells were culture with RPMI, 10% FBS (Hyclone), 1% penicillin/streptomycin, and 1% Glutamax. Finally, K562 cells were cultured with RPMI, 10% FBS (Hyclone), 1% penicillin/streptomycin, 1% Glutamax, 1% NEAA, and 1% sodium pyruvate.

### Hematopoietic Stem Cell (HSC) Differentiation Protocol into NK Cells

Umbilical cord blood mononuclear cells were obtained by density gradient using Ficoll-Paque™ PLUS (GE Healthcare). Then, HSCs were isolated by MACS sorting, using the CD34 MicroBead kit from Miltenyi Biotec. $CD34^{+}$ cells (5,000 cells/well) were plated onto 6-well plates coated with OP9 or M2-10B4 cells inactivated with Mitomycin C (10 µg/ml) (Sigma) and plated in feeder-free system and cultured with the media described by Ni et al. (23): Ham $F12^{+}$ DMEM (1:2), 20% human serum (AB serum-Invitrogen, Life Technologies), 1% penicillin/streptomycin, 2-mercaptoethanol (25 µM), ascorbic acid (20 µg/ml), and sodium selenite (5 ng/ml). At the beginning of the differentiation process, IL-3 (5 ng/ml), IL-7 (20 ng/ml), IL-15 (10 ng/ml), SCF (20 ng/ml), and FLT3 ligand (10 ng/ml) (Miltenyi Biotec) were added to the medium. Half of the medium was changed every week. From the second week of differentiation, IL-3 was no longer added to the medium as was described by Cichocki and Miller (24) and Grzywacz et al. (25). The differentiation protocol that we carried out consisted of plating purified $CD34^{+}$ cells over two different culture conditions using two feeder cells layers, OP9 and M2-10B4, and cultured for 42 days with the differentiation medium previously described. From day 14 up to day 42 of differentiation, immunophenotype analyses were performed, along with cytotoxicity and degranulation assays at 28, 35, and 42 days of differentiation.

### Flow Cytometry Analysis

Purity of $CD34^{+}$ sorted cells from UCB samples was analyzed with CD34-PE antibody (BD Biosciences, clone 581) in a FACS Canto II (BD Biosciences). Purity of the $CD34^{+}$ cells isolated had to be higher than 80% in order to perform our protocol of differentiation (Figure S1A in Supplementary Material). The

number of remaining CD56$^+$ cells in the purified sample was not significant (Figure S1B in Supplementary Material).

Different populations and maturation stages of *in vitro* differentiated NK cells were analyzed by flow cytometry at 14, 21, 28, 35, and 42 days in culture. Cells were washed with PBS/10% FBS and incubated for 30 min at 4°C for labeling with anti-CD94-FITC (BD Biosciences, clone HP-3D9), anti-CD117-PE (Miltenyi Biotec, clone A3C6E2), anti-CD56-APC (Biolegend, clone MEM-188), and anti-CD16-BV421 (BD Biosciences, clone 3G8). Next, cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Plus in order to label them with anti-Perforin-PerCP-eF710 (BD Biosciences, clone δG9) and anti-Granzyme B-BV510 (BD Biosciences, clone GB11). 50,000–100,000 events were acquired for analyses. Populations were analyzed using FlowJo v.X.0.7 (TreeStar Inc.).

## Cytotoxicity Assay

In order to check the *in vitro* lytic activity of the differentiated NK cell against the K562 target cell line, we performed a calcein-AM-based cytotoxicity assay (26). K562 cell line was used as target cells. $10^6$ cells were incubated for 30 min at 37°C with 15 μM of calcein-AM (Life technologies C3099). These cells were washed twice after incubation. Calcein-AM-labeled K562 cells were cocultured with NK cells differentiated from CD34$^+$ progenitors from UCB in a U-bottom 96-well plate for 4 h at 37°C at different ratios (25:1, 12.5:1, 6.25:1, and 3.125:1). As a control, we used NK cells from adult healthy donors' blood isolated with the NK Cell Isolation Kit from Miltenyi Biotec (catalog number 130-092-657). These adult PB-NK cells were stimulated overnight under the same conditions as our UCB CD34$^+$ *in vitro* differentiation protocol (IL-7, IL-15, SCF, and FLT3). Adult PB-NK cells were purified by MACS sorting, using the NK Cell Isolation Kit from Miltenyi Biotec (130-092-657). For measurement of spontaneous release, K562 target cells were incubated with no NK cells. Total released was achieved by adding 4% Triton™ X-100 (Sigma-Aldrich) to the target cells. Each condition was performed in triplicates. After the incubation, 100 μl of supernatant was collected and transferred to a black 96-well plate to measure the calcein-AM release in a Fluoroskan Ascent (Thermo Fisher) (excitation filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm). The percentage of specific lysis is calculated according to the following formula: [(Test release) − (Medium fluorescence)] − [(Spontaneous release) − (Medium fluorescence)]/[(Total release) − (Triton fluorescence)] − [(Spontaneous release) − (Medium fluorescence)] × 100.

## Degranulation Assay

Natural killer cells were cocultured with K562 target cells at ratio 1:1 in a 24-well plate for 6 h at 37°C. At the beginning of the assay, anti-CD107a BV421 (BD Biosciences, clone H4A3) was added in order to detect the degranulation activity of the effector cells against the target cells. Golgi Stop™ (BD Biosciences) (monensin) was added following the manufacturer's protocol. After the incubation, cells were collected, washed, and labeled with anti-CD94-FITC and anti-CD56-APC. Degranulating NK cells (CD107a$^+$) were determined in the CD56$^+$ cells, both on stage 3 (CD56$^+$CD94$^-$) and stages 4–5 (CD56$^+$CD94$^+$) cells.

## Data Analysis

Differences between groups were evaluated using paired Student's *t*-test. *p*-Values <0.05 were considered significant. Statistical calculations were done using GraphPad Prism 6 (GraphPad Software, Inc.) Bars represent the mean and error bars represent the SEM.

# RESULTS

## OP9 Cell-Based Coculture System Generates Higher Numbers of CD56$^+$ NK Cells than M2-10B4 Cell-Based Coculture System

CD34$^+$ UCB cells were cultured up to 42 days using the protocol described in Section "Materials and Methods." Cell number and percentage of CD56$^+$ NK cells were checked weekly. CD34$^+$ UCB cocultured with OP9 cells feeder layer exhibited a better proliferative capacity as compared with CD34$^+$ UCB cocultured with M2-10B4 cells feeder layer at day 21. In addition, we found a higher number of CD56$^+$ NK cells with OP9 cells coculture at 28 days of differentiation ($p < 0.05$), reaching $2 \times 10^7$ NK cells on average, while this number dropped in the next days. Meanwhile, the number of CD56$^+$ cells in the M2-10B4 cells coculture condition increased gradually until 35 days of differentiation, reaching $1 \times 10^7$ NK cells, and dropped slightly at 42 days of differentiation (**Figure 1A**). Similarly, we observed a higher frequency of CD56$^+$ cells in the OP9 cells coculture than in the M2-10B4 cells coculture, with significant differences at day 21 ($p < 0.001$) and day 28 ($p < 0.01$) (**Figure 1B**). Likewise, we observed a higher fold expansion of CD56$^+$ cells in the OP9 cells coculture condition, especially at day 28 of differentiation ($p < 0.05$) which correlates with the pattern followed by the number of CD56$^+$ cells obtained (**Figure 1C**). Also, we perform a feeder-free culture condition. Our results showed that a very small number of NK cells were obtained in this culture condition (Figure S2 in Supplementary Material). We believe that the presence of the feeder layer is crucial for the correct differentiation of NK cells from CD34$^+$ cells.

## The *In Vitro* Differentiation Pattern of NK Cells Resembles the *In Vivo* Differentiation Pattern

According to the differentiation pattern from CD34$^+$ UCB to mature CD56$^+$ CD3$^-$ NK cells *in vivo* (Figures S3 and S4 in Supplementary Material), key markers were selected in order to determine the *in vitro* differentiation pattern obtained under our differentiation protocols (27). Depending on the presence or absence of different markers, NK cells were classified in different stages (*Stage < 3*: CD56$^-$, CD94$^-$, CD117$^{low}$; *Stage 3*: CD56$^+$, CD94$^-$, CD16$^-$, CD117$^{high}$; *Stage 4:* CD56$^+$, CD94$^+$, CD16$^-$, CD117$^{low}$, and *Stage 5*: CD56$^+$, CD94$^+$, CD16$^+$, CD117$^{low}$), which were determined according to the expression of the NK cell markers CD56 and CD94. The correct analysis of the stages was assured by the expression of the CD117 marker on NK cells. CD56$^+$/CD94$^+$ population was divided in stages 4 and 5 based on the presence or absence of the CD16 marker. In this manuscript,

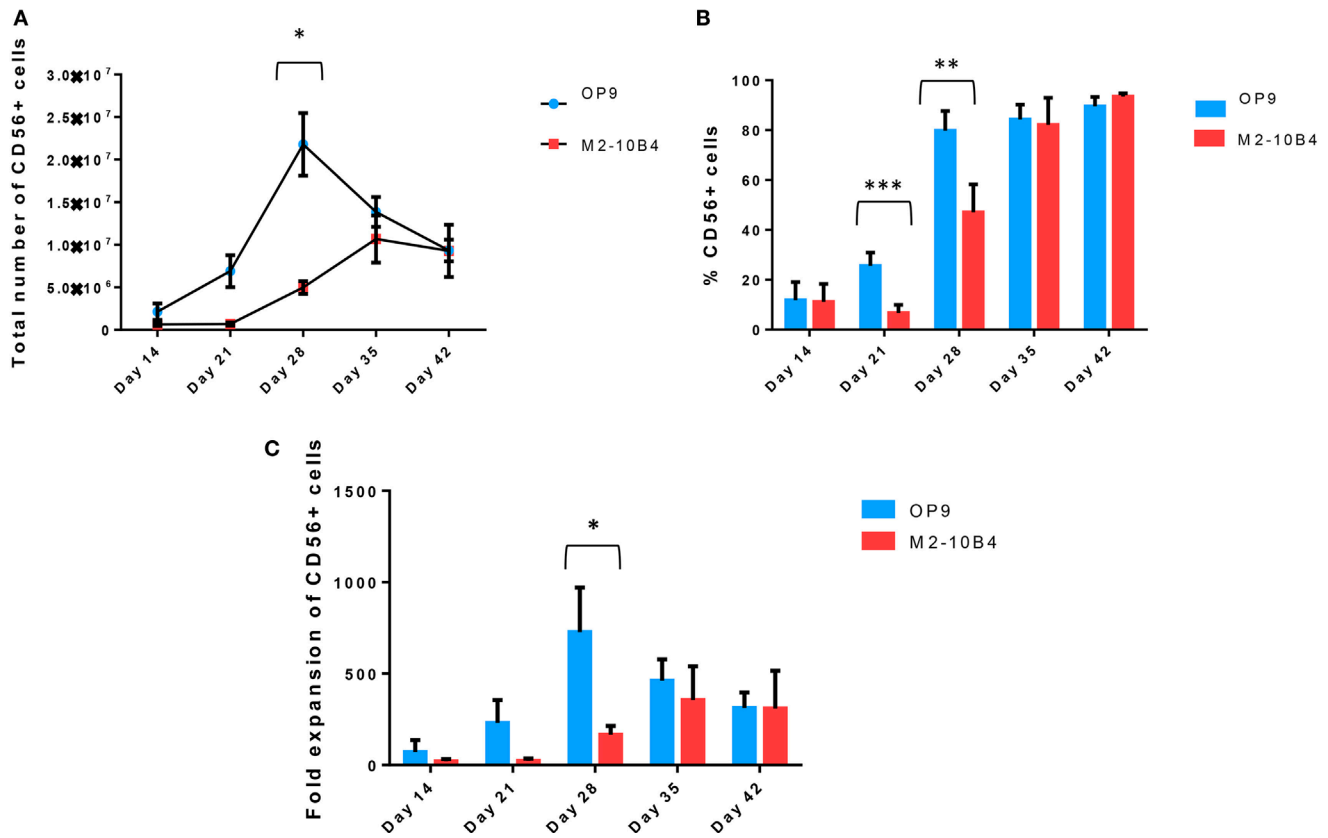


**FIGURE 1** | **(A)** Total number of CD56⁺ cells at different time points during the *in vitro* differentiation protocol with the two different culture conditions: OP9 and M2-10B4 cells coculture. **(B)** Percentage of CD56⁺ cells at different time points of the differentiation protocol with the two different culture conditions: OP9 and M2-10B4 cells coculture. **(C)** Fold expansion of the CD56⁺ cells regarding the initial number of CD34⁺ cells plated in each experiment ($n = 4$). The bars represent the mean and error bars represent SEM. $p$-Value: $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$. The absence of any asterisk indication means there are no significant differences.

stages 4 and 5 were represented as stages 4–5 in order to include mature NK cells in a single population.

The percentage of more mature NK cells increased over time during the *in vitro* differentiation protocol (**Figure 2A**), with higher percentage of more mature stages in culture conditions in the presence of OP9 cells in comparison with cultures in the presence of M2-10B4 cells. On the one hand, these more mature stages are reached earlier by the cells cocultured with the OP9 cell line than with the M2-10B4 cell line and the percentage of cells in the stages 4–5 is maintained over time with the OP9 cell line, while it gets higher with the M2-10B4 (**Figure 2B**). On the other hand, the total number of cells at stages 4–5 of maturation equalizes between the two culture conditions at the end of the differentiation protocol, having more cells at day 28 with the culture condition using OP9 cells (**Figure 2C**).

## *In Vitro*-Generated NK Cells Exhibit Cytotoxic Activity

Mature NK cells (stages 4 and 5) express cytolytic granules containing perforin and granzyme (28). We determined the expression of perforin and granzyme B in developing NK cells at days 28, 35, and 42 of the differentiation protocol. No significant differences in the expression of these two cytolytic markers were found between cells cultured with the OP9 cells layer in comparison with cells cultured with the M2-10B4 feeder cells. In both culture conditions, the percentage and the intensity of perforin expression in positive cells was higher than the expression of granzyme-B (**Figures 3A,B**). Moreover, the frequency of cells expressing perforin at days 28, 35, and 42 with OP9 cells feeder layer was very similar to the percentage of CD56⁺ NK cells, as well as to the condition with M2-10B4 cells feeder layer at days 35 and 42. We also found that *in vitro* differentiated NK cells did not show a significant difference in the expression of perforin and granzyme B between stage 3 and stages 4–5 in both culture conditions (data not shown).

Next, we determined the degranulation capacity of the *in vitro*-generated NK cells. To do that, we measured the expression of CD107a on NK cells after being stimulated with K562 target cells, according to the protocol described in Section "Materials and Methods." Activated NK cells from PB of healthy adult donors were used as a control (data not shown). At days 28, 35, and 42, the degranulation tended to be higher in NK cells generated with M2-10B4 feeder cells than in the NK cells generated in the

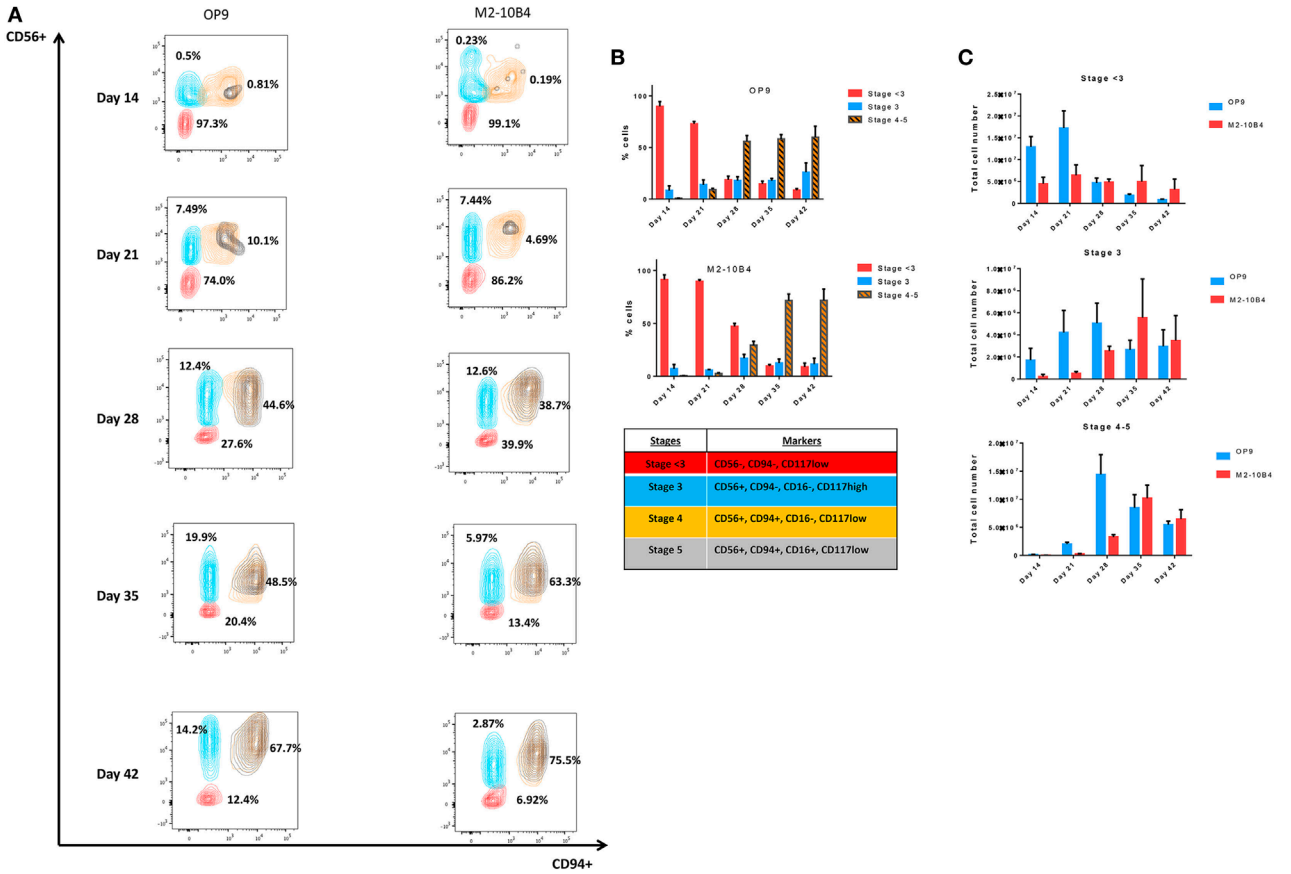


**FIGURE 2** | **(A)** Contour plots representing the different natural killer (NK) cell maturation stages during the differentiation protocol using OP9 and M2-10B4 cells as feeder layers. CD94, CD56, and CD16 markers were analyzed to determine the maturation stage as described in the text. Numbers represent the percentage of cells in stage <3 (red), stage 3 (turquoise), and stages 4–5 (orange and black). **(B)** Percentage of NK cells in the different stages of maturation (<3, 3, and 4–5) along the differentiation time course. **(C)** Total number of cells in the different stages (<3, 3, and 4–5) cultured with the two conditions: OP9 and M2-10B4 cells coculture. The bars represent the mean and error bars represent SEM (*Stage <3*: $CD56^-$, $CD94^-$, $CD117^{low}$; *Stage 3*: $CD56^+$, $CD94^-$, $CD16^-$, $CD117^{high}$; *Stage 4:* $CD56^+$, $CD94^+$, $CD16^-$, $CD117^{low}$, and *Stage 5*: $CD56^+$, $CD94^+$, $CD16^+$, $CD117^{low}$).

presence of OP9 feeder cells, although there were no significant differences (**Figure 4A**). We also analyzed the degranulation in the stage 3 and stages 4–5 of the *in vitro*-generated NK cells. The degranulation in both culture conditions exhibited a very significant difference between stage 3 and stages 4–5 (**Figure 4B**). Finally, we tested the cytolytic activity of NK cells against the K562 target cells. NK cells purified and activated from adult PB were used as a control. At day 28, NK cells differentiated in the presence of OP9 cells were more cytotoxic than NK cells differentiated in the presence of M2-10B4 cells (**Figure 4C**). At days 35 and 42, the cytotoxic activity was similar in both culture conditions at several effector:target ratios (**Figure 4C**). However, at day 42, at ratio 25:1, NK cells differentiated in the presence of M2-10B4 cells were significantly more cytotoxic than the NK cells differentiated in the presence of OP9 cells.

## DISCUSSION

In this study, we tested and compared the effect of two different culture conditions during the generation of *in vitro* functional and mature NK cells from HSC precursors from UCB. We show that by using OP9 cells as a feeder layer we obtained higher number of $CD56^+$ mature NK cells in comparison with M2-10B4 cells as a feeder layer. To date, several articles have described different protocols for *in vitro* NK cell differentiation from hematopoietic progenitors from UCB ($CD34^+$ cells) including the usage of different feeder cells, cytokine cocktails, and time of culture (16, 17, 24, 25, 29–40). However, we believe that our study for the first time describes in detail the maturation stages of NK cells during the *in vitro* differentiation process in which a high number of functional NK cells are achieved with the possibility for using them in future immunotherapies against cancer.

We have described a new culture condition, using two cell lines as feeder layers, i.e., OP9 cells and M2-10B4 cells, to generate NK cells from UCB $CD34^+$ hematopoietic precursors.

Other authors have described the use of these two cell lines to differentiate $CD34^+$ cells from pluripotent stem cells, such as ESCs and induced pluripotent stem cells (12, 18, 19, 21) and also to support and maintain $CD34^+$ cells in a long-term culture (41, 42).

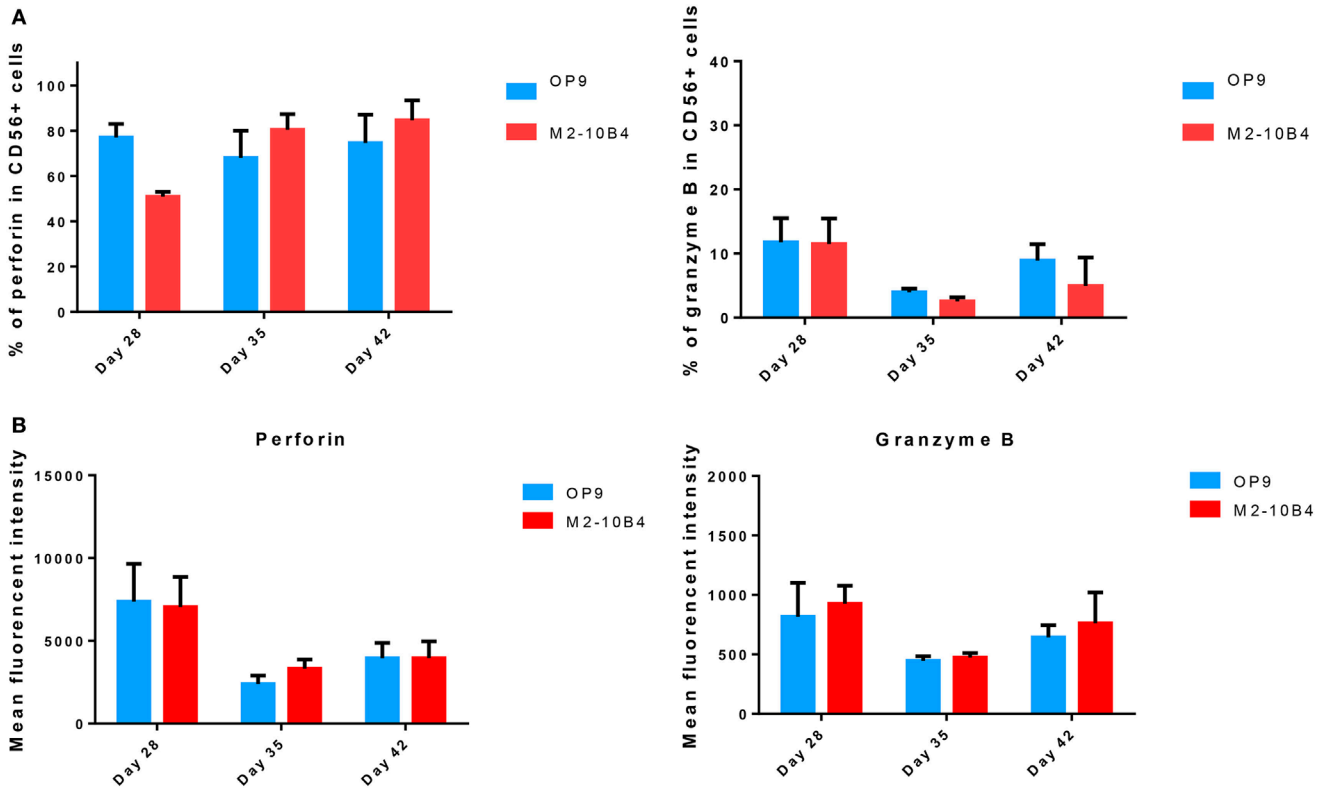


**FIGURE 3** | **(A)** Percentage of CD56⁺ natural killer (NK) cells expressing perforin and granzyme B cultured with OP9 and M2-10B4 feeder cells at days 28, 35, and 42 of the differentiation protocol. **(B)** Mean fluorescence intensity of perforin and granzyme B of CD56⁺ NK cells generated in the presence of OP9 and M2-10B4 feeder cells at days 28, 35, and 42 of differentiation. *p*-Value: $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$. The absence of any asterisk indication means there are no significant differences.

The use of feeder cell lines with the aim of maintenance and differentiation of stem cells toward blood lineage cells is an usual practice. First, the cell line AFT024 was described by Moore et al., immortalized with SV-40 T antigen, and derived from murine fetal liver stromal cells (43). Specifically, to study human NK cell ontogeny, Miller and McCullar suggested that NK cell differentiation from CD34⁺ cells and receptor acquisition was contact dependent with the feeder layer AFT024 (44). Other groups investigated the AFT024 and EL08-1D2 potential to generate *in vitro* NK cells and found that EL08-1D2 is significantly better at recapitulating NK cell development (39).

M2-10B4 is the other feeder cell line commonly used, and it derives from murine bone marrow stromal cells. M2-10B4 has been used earlier for NK cell expansion (45, 46) and others determined its differentiation potential for NK cell generation from hESCs (47). Remarkably, these findings suggest the need of stromal cell microenvironment, and the importance of direct contact with the feeder layer.

Finally, few reports have used the OP9 cell line (38, 40) to differentiate NK cells from bone marrow CD34⁺ cells. Also, OP9-DL1 (OP9 modified with Notch ligand delta-like 1) is used to develop T lymphocytes (48) and NK cells from hematopoietic precursors (49). To summarize, the majority of published data indicate the need of a microenvironment supported by stromal cells. This microenvironment provides necessary factors for the correct maturation of NK cells. Therefore, we wanted to explore new culture conditions to obtain high number of mature and functional NK cells from UCB CD34⁺ cells, to improve the *in vitro* differentiation protocols previously published, and also to in detail characterize there *in vitro* development in comparison with the maturation stages described *in vivo*.

To do this, we have compared both cell lines (OP9 and M2-10B4) in terms of differentiation capacity, number of CD56⁺ cells and NK fold expansion from the first week up to 6 weeks of differentiation. In general, we have obtained higher number, fold expansion, and frequencies of CD56⁺ NK cells, specifically when they are generated in the presence of OP9 feeder cells, than other authors who have used similar protocols. For example, with the EL08.1D2 cell line as feeder cells, several groups have obtained a similar fold of expansion (16) or a lower number and frequency of CD56⁺ NK cells (25, 31, 39) than us. Others have also obtained lower number or lower fold expansion when the AFT024 cell line (39) or Stro-1⁺ cell line (32) were used as feeder layers. In our study, using both cell lines (OP9 and M2-10B4), we get a higher number of NK cells with OP9 cell line. Importantly, we have also obtained higher number and frequencies of CD56⁺ NK cells than others who have also used OP9 cells as feeder layer (38, 40).

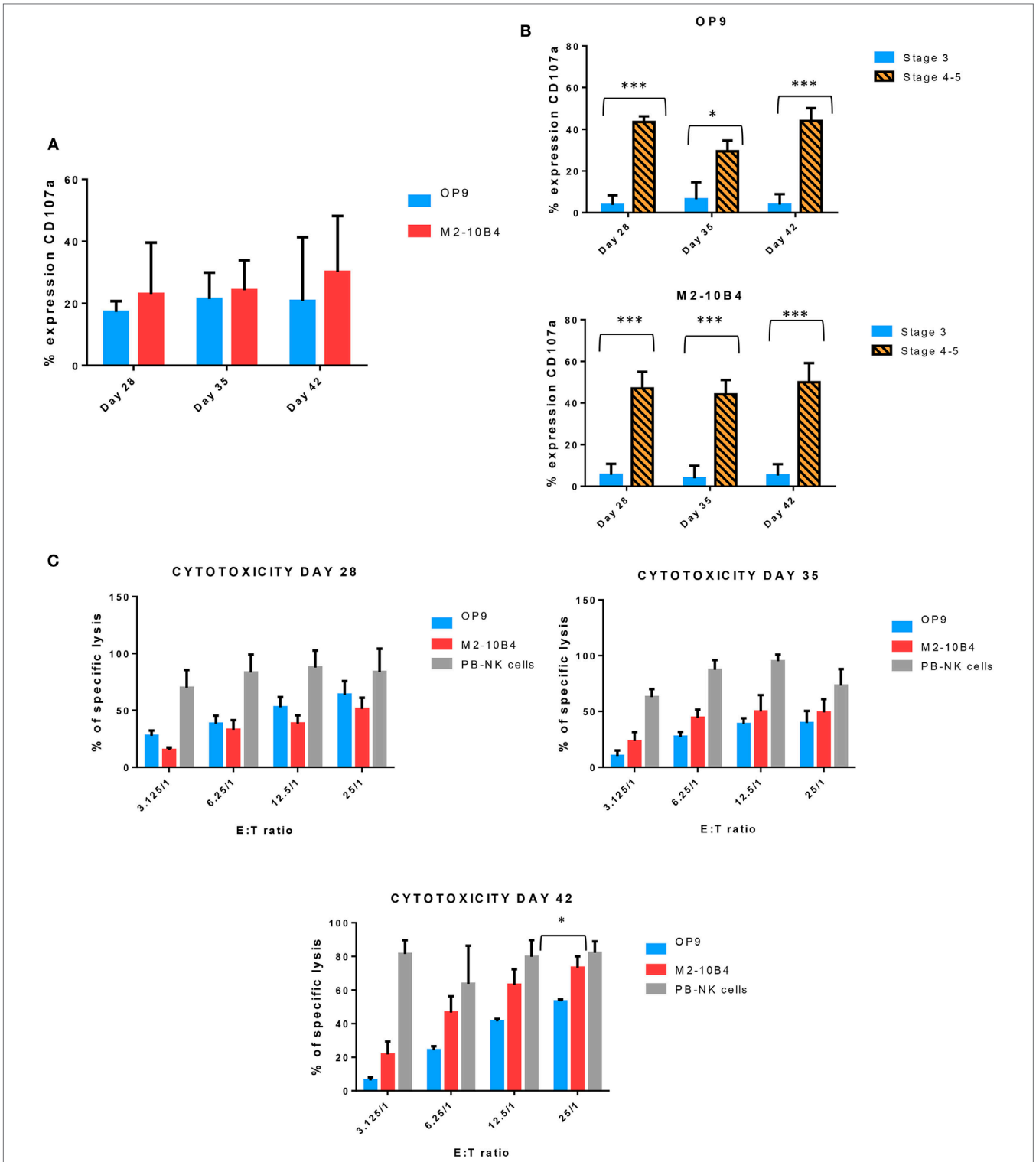


**FIGURE 4** | **(A)** Expression of CD107a, a marker of degranulation, in the *in vitro*-generated natural killer (NK) cells ($CD56^+$) at different time points of the differentiation protocol in response to the stimulation with K562 target cells. **(B)** Expression of CD107a in both conditions (with OP9 and M2-10B4 feeder cell layers) at stage 3 and stages 4–5 at different times of the differentiation protocol. **(C)** Cytotoxicity activity of NK cells against K562 target cells at days 28, 35, and 42 of the differentiation protocol. Overnight cultured NK cells obtained from adult peripheral blood stimulated with the same cytokines as our *in vitro*-generated NK cells were used as a control. The bars represent the mean and error bars represent SEM. *p*-Value: $*p < 0.05$; $***p < 0.001$. The absence of any asterisk indication means there are no significant differences (*Stage <3*: $CD56^-$, $CD94^-$, $CD117^{low}$; *Stage 3*: $CD56^+$, $CD94^-$, $CD16^-$, $CD117^{high}$; *Stage 4:* $CD56^+$, $CD94^+$, $CD16^-$, $CD117^{low}$, and *Stage 5*: $CD56^+$, $CD94^+$, $CD16^+$, $CD117^{low}$).

Currently, the vast majority of researchers accept a linear model of differentiation of human NK cells with five stages of maturation, each characterized by a pattern of expression of surface receptors, functional capabilities, and differentiation potential (27). In stage 3, cells have variable expression of the markers CD161 and CD56, typical of mature NK cells, but they do not express inhibitory receptors for MHC class I molecules, i.e., KIR and CD94/NKG2A, which they are characteristic of mature NK cells. In addition, cells in stage 3 have the two signs of functional identity of mature NK cells: IFN-γ production and perforin-dependent cytotoxic activity. CD94 expression marks the transition to stage 4 in the development of human NK cells. Cells at this stage are characterized by high CD56 expression. The acquisition of CD16 in some $CD94^+$ cells is considered a marker of cells in stage 5, the group defined by the phenotype $CD94^{high}CD56^{dim}CD16^+$ cells. Therefore, $CD56^{dim}$ and $CD56^{bright}$ cells in PB represent the two terminal stages of differentiation of human NK cells (50).

We have observed that our *in vitro* differentiation and maturation process of NK cells follows a similar pattern regarding the surface markers acquisition, which are observed *in vivo*. For example, CD117 expression in our *in vitro* NK cells is always present in late stages (4, 5), whereas is downregulated in *in vivo* differentiated NK cells at stage 5. We believe this is due to the presence of SCF in our culture system, as it has been reported that the presence of this cytokine upregulates the expression of CD117 in $CD56^+$ NK cells and significantly increases the capacity of $CD56^{bright}$ NK cells to degranulate (51). In our cultures, we cannot distinguish between $CD56^{dim}$ and $CD56^{bright}$ NK cells, which probably are due to the fact that this *in vitro* phenotype is slightly different to the *in vivo* due to the culture conditions.

We observed differences in maturation stages between the two cell lines used as feeder layers. More mature stages are reached earlier by using OP9 cell line than with the M2-10B4 cell line. Otherwise, the total number of cells at stages 4–5 of maturation equalizes between the two conditions at the end of the differentiation protocol, having more NK cells at day 28 when the culture conditions include the OP9 feeder cells, which also may be responsible for the observed higher cytotoxicity activity. We think that these differences in timing of maturation and number is due to the NK cell differentiation potential properties among stromal cell lines used in this study, because it has been reported that depending of the origin of the stromal cell lines used in hematopoietic differentiation, the features of maturation, and functionality of the terminal cell type could be different (52).

Few groups have described the *in vitro* developed NK cells in the presence of OP9 feeder cells. First, it has been reported that using OP9 cell line (38) during the *in vitro* development of NK cells from bone marrow or UCB $CD34^+$ cells, the levels of TGF-β may influence the developmental progression and subset formation of NK cells, like $CD56^{bright}CD16^-$ subset, but there are no studies in the progression of NK stages *in vitro*. Second, other group (40) also using OP9 feeder cells, obtained 80% of $CD56^+$ cells at 28 days, but they did not distinguish between different stages of maturation. In our case, we are able to achieve 70–80% of $CD56^+$ cells at the same day of differentiation but we studied in detail that around 38–45% of $CD56^+$ cells are already in stages 4–5, showing the typical markers of mature NK cells. Besides, other groups using other culture conditions achieved a minor percentage of $CD56^+$ cells in stages more immature than we did (16, 36).

In general, we have also obtained better or similar results than others when we look at the expression of cytolytic markers, degranulation properties, and killing activity of *in vitro*-generated NK cells. For example, in feeder-free systems, *in vitro*-generated NK cells expressed lower levels of perforin (34, 36), lower (36) or equal (35) cytotoxic activity, and equal levels of granzyme B (36) and degranulation potential (29, 30). When NK cells were generated in the presence of the EL08.1D2, they exhibited similar levels of perforin and granzyme B (16), similar degranulation levels (16, 33), and lower cytotoxic activity (25). Finally, in a study where OP9 feeder cells were used for the differentiation of NK cells, the authors found that they expressed lower levels of perforin (40) compared with the NK cells we obtained in our study.

In conclusion, we found that the use of OP9 and M2-10B4 cell lines to generate NK cells *in vitro* from UCB $CD34^+$ is a feasible option that offers the advantage of obtaining higher functional and mature NK cell numbers with enhanced killing capacity. To the best of our knowledge, this is the first and the most comprehensive study comparing these two culture conditions for the generation of NK cells from fresh UCB $CD34^+$ cells, being OP9 cells culture condition better than M2-10B4 cells, highlighting the great potential for UCB $CD34^+$ for future NK cell-based immunotherapy.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations and approval of "Basque Ethics Committee for Clinical Research [Comité Etico de Investigación Clinica de Euskadi-CEIC-E (PI2014138)]" with written informed consent from all subjects in accordance with the Declaration of Helsinki.

## AUTHOR CONTRIBUTIONS

LH: collection and/or assembly of data, data analysis and interpretation, and manuscript writing. JS: collection and/or assembly of data and data analysis. SS: data analysis and interpretation. MV: final approval of manuscript and financial support. FB: conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript. CE: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00755/full#supplementary-material.

**TABLE S1** | Number of fresh umbilical cord blood (UCB) units, volume in milliliters, number of mononuclear cells per UCB, and CD34+ cells remaining after purification for each experiment. UCB were fresh, being not longer than 30 h after the extraction.

**FIGURE S1** | **(A)** Purity of CD34+ cell population from umbilical cord blood (UCB) obtained by immunomagnetic isolation. **(B)** Remaining percentage of CD56+ cells after purification of CD34+ cells from UCB.

**FIGURE S2** | Total number of CD56+ cells at different time points during the *in vitro* differentiation protocol with three different culture conditions: OP9 and M2-10B4 cells coculture (N:4) and feeder-free (N:2).

**FIGURE S3** | Key markers expressed at different stages through natural killer cell differentiation/maturation *in vivo* and *in vitro*.

**FIGURE S4** | Dot-plots of the expression of CD3 at different stages of natural killer cells during the differentiation protocol.

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# Selection of Shared and Neoantigen-Reactive T Cells for Adoptive Cell Therapy Based on CD137 Separation

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Adoptive cell therapy (ACT) of autologous tumor infiltrating lymphocytes (TIL) is an effective immunotherapy for patients with solid tumors, yielding objective response rates of around 40% in refractory patients with metastatic melanoma. Most clinical centers utilize bulk, randomly isolated TIL from the tumor tissue for *ex vivo* expansion and infusion. Only a minor fraction of the administered T cells recognizes tumor antigens, such as shared and mutation-derived neoantigens, and consequently eliminates the tumor. Thus, there are many ongoing effects to identify and select tumor-specific TIL for therapy; however, those approaches are very costly and require months, which is unreasonable for most metastatic patients. CD137 (4-1BB) has been identified as a co-stimulatory marker, which is induced upon the specific interaction of T cells with their target cell. Therefore, CD137 can be a useful biomarker and an important tool for the selection of tumor-reactive T cells. Here, we developed and validated a simple and time efficient method for the selection of CD137-expressing T cells for therapy based on magnetic bead separation. CD137 selection was performed with clinical grade compliant reagents, and TIL were expanded in a large-scale manner to meet cell numbers required for the patient setting in a GMP facility. For the first time, the methodology was designed to comply with both clinical needs and limitations, and its feasibility was assessed. CD137-selected TIL demonstrated significantly increased antitumor reactivity and were enriched for T cells recognizing neoantigens as well as shared tumor antigens. CD137-based selection enabled the enrichment of tumor-reactive T cells without the necessity of knowing the epitope specificity or the antigen type. The direct implementation of the CD137 separation method to the cell production of TIL may provide a simple way to improve the clinical efficiency of TIL ACT.

**Keywords: adoptive cell therapy, melanoma, tumor infiltrating lymphocytes (TIL), CD137, neoantigen, tumor antigen**

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## INTRODUCTION

Adoptive cell therapy (ACT) of tumor infiltrating lymphocytes (TIL) is an effective therapy for patients with metastatic melanoma (1), leading to objective response rates of 40–50%, with some of those patients experiencing durable complete regression of their metastatic tumors (2–4). This form of ACT utilizes T cells that are isolated from fresh tumors, rapidly expanded and reactivated *ex vivo*

and then transferred back into the patient to eliminate the cancer cells (2, 5–8).

T cell responses rely on T cell receptor (TCR)-mediated recognition of tumor antigen derived from shared tumor-associated antigens (TAA) or neoantigens presented by self-MHC molecules (9–15). Neoantigenic peptides arise from somatic mutations occurring during neoplastic transformation and are mostly tumor, and even patient specific. The presence of tumor-specific MHC-neoantigen complexes on the surface of malignant cells represents a unique and specific target for T cells (16, 17). Shared/TAA, such as NY-ESO-1, MART-1, and gp100, are typically over-expressed in malignant cells, but also exist in normal cells (10–12).

T cells that target tumor neoantigens have been suggested to be the main mediators of effective cancer immunotherapies, not only in the context of adoptive T cell therapy, but also for successful treatment with checkpoint modulators against CTLA-4 and PD-1 (18, 19). Neoantigen-reactive TIL have been identified in the infusion products of metastatic melanoma patients who achieved durable cancer regression following ACT. As a result, multiple research efforts are currently being invested in the identification and selection of tumor mutation-specific TIL for therapy (20–22); however, these approaches are still very complex. Whole-exome sequencing (WES) of tumor DNA in combination with RNAseq and *in silico* HLA-binding prediction has been applied to identify non-synonymous cancer mutations recognized by T cells. This analysis can result in dozens or even hundreds of potential candidate peptides in highly mutated tumor types, such as melanoma (20, 22, 23). Candidate peptides, tetramers or tandems of minigenes (TMG) of those peptides are then expressed on MHC matched antigen-presenting cells (APC) and co-incubated with TIL cultures (22, 24). Neoantigen-reactive T cell cultures can be identified, as they specifically secrete interferon (IFN) γ or upregulate co-stimulatory molecules, such as CD134OX-40 or CD137 (4-1BB) upon peptide recognition (17, 25).

We have recently developed an alternative analytical tool that combines WES with HLA peptidome mass spectrometry, to identify neoantigenic peptides that are actually processed and presented by the tumor HLA molecules (26). Although the latter method is already more cost and time effective, all approaches still require sophisticated equipment and a period of several months. For most metastatic patients, this timeframe is unreasonable. Therefore, a quick and easy method for the identification of antitumor-reactive TIL is required, to make this approach clinically applicable.

Following antigen recognition, T cells undergo a wide range of phenotypic and functional changes including cytokines secretion and upregulation of multiple activation markers such as CD25, CD38, and CD69. The specific upregulation of co-stimulatory molecules, such as CD137 or CD134 or co-inhibitory molecules, such as CD279 (PD-1), provides an opportunity of using those molecules as biomarkers to detect and select tumor-reactive T cells for therapy (18, 27, 28).

CD137, a member of the TNF receptor superfamily, is an activation induced T cell co-stimulatory molecule. Signaling with CD137 upregulates survival genes, enhances cell division, induces cytokine production and prevents activation induced cell death of T cells. Others and our institute have shown that T cells co-incubated with APC loaded with neoantigenic or shared tumor peptides upregulate CD137 expression (21, 29, 30). This upregulation is highly specific and occurs only in T cells that recognize the antigenic tumor peptide. Ye et al. evaluated the immunobiology of CD137 in human ovarian cancer and showed that $CD137^+$ T cells when cocultured with autologous tumor cells, demonstrated increased reactivity against shared antigens (31). Importantly, this fraction of cells demonstrated enhanced *in vitro* and *in vivo* antitumor reactivity. Recently, Parkhurst et al. isolated $CD137^+$ TIL by FACS sorting after stimulation with dendritic cells transfected with mutated TMG RNA and could show that expanded $CD137^+$ cells are enriched for neoantigen-specific T cells (32). TCRs, isolated from those cells, were then introduced into autologous peripheral blood lymphocytes (PBL) to induce tumor rejection *in vitro*. Therefore, CD137 serves as a potential biomarker to isolate shared, as well as neoantigen-reactive TIL.

Here, we describe systematically a novel and time efficient method for the identification and selection of CD137-expressing T cells for therapy based on magnetic bead separation, using clinical grade compliant reagents. The method was adjusted to meet clinical limitations and needs. As expected, CD137-selected TIL showed significantly increased antitumor reactivity. This approach can be directly implemented to the clinical cell production of TIL and may provide a simple way to improve the efficiency of TIL ACT.

## MATERIALS AND METHODS

### Generation of TIL and Autologous Melanoma Lines

TIL and autologous melanoma lines were isolated from tumor biopsies of metastatic melanoma patients, enrolled to a phase II TIL ACT trial at the Sheba Medical Center (NCT00287131). Patients signed an informed consent approved by the Israeli Ministry of Health (Helsinki approval no. 3518/2004), which allowed enrollment to the clinical trial and the use of excess cell material for research purpose. The generation of TIL was conducted precisely as for the clinical setting and has been described in detail before (5). In short, fragmentation, enzymatic digestion, fine needle aspiration (FNA), and tissue remnant culture (TRC) techniques were used to isolate TIL and melanoma cells from surgically resected metastatic lesions. TIL were cultured in complete medium (CM) containing 10% human serum (Cat. no. 100-512, Gemini Bio-Products), 25 mmol/l HEPES (Cat. no. 03-025-1B, Biological Industries), 100 U/ml penicillin, 100 µg/ml streptomycin (penicillin–streptomycin mixture, Cat. no. BE-17-602E, Lonza), 50 µg/ml gentamicin (Cat. no. PZN-3928180, TEVA Pharmaceutical), and 5 × 10e (−5) M 2-mercaptoethanol (Cat. no. 31350-010, Thermo Fisher Scientific) in RPMI 1640 (Cat. no. 12-702F, Lonza). Melanoma cells were cultures in MEL medium contained 10% human serum, 25 mmol/l HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol L-glutamine (Cat. no. BE-17-605E, Lonza), and 1 mM

sodium pyruvate (Cat. no. BE-13-115E, Lonza) in RPMI 1640. TIL cultures were *ex vivo* expanded in three major steps: (1) pre-rapid expansion procedure (Pre-REP); TIL were cultured in 24-well plates, and CM with 3,000 IU/ml IL-2 (Cat. no. CH1001, Proleukin, Novartis Pharmaceuticals) was added every 2–3 days to keep the cell concentration at 0.5–2 × 10e6/ml. TIL cultures typically reached a total number of about 200 × 10e6 within 3–4 weeks, (2) CD137 selection; established TIL were cocultured with autologous melanoma cells followed by a magnetic bead separation of CD137-expressing cells; detailed below, (3) REP; unselected or CD137-selected TIL were expanded in a large-scale expansion procedure utilizing 30ng/ml anti-CD3 GMP antibody (Cat. no. 176-076-116; Miltenyi), 3,000 IU IL-2, and 50 Gy irradiated feeder cells of healthy donors (feeder cells to TIL = 100:1) in GRex flasks as detailed in Besser et al. (2). Feeder cells consist of a mixture, approximately equal proportions, of peripheral blood mononuclear cells (PBMC) isolated by Ficoll gradient from the apheresis product of three healthy donors. The intention is to compensate for a possible poor source of feeder cells. Within 14 days, cultures expanded by about 1,000-fold. Day 14 is the potential day of infusion.

## Generation of Autologous EBV Transformed B Cell Lines

PBL were separated by centrifugation on a Ficoll/Hypaque cushion. 10e7 mononuclear cells were centrifuged at 400 *g* for 10 min, and supernatant was carefully aspirated; the cells were resuspended in 1 ml B cell medium (10% human serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mmol L-glutamine in RPMI 1640), and 1 ml of Epstein-Barr virus (EBV) particles (kindly provided by Prof. E. Gazit—Sheba Medical Center, Israel) was added in a 15 ml conical tube. After careful mixing, the cells were incubated for 2 h at 37°C. Another 3 ml of B cell medium containing 1 µg/ml cyclosporine (Cat. no. tlrl-cyca, InvivoGen) were then added to the tube. The cells were transferred to a 25 $cm^2$ tissue culture flask and placed horizontally in an incubator at 37°C in a 5% $CO_2$ atmosphere for 3 weeks. Fresh B cell medium with cyclosporine was added whenever the medium turned yellow.

## Coculture and CD137 Separation

After completion of the Pre-REP step, TIL were cocultured with autologous melanoma cells at an effector to target (E:T) ratio of 8:1 (or as indicated). For this purpose 0.5 × 10e6 melanoma cells were resuspended in 2 ml MEL medium and transferred to one well of a 24-well plate. Typically, 48 wells (24 × 10e6 melanoma cells) were set up per experiment. Plates were incubated overnight at 37°C to allow adhesion of the tumor cells. The following day plates were rinsed with RPMI to remove non-adhered tumor cells. 4 × 10e6 TIL (in 2 ml MEL medium) were added per well and co-incubated with the tumor cells at 37°C. After 6–8 h (or as indicated), TIL were collected, centrifuged at 300 *g* for 10 min, and resuspended in CliniMACS PBS/EDTA buffer (Cat. no. 700-25, Miltenyi Biotech). To determine the number of $CD137^+$ cells, a small sample of the cells was removed to perform CD137 flow cytometry (detailed below). In the meantime, cells were centrifuged, and the pellet was resuspended with biotinylated anti-CD137 antibody (Cat. no. 701-30, CliniMACS CD137-Biotin reagent, Miltenyi Biotech, CE approved) (37.5 µl anti-CD137 antibody in 1 ml CliniMACS Buffer per 5 × 10e6 total cells), independent of the frequency of $CD137^+$ cells. The cell suspension was incubated for 30 min in the dark at 2–8°C and washed by adding 10 ml of CliniMACS PBS/EDTA buffer and centrifugation (300 *g* for 10 min). The cell pellet was resuspend with CliniMACS Anti-Biotin Reagent (Cat. no. 173-01, Miltenyi Biotech, CE approved) (37.5 µl anti-biotin MicroBeads in 1 ml CliniMACS Buffer per 5 × 10e6 total cells), incubated for 30 min in the dark at 2–8°C and washed by adding 10 ml of CliniMACS PBS/EDTA buffer followed by centrifugation (300 *g* for 10 min).

During the second incubation step, MACS ART MS Columns (Cat. no. 200-070-500; Miltenyi Biotech, CE approved) were prepared by connecting the columns to the MACS ART Separation Unit (Cat. no. 200-070-501, Miltenyi Biotech, see the manufacture's instruction; CE approved) and rinsing each column with 500 µl of MACS buffer. Results of the CD137 flow cytometry were obtained.

According to the manufacture's instruction, the capacity of one MS ART column is 10e7-labeled cells and up to 20e8 total cells. The cell pellet was resuspended in 500 µl MACS buffer for up to 10e8 total cells and applied onto the prepared column.

Unlabeled, $CD137^-$ cells passing through the column were collected after rinsing the column ones with 500 µl CliniMACS Buffer, and $CD137^+$ cells were collected after removal of the column from the magnetic separator and rinsing the column twice with 500 µl CliniMACS Buffer (see the manufacture's instruction).

## Coculture of TIL with Autologous EBV Transformed B Cell Lines (B-LCL)

B-LCL were resuspended in B cell medium at concentration of 2 × 10e6 per ml. 2 × 10e5 B-LCL were seeded in a well of a 96-well plates, and 5 µl of 25-mer peptide (stock: 0.2 mg/ml) was added. After an overnight incubation at 37°C, B-LCL were washed twice by centrifugation at 300 *g* for 6 min and resuspended in 100 µl MEL medium. A coculture was performed by adding 0.5 × 10e5 T cells suspended in 100 µl MEL medium to each well, followed by an overnight incubation at 37°C.

## Flow Cytometry

The following antibodies were used for flow cytometry: APC-labeled antihuman CD137 (Cat. no. 130-094-821, clone 4B4-1, Miltenyi Biotech), FITC-labeled antihuman CD4 FITC (Cat. no. PMG555346 BD Bioscience), PE-Cy7-labeled antihuman CD8 (Cat. no. 344712, BioLegend), APC-labeled antihuman CD45 (Cat. no. 340910 BD Bioscience), PE-labeled anti-MCSP (Cat. no. 130-091-225 Miltenyi Biotec), and PE-labeled antihuman CD3 (Cat. no. 555340 BD Bioscience).

TIL were washed and resuspended in FACS buffer consisting of 0.5% BSA in PBS. Cells were incubated for 30 min with the antibodies on ice, washed in FACS buffer, and read as the relative log fluorescence of live cells using MACSQuant flow cytometer

(Miltenyi Biotech). Samples were analyzed using FlowJo software (BD Bioscience). Cells were gated on viable lymphocytes, according to FSC and SSC, as well as singlets. If indicated, the cells were further gated on CD3 T cells (Figure S1 in Supplementary Material).

## IFNγ Release Assay

TIL were cocultured overnight with autologous melanoma cells in 96-well plates at an E:T ratio of 1:1 (5 × 10e5 each in a total of 200 µl MEL medium) or as indicated. Cells were centrifuged, supernatant was collected, and secreted IFNγ levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Cat. no. 430104, BioLegend). Measurements were performed in triplicates.

## Cell-Mediated Cytotoxicity Assay

TIL were cocultured with autologous melanoma cells overnight at 37°C, at an E:T ratio of 1:1 (1×10e5 each in a total of 200 µl MEL medium). Cells were centrifuged, supernatant was collected, and the levels of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis, were determined by CytoTox 96® Assay according to the manufacturer's instructions (Cat. no. G1780, Promega). Measurements were performed in triplicates.

## Statistical Analysis

Significance of variation between groups was evaluated using a non-parametric two-tailed Student's *t*-test. Test for differences between proportions was performed using two-sided Fisher's exact test with $p \leq 0.05$ considered significant and $p \leq 0.01$ highly significant. To strengthen the trend of dependence between two kinds of variables, we used the Spearman rank correlation coefficient.

# RESULTS

## Establishment of TIL and Autologous Melanoma Lines

Twelve metastatic melanoma patients, age 49 ± 14 years, underwent resection of a metastatic lesion. TIL isolation and generation was performed precisely as for the clinical setting (5–7).

Baseline characteristics, origin of metastasis and the size of metastasis are summarized in **Table 1**. Fragmentation (typically 12 fragments per patient), enzymatic digestion, fine needle aspiration (FNA) and tissue remnant culture (TRC) techniques were applied to generate TIL and melanoma cultures from surgically resected metastatic lesions (2, 5–7). In one patient, TIL and melanoma cultures could not be established (patient #12) and in three more patients autologous melanoma cells failed to grow (patients #9, #10, and #11, **Table 1**). Those four patients were excluded from further analysis. For the other eight patients (patients #1–#8), an average of 304 × 10e6 ± 87 × 10e6 TIL was established in an average of 14 days (range 7–21 days).

Retrospective analyses of TIL and melanoma cultures in 203 patients enrolled between the years 2005 and 2016 to our TIL ACT trial, showed that in 20 (10%) of the patients TIL numbers of over 200 × 10e6 TIL could not be reached and in 97 (48%) of the patients autologous melanoma lines could not be established within 1–2 months (or both). Since the co-incubation of both cell types is required prior to CD137 separation (see below), the method seems feasible for about half of enrolled patients.

## CD137 Expression and Correlation to *In Vitro* Reactivity

Baseline CD137 expression in established TIL cultures before and following co-incubation with autologous melanoma lines was determined in patients #1–#8. For each patient, one of multiple TIL cultures or a pool of different TIL cultures were analyzed. As expected, baseline CD137 expression was low in TIL cultures before coculture (3.4 ± 2.4%) or TIL cultures co-incubated with HLA-mismatched melanoma lines (data not shown) and significantly increased after coculture with autologous melanoma lines (30.1 ± 25.9%; $p = 0.011$) (**Table 1**), supporting the understanding that CD137 serves as a marker for tumor recognition.

Nine independently grown TIL cultures [four cultures derived from enzymatic digestion (Dig 1–4) and five cultures (Frg 1–5) derived from different fragments] were established from the same biopsy of patient #4 and analyzed for CD137 expression following co-incubation with autologous tumor cells. As shown in **Figure 1A**, even among different TIL cultures derived from the same patient, there is a large variation of CD137 expression. CD137 expression was significantly correlated with *in vitro* reactivity measured as IFNγ secretion following co-incubation with target cells (**Figures 1B,C**; $R^2 = 0.85$, $p \leq 0.01$).

## Adaptation of the Coculture Assay to the Clinical Setting

Coculture assays for experimental purposes are often conducted in 96-well plates at an effector to target ratio of 1:1 by mixing 1 × 10e5 TIL with 1 × 10e5 target cells (25).

Four major adaptations had to be to done to make this approach feasible for the clinical setting: (1) Decreasing the number of autologous tumor cells, as they are often the limiting factor in the TIL to tumor cell ratio; (2) Up scaling of the coculture assay; (3) Defining the time point of maximal CD137 expression; and (4) Separation of tumor cells from TIL after coculture, as CD137-selected TIL are directly used for large-scale expansion and sequential infusion.

To address those issues, pre-REPed TIL were cocultured overnight with autologous melanoma at different E:T ratios in 24-well plates by plating 0.5 × 10e6 tumor cells per well and addition of TIL at various E:T ratios in a final volume of 2 ml MEL medium. As shown before, for 90% of the patients TIL cultures can be established within 2–4 weeks, whereas melanoma cells grow often slowly. Therefore, we aimed to define an E:T ratio that requires low melanoma cell numbers. As shown in **Figure 2A**, E:T ratio of 8 TIL to 1 melanoma cell induced similar CD137 expression as for E:T of 1:1 ($p = 0.3$, normalized to 1:1 at the various time points). **Figure 2A** represents one of

**TABLE 1** | Baseline characteristics and CD137 expression of TIL patients.

| Pt. | Age/ gender | Origin of Met. | Size ($cm^3$) | Days and method to reach at least 200 × 10e6 TIL in total | % $CD8^+$ | Autol. mel. availed | CC feasible[a] | CD137 expression before CC (%) | CD137 expression after CC (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 57/M | SC | 2.3 | Day 14 (total 301 × 10e6)<br>Digest 130 × 10e6<br>TRC 63 × 10e6<br>Frag. 108 × 10e6<br>FNA not performed | 99 | Yes | Yes | 1.4 | 72.3 |
| 2 | 25/M | LN | 1.4 | Day 7 (total 325 × 10e6)<br>Digest 300 × 10e6<br>TRC 25 × 10e6<br>Frag. None<br>FNA none | 55 | Yes | Yes | 4.2 | 17.1 |
| 3 | 41/M | SC | 7.8 | Day 11 (total: 406 × 10e6)<br>Digest 320 × 10e6<br>TRC 70 × 10e6<br>Frag. 10 × 10e6<br>FNA 6 × 10e6 | 92 | Yes | Yes | 3.0 | 43.2 |
| 4 | 61/F | SC | 6.1 | Day 21 (total 200 × 10e6)<br>Digest 120 × 10e6<br>TRC None<br>Frag. 80 × 10e6<br>FNA none | 40 | Yes | Yes | 8.3 | 14.1 |
| 5 | 41/M | SC | 6.4 | Day 17 (total 240 × 10e6)<br>Digest 50 × 10e6<br>TRC: 50 × 10e6<br>Frag. 140 × 10e6<br>FNA none | 87 | Yes | Yes | 2.3 | 3.3 |
| 6 | 50/M | Visceral | 5.2 | Day 14 (total 432 × 10e6)<br>Digest 25 × 10e6<br>TRC: 250 × 10e6<br>Frag. 25 × 10e6<br>FNA: 132 × 10e6 | 84 | Yes | Yes | 3.8 | 33.7 |
| 7 | 36/M | LN | 20 | Day 13 (total 231 × 10e6)<br>Digest 14 × 10e6<br>TRC 159 × 10e6<br>Frag. 47 × 10e6<br>FNA 11 × 106 | 76 | Yes | Yes | 3.6 | 56.8 |
| 8 | 45/M | SC | 34 | Day 17 (total 248 × 10e6)<br>Digest 85 × 10e6<br>TRC 21 × 10e6<br>Frag. 142 × 10e6<br>FNA none | 54 | Yes | Yes | 0.3 | 0.5 |
| 9 | 74/M | LN | 162 | Day 13 (total 350 × 10e6)<br>Digest 100 × 10e6<br>TRC 110 × 10e6<br>Frag. 100 × 10e6<br>FNA 40 × 106 | 75 | No | No | nd | nd |
| 10 | 64/M | visceral | 8.0 | Day 13 (total 246 × 10e6)<br>Digest 158 × 10e6<br>TRC 26 × 10e6<br>Frag. 32 × 10e6<br>FNA 30 × 106 | 96 | No | No | nd | nd |
| 11 | 50/F | SC | 16 | Day 25 (total 202 × 10e6)<br>Digest 126 × 10e6<br>TRC 32 × 10e6<br>Frag. 32 × 10e6<br>FNA none | 98 | No | No | nd | nd |

(*Continued*)

**TABLE 1** | Continued

| Pt. | Age/ gender | Origin of Met. | Size ($cm^3$) | Days and method to reach at least 200 × 10e6 TIL in total | % $CD8^+$ | Autol. mel. availed | CC feasible[a] | CD137 expression before CC (%) | CD137 expression after CC (%) |
|---|---|---|---|---|---|---|---|---|---|
| 12 | 61/F | SC | 3.8 | Day 23 (not reached)<br>Digest 15 × 10e6<br>TRC 2 × 10e6<br>Frag. 3 × 10e6<br>FNA none | nd | No | No | nd | nd |

*Met., metastasis; Autol. mel., autologous melanoma line; CC, coculture; M, male; F, female; LN, lymph node; SC, subcutaneous; Digest, enzymatic digestion; TRC, tissue remnant culture; Frag., fragments (cell number indicates the total sum of TIL in 6–12 initiated fragment cultures); FNA, fine needle aspiration; nd, not determined.*
*% $CD8^+$ within the $CD3^+$ T cell population (all others are $CD4^+$ cells).*
*[a]A coculture was considered feasible when a minimum total number of 200 × 10e6 TIL was available and autologous melanoma lines were successfully established.*

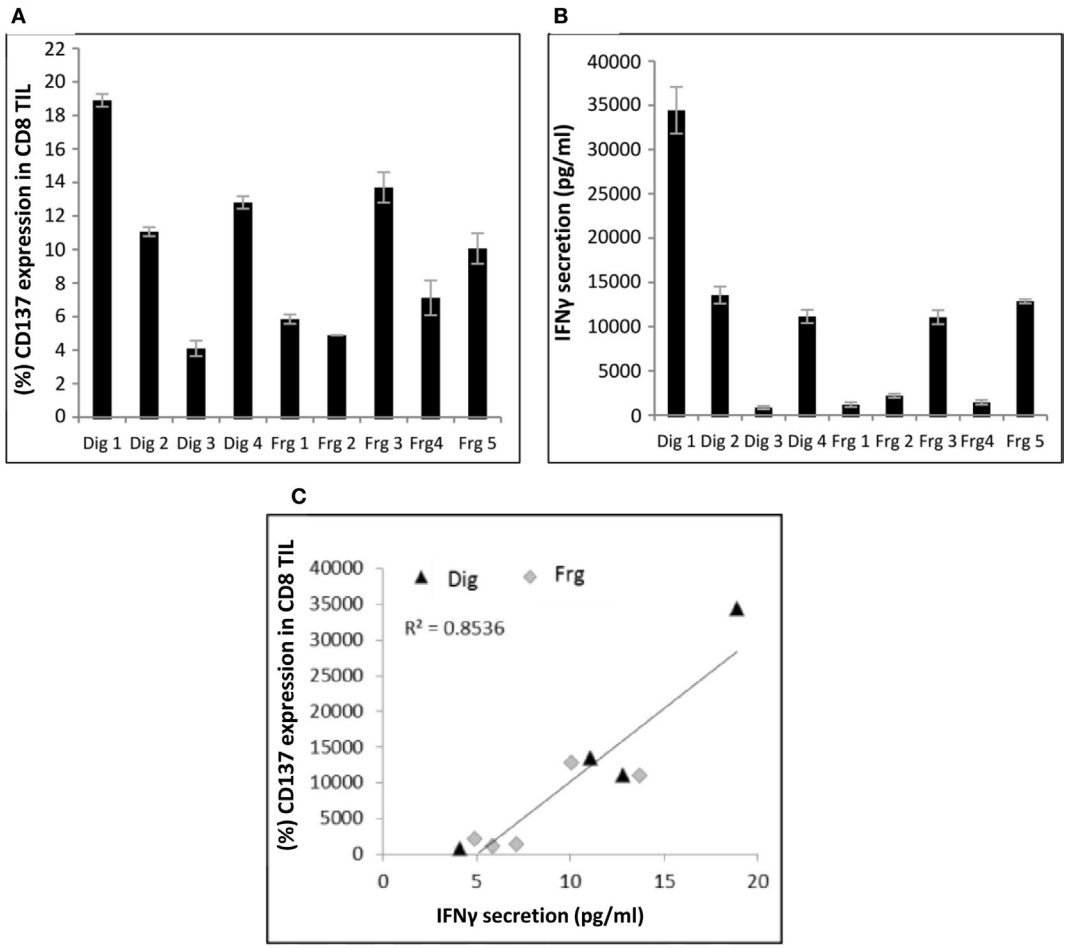


**FIGURE 1** | Correlation between CD137 expression and interferon (IFN) γ secretion. Multiple TIL cultures derived from the same melanoma biopsy of patient #4 were cocultured overnight with autologous melanoma cells at an E:T ratio of 1:1 (10e5 cells each). CD137 expression **(A)** and IFNγ secretion **(B)** were measured. Experiments were performed in triplicates. Correlation between the two is shown in panel **(C)**. Dig, enzymatic digest; Frg, fragments.

three experiments comparing different time points and E:T ratio, which all showed the same results. Consequently, cocultures can be conducted in two plates of 24-wells, by plating per well 0.5 × 10e6 melanoma cells and 4 × 10e6 TIL in a final volume of 2 ml media. This procedure requires altogether a total amount of 24 × 10e6 melanoma cells and 192 × 10e6 TIL cells in 96 ml medium.

To determine the time point of maximum CD137 expression, TIL were cocultured with target cells and CD137 expression was evaluated by flow cytometry at multiple time points. As shown in **Figure 2B**, the peak of CD137 expression was after 6–8 h.

As seen in **Figure 2C**, T cell reactivity measured by IFNγ secretion was detectable at all E:T ratios and specific, as

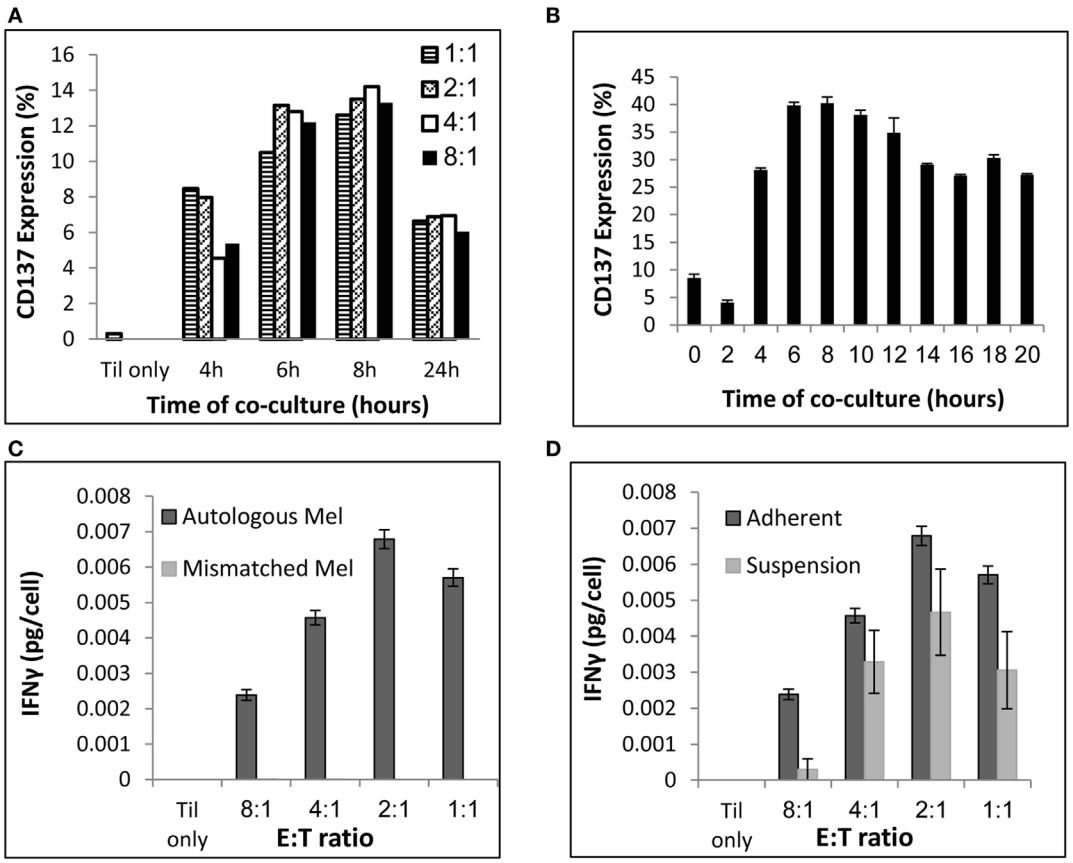


**FIGURE 2** | Coculture conditions. **(A)** Evaluation of CD137 expression after coculture at various E (TIL):T (melanoma) ratios and time points. **(B)** Evaluation of CD137 expression after coculture at E:T of 8:1 at various time points. **(C)** Interferon (IFN) γ secretion (pg/ml) per TIL cell following a 24 h coculture with autologous or HLA-mismatched melanoma cells at the indicated E:T ratios. **(D)** IFNγ secretion (pg/ml) per TIL cell following a 24 h coculture with autologous melanoma cells at the indicated E:T ratios. Melanoma cells were seeded 24 h before addition of TIL ("Adherent") or simultaneously to TIL ("Suspension"). Experiments were performed in triplicates **(B,C,D)**.

HLA-mismatched melanoma did not induce cytokine secretion. The total concentration of IFNγ was obviously higher at 8:1 compared with 1:1, as those cultures contained eight times more TIL. To consider this difference, **Figures 2C,D** demonstrate IFNγ levels per T cell [measured as IFNγ secretion (pg/ml)/number of TIL]. Although the IFNγ levels per T cell was lower at E:T of 8:1 compared with 1:1 ($p \leq 0.004$), an E:T of 8:1 was clearly sufficient to measure specific IFNγ secretion.

To avoid the passage over of tumor cells after coculture, melanoma cells were seeded for 16–24 h before addition of TIL to allow their attachment to the 24-well plate. Plates were rinsed twice with RPMI, to remove non-adhered tumor cell before adding TIL. As shown in **Figure 2D**, TIL cocultured with adhered melanoma cells secreted similar or higher IFNγ levels per T cell than TILs that were cocultured with non-adherent melanoma cells, which were added simultaneously with TIL ($p \leq 0.05$ at 8:1 and $p = 0.06$ at 1:1). Importantly, immunocytology with hematoxylin/eosin stain and antibodies against pan-cytokeratin and CD45 was performed and analyzed by a certified pathologist on four post-REP TIL (the potential infusion product) and confirmed no evidence of melanoma cells (data not shown). This was further supported by FACS analysis with antibodies against the melanoma-associated chondroitin sulfate proteoglycan (MCSP) antigen and the leukocyte marker CD45 (data not shown). Immunocytology is the standard methodology of most regulatory bodies for the detection of cellular impurities. Theoretically, the end product (also without coculture and CD137 selection) could still contain tumor cells, although this is very unlikely due to the magnetic bead separation and culture conditions that strongly promote T cell growth and T cell-mediated killing of tumor cells.

In conclusion, the final coculture protocol was defined as follows: (1) transfer of 0.5 × 10e6 melanoma cells per 1 ml MEL medium to each well of two 24-well plates (total of 24 × 10e6 melanoma cells in 48 wells); (2) overnight incubation at 37°C; (3) rinse twice to remove non-adhered tumor cells; (4) add 4 × 10e6 TIL per 1 ml to each well (8:1 E:T ratio; resulting in a total of 192 × 10e6 TIL in 48 wells); (5) co-incubation for 6–8 h at 37°C; and (6) collection of TIL.

As shown in **Table 1**, the average frequency of CD137-expressing TIL following coculture was 30.1 ± 25.9%. Performing coculture in two 24-well plates (192 × 10e6 TIL in total) would

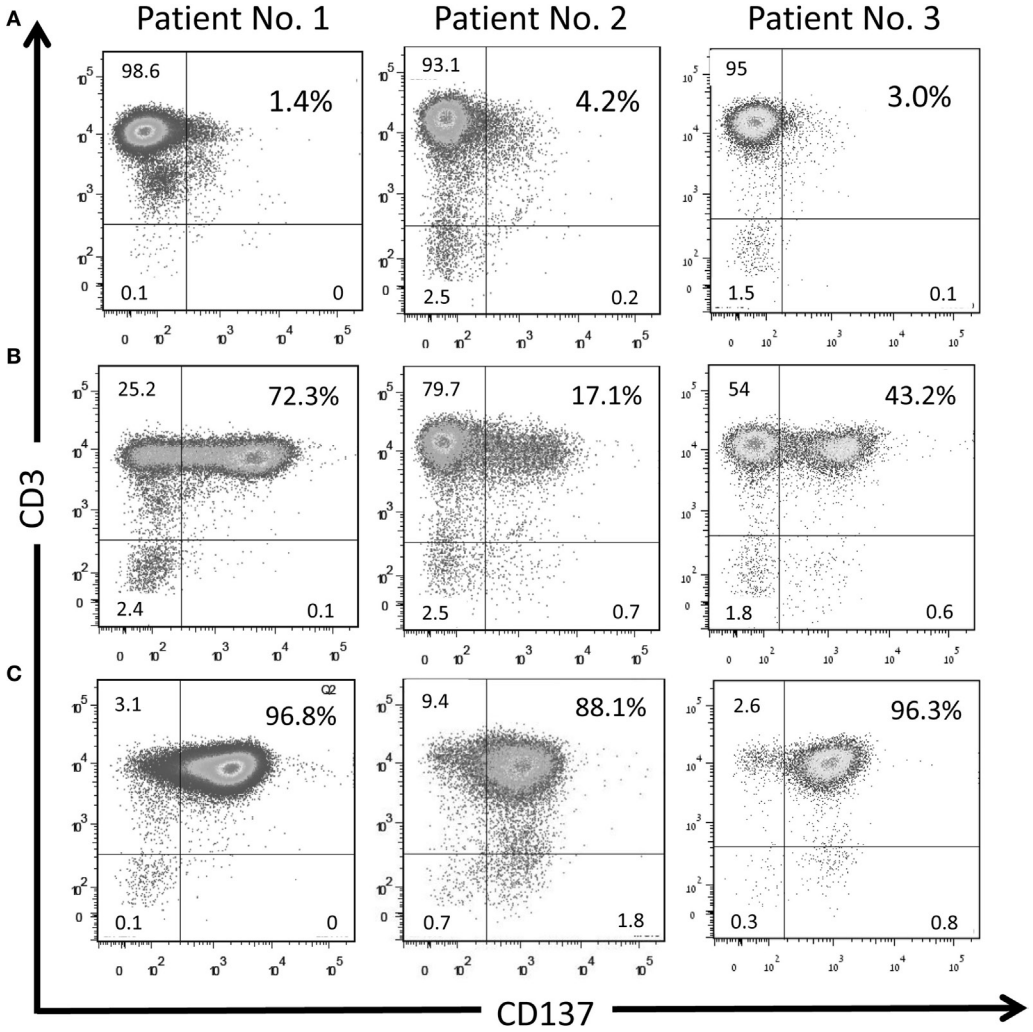


**FIGURE 3** | CD137 separation with magnetic beads enriches TIL population with activated TILs. CD137 expression on TIL cultures derived from three patients at baseline level **(A)**, after 6-h of co-incubation with autologous target cells (E:T 8:1) **(B)** and in the CD137$^+$ fraction after magnetic bead separation **(C)**. Singlet cells were gated on lymphocytes according to FSC and SSC.

**TABLE 2** | CD8, CD4, and PD-1 frequency of CD137 separated cells.

| | % CD8 | | % CD4 | | % PD-1 | | |
|---|---|---|---|---|---|---|---|
| | **Before separation** | **CD137 fraction** | **Before separation** | **CD137 fraction** | **Before CC** | **After CC** | **CD137 fraction** |
| Pt. 1 | 98.5 | 99.6 | 1.5 | 0.4 | 9.7 | 36.2 | 49.8 |
| Pt. 2 | 54.6 | 71.1 | 45.4 | 28.9 | 36.1 | 45.1 | 42.3 |
| Pt. 3 | 92.0 | 95.2 | 8.0 | 4.8 | 22.9 | 49.2 | 91.3 |
| Pt. 4 | 39.9 | 67.9 | 60.1 | 32.1 | nd | nd | nd |
| Av. | 71.3 ± 28.5 | 83.5 ± 16.3 | 28.8 ± 28.5 | 16.6 ± 16.3 | 22.9 ± 13.2 | 43.5 ± 6.6 | 61.1 ± 26.4 |
| *p*-Value | 0.48 | | 0.48 | | | 0.07 | 0.32 |

*CC, coculture with autologous melanoma cells; nd, not determined.*
*Cells were gated on viable, singlet CD3 T cells.*

therefore result in an average of 61 × 10e6 CD137-expressing TIL being availed for the next expansion step. In the clinical setting, the large-scale expansion of TIL (rapid expansion process, REP) is typically initiated with 30 to 50 × 10e6 TIL. For TIL cultures with a low frequency of CD137-expressing cells, coculture may be performed with additional 24-well plates.

## Magnetic Bead Separation of CD137-Expressing Cells

To allow the implementation of the CD137 selection process to the clinical setting, the enrichment process must be performed with clinical grade compliant reagents.

Following coculture, CD137-expressing cells were positively enriched by magnetic bead separation, by incubating 5 × 10e6 TIL first for 30 min with 37.5 µl/ml CliniMACS CD137-Biotin antibody, then for 30 min with 37.5 µl/ml CliniMACS Anti-Biotin Reagent and next transferred to MACS ART MS Columns, connected to a MACS ART Separation Unit (detailed in the Section "Materials and Methods"). Consequently, 192 × 10e6 TIL were incubated with 1.44 ml of each antibody and transferred to two to eight MACS ART MS Columns, depending on the percent of CD137-expressing cells following coculture (the column capacity is 1 × 10e7-labeled cells).

TIL cultures from three patients (patients #1–#3) were enriched for CD137-expressing cells. As shown in **Figure 3**, in those three patients the baseline level of CD137-expressing cells was 2.9 ± 1.4%, increased to 44.2 ± 27.6% after coculture and further increased to 93.3 ± 4.9% in the CD137$^+$ fraction after magnetic bead separation. The frequencies of CD4$^+$, CD8$^+$, and PD1+ cells within CD3 gated T cells are shown in **Table 2**. Since all TIL cultures of patient #1 were exclusively CD8 T cells (≥98%), the CD4/CD8 analysis was also performed on TIL of patient #4. The coculture assay itself does not affect the CD4/CD8 content. As shown in **Table 2**, although not significant, the percentage of CD8$^+$ cytotoxic T cells in the CD137 fraction was higher in all four patients compared with unseparated TIL; however, also the CD137$^+$ fraction contained CD4$^+$ T cells (16.6 ± 16.3%).

The antitumor *in vitro* reactivity of TILs was determined post separation by a cytotoxicity assay. As shown in **Figure 4A**, the CD137$^+$ fraction demonstrates significant increased antitumor reactivity compared with unseparated TIL (CD137$^+$ fraction 52.3 ± 10.6%; unseparated 6.6 ± 6.6%; $p \leq 0.007$).

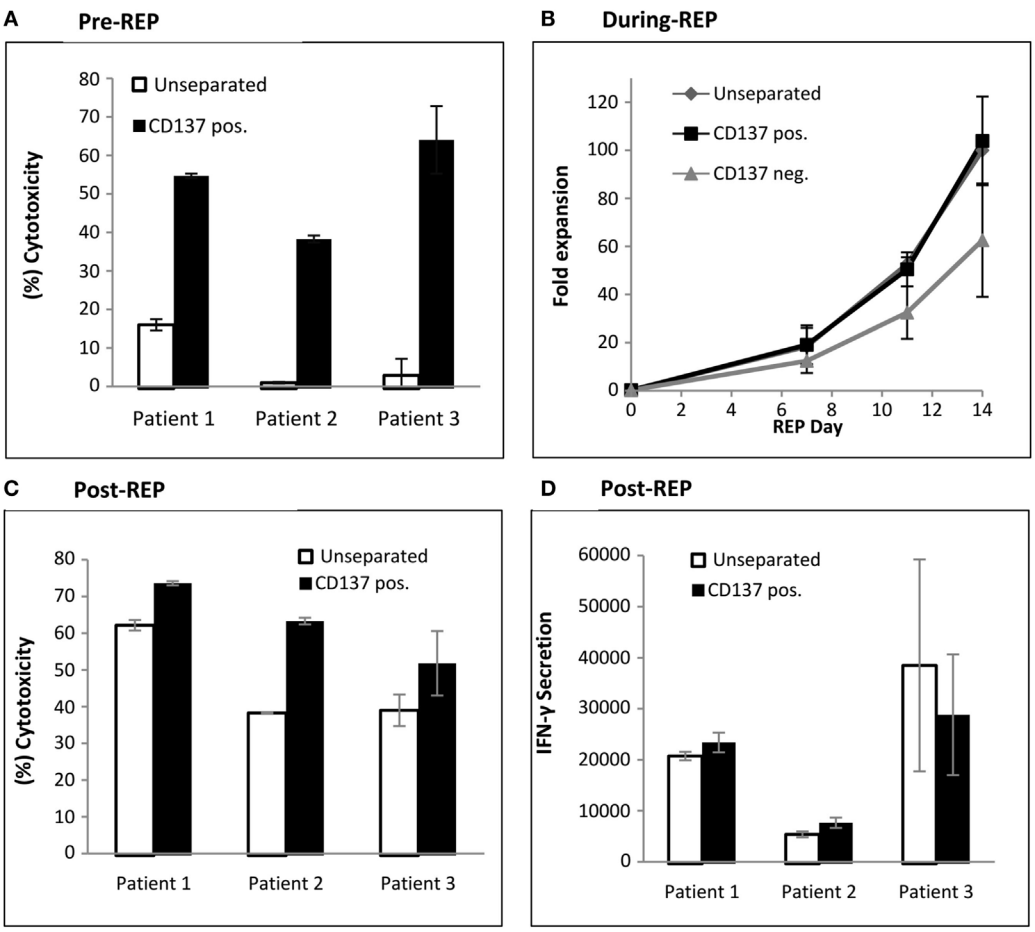


**FIGURE 4** | Antitumor reactivity and fold expansion of CD137 separated TIL compared with unseparated TIL. **(A)** Following magnetic bead separation, TIL of patients #1, #2, and #3 were cocultured with autologous melanoma cells and cytotoxicity was determined by lactate dehydrogenase (LDH) release. **(B)** Fold expansion of the CD137$^+$ fraction, CD137$^-$ fraction, and unseparated TIL during a 14-day rapid expansion procedure. Numbers were normalized to fold expansion on unseparated TIL on day 14. **(C,D)** *In vitro* reactivity of large-scale expanded TIL. On day 14 of REP, TIL were cocultured with autologous tumor cells, and cytotoxicity assay by LDH release **(C)** and interferon γ enzyme-linked immunosorbent assay (pg/ml) were performed **(D)**. All experiments were performed in triplicates.

**TABLE 3** | Memory phenotype and expression of co-inhibitory molecules on rapid expanded TIL.

| | Patient 1 | | Patient 2 | | Patient 3 | | *p*-Value |
|---|---|---|---|---|---|---|---|
| | Unsepa. | CD137 fraction | Unsepa. | CD137 fraction | Unsepa. | CD137 fraction | |
| **Subpopulations** | | | | | | | |
| CD4 | 4.7 | 3.9 | 51.0 | 28.3 | 0.4 | 0.3 | 0.69 |
| CD8 | 95.3 | 96.1 | 49.0 | 71.6 | 96.6 | 96.7 | 0.69 |
| **Memory phenotype** | | | | | | | |
| $T_N$ | 0.22 | 0.32 | 0.88 | 0.95 | 0.41 | 0.27 | 0.98 |
| $T_{CM}$ | 1.01 | 1.14 | 2.43 | 3.68 | 2.85 | 1.70 | 0.94 |
| $T_{EM}$ | 98.7 | 98.6 | 89.8 | 93.4 | 94.5 | 96.8 | 0.56 |
| $T_{EMRA}$ | 0.04 | 0.02 | 6.88 | 2.02 | 2.30 | 1.27 | 0.40 |
| **Co-inhibitory molecules** | | | | | | | |
| PD-1 | 14.3 | 14.2 | 32.3 | 33.0 | 23.1 | 20.0 | 0.88 |
| TIM-3 | 61.8 | 63.1 | 70.9 | 71.6 | 76.8 | 77.4 | 0.86 |
| LAG-3 | 4.94 | 4.79 | 70.6 | 63.3 | 8.35 | 8.84 | 0.88 |

*$T_N$ (naïve), $CD3^+CD45RA^+CCR7^+$; $T_{CM}$ (central memory), $CD3^+CD45RA^-CCR7^+$; $T_{EM}$ (effector memory), $CD3^+CD45RA^-CCR7^-$; $T_{EMRA}$ (effector), $CD3^+CD45RA^+CCR7^-$. Cells were gated on viable, singlet CD3 T cells.*

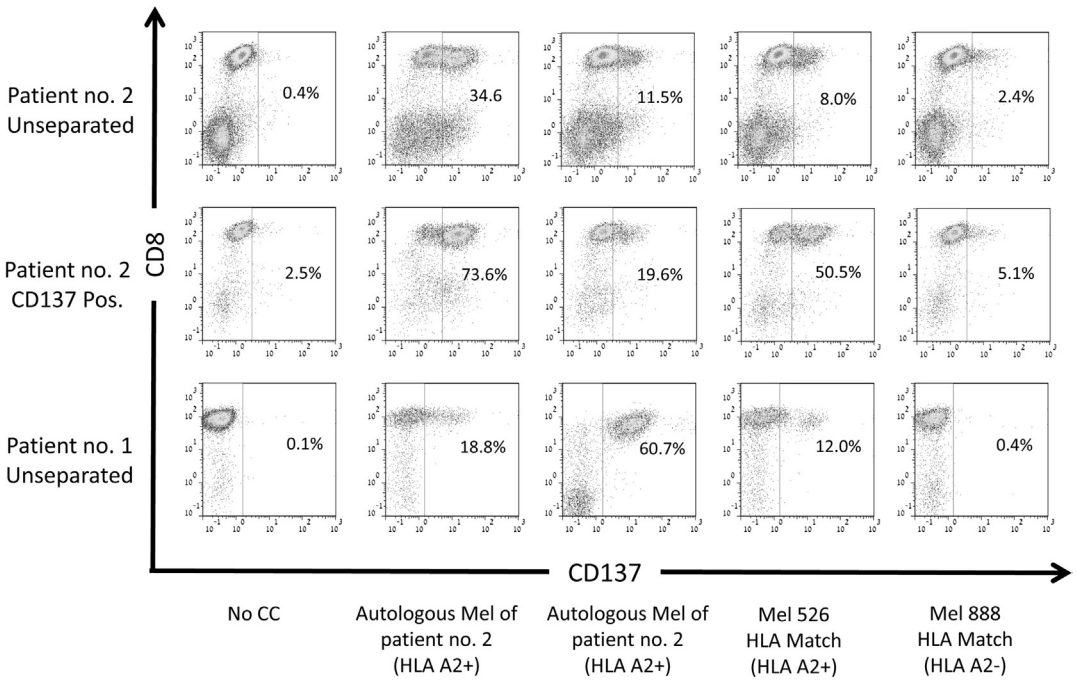


**FIGURE 5** | Reactivity of CD137-enriched TIL against shared tumor antigens. CD137 expression of TIL cultures derived from two patients post rapid expansion. Both patients carry the HLA-A0201 allele (HLA-$A2^+$). CD137 expression was evaluated before coculture and after a 6-h coculture with autologous melanoma (mel) lines of the patients, HLA-A0201-expressing melanoma line 526 or HLA-$A0201^-$ melanoma line 888. Cells were gated on viable, singlet CD3 T cells.

## Large-scale Expansion of CD137 Selected TIL

To produce large numbers of TIL for infusion, TIL are expanded in a standard 14-day rapid expansion protocol resulting in the final drug product for infusion. Unseparated cells, $CD137^+$ and $CD137^-$ TIL from three patients were expanded. The fold expansions during REP, normalized to unseparated TIL on day 14 are shown in **Figure 4B**. The $CD137^+$ fraction (103 ± 18%) reached on day 14 a similar fold expansion as unseparated TIL (100%; $p = 0.78$) or $CD137^-$ TIL (63 ± 24%; $p = 0.12$). *In vitro* tumor reactivity was determined on day 14 of REP, the potential day of infusion and demonstrated significantly increased cytotoxicity ($CD137^+$ fraction 62.9 ± 11.0%; unseparated 47.5 ± 12.6%; $p \leq 0.021$) (**Figure 4C**). Although not significant ($p \leq 0.23$), increased IFNγ secretion was observed in two out of three patients (**Figure 4D**).

Large-scale expanded TIL were further analyzed for CD4 and CD8 expression, their memory phenotype and expression of co-inhibitory molecules, such as PD-1, TIM-3, and LAG-3 (**Table 3**). Over 99% of the cells were $CD3^+$ T cells. Unseparated TIL and the $CD137^+$ fraction demonstrated a similar phenotype ($p$ values $\geq 0.4$).

## CD137$^+$ Cells Recognized Shared Tumor Antigens and Neoantigens

Previously, it has been described that CD137 is upregulated upon coculture with cells that present shared and neoantigenic tumor peptides (31, 32). To evaluate, if the CD137$^+$ fraction after REP, ready for infusion, is indeed enriched for TIL directed against shared antigens and/or neoantigens, we analyzed unseparated TIL or the post-REP CD137$^+$ fraction of patient #2. Patient #2 was chosen for this analysis, as the patient's cells express HLA-A0201$^+$ and the mutated neoantigenic peptide recognized by the patient's TIL was known. Unseparated TIL and the post-REP CD137$^+$ fraction were cocultured with four different target cells: (1) autologous, low passage melanoma cells; (2) allogeneic, low passage melanoma cells of patient #1 (HLA-A0201$^+$); (3) established melanoma line mel 526 (HLA-A0201$^+$); and (4) established melanoma line mel 888 (HLA-A0201$^-$). As shown in **Figure 5**, the frequency of CD137-expressing cells was increased post-REP from 34.6% in unseparated cells to 73.6% in the CD137$^+$ fraction when cocultured with autologous tumor cells. An increase was also observed after coculture with allogeneic HLA-A0201 matched tumor cell lines (11.5% unseparated to 19.6% in CD137 fraction after coculture with melanoma of patient #1; and from 8.0% to 38.9% after coculture with mel 526). On the other hand, CD137 expression was low after coculture with the HLA-mismatched cells mel 888 (2.4 and 5.1% for unseparated and CD137$^+$, respectively). Also when TIL of patient #1 were cocultured with autologous (60.7%) or HLA-A2 matched allogeneic tumor cells (mel patient #2, 18.8%; mel 526, 12.0%) CD137 expression was high, but low after co-incubation with HLA-mismatched tumor cells (mel 888, 0.4%). This indicates that CD137-enriched TIL exhibit the ability of recognizing shared tumor antigens, presented by autologous or allogeneic tumor cells in an HLA-dependent manner.

To evaluate, if the CD137-selected fraction is also enriched for tumor mutation-specific TIL, we determined the neoantigen reactivity of post-REP TIL of patient #2. Comparing WES data of the tumor tissue and PBMC revealed 3,256 non-synonymous cancer mutations, which were narrowed down to 223 potentially expressed peptides following RNAseq. The number of potential neoepitopes was further reduced to 162 after applying the NetMHCpan predication tool. EBV transformed B cell lines of patient #2 were pulsed with 25-mer peptides of each candidate peptide and cocultured with post-REP TIL of patient #2. CD137 expression was determined. Peptide #29 demonstrate increased CD137 expression (2.6%) compared with other peptides (around 1%, not shown) or the wild-type peptide (1.1%) (**Figure 6A**).

As shown in **Figure 6A**, following CD137 separation the fraction of TIL recognizing the mutated peptide increase by 3.2-fold from 2.6% in the unseparated TIL to 8.4% in the CD137$^+$ fraction. Antitumor reactivity was further examined by IFNγ ELISA. The secretion of IFNγ was significantly increased from 5,687 ± 224 pg/ml in unseparated TIL to 20,264 ± 2,043 pg/ml in CD137-enriched TIL ($p \leq 0.01$) after cocultured with neoantigen pulsed B-LCL (**Figure 6B**). These results demonstrate that CD137 bead separation increases the fraction of neoantigen-reactive TIL.

## DISCUSSION

The nature of antigens that allow specific tumor recognition and rejection by TIL cells has long remained obscure. T cells

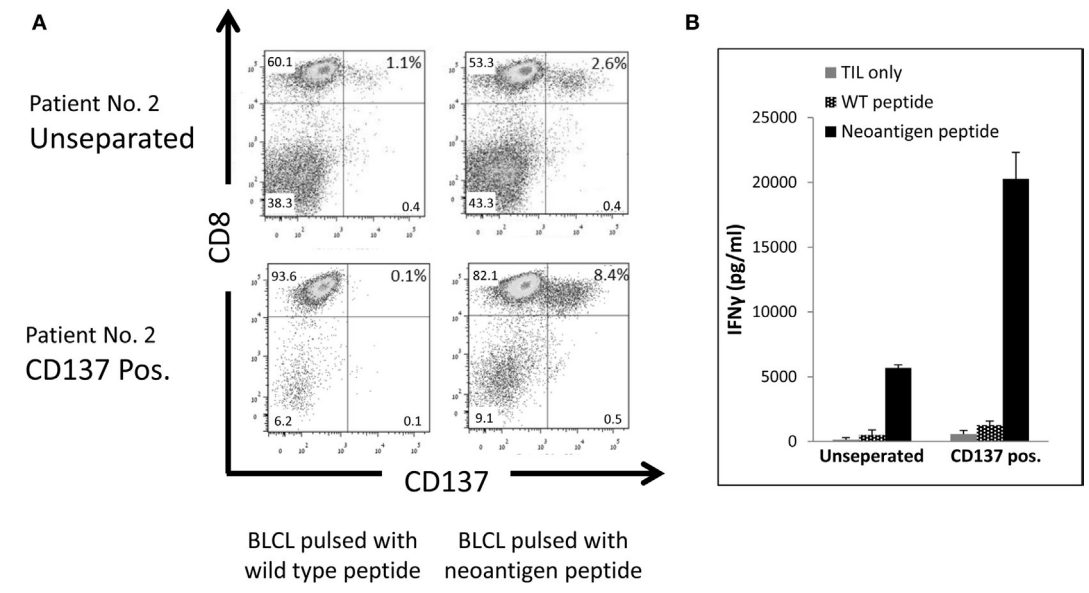


**FIGURE 6** | Reactivity of CD137-enriched TIL against neo antigens. Comparison of unseparated and CD137 positively enriched TIL of patient #2 post rapid expansion. TIL were tested after an overnight coculture with autologous B-LCL pulsed with a neoantigen peptide or control peptide at ratio of TIL to B-LCL of 1:4. CD137 expression by FACS. Cells were gated on viable, singlet CD3 T cells **(A)**, and interferon (IFN) γ secretion by enzyme-linked immunosorbent assay (ELISA) **(B)** was tested. ELISA was performed in triplicates.

reactive against tumor-specific mutations have been described as the key players for successful tumor regression. In a first clinical trial with neoantigen-enriched TIL, Tran et al. could demonstrate objective response in a metastatic epithelial cancer patient treated with a 95% population of mutation-reactive TIL (16). In addition, neoantigen-specific T cells were identified in the infusion products of melanoma patients achieving a durable response with TIL therapy (18, 19) or in the blood of patients following treatment with immune checkpoint antibodies (20, 21).

Many efforts are being made to identify and isolate tumor-specific T cells that will potentially allow tumor recognition and cancer regression. Detection of rare neoantigen-specific T cells relies on the identification of somatic mutations specific for tumor cells. These mutations exhibit patient-to-patient variability even in the same type of cancer. The process requires advanced technology such as WES, RNA sequencing, *in silico* analysis followed by synthetic peptides or tandem minigenes screening.

The identification of patient-specific, neoantigen-reactive TIL is not only labor intensive, but also requires several weeks in addition to the regular TIL production, which by itself can take up to 2 months. As most of the metastatic melanoma patients enrolled to TIL ACT have highly advanced metastatic disease, this period is unreasonable for many patients. Quicker and easier methods for the isolation of antitumor-specific TIL are therefore required to make this approach clinically applicable (5, 26).

In addition, T cells reactive against shared antigens, such as differentiation antigens in melanoma, have also demonstrated clinical activity. In adoptive cell transfer trials with gp100 or MART-1 TCR-transduced T cells, objective response rates of 19 and 30%, respectively, have been reported (12). These results indicate the importance of finding a biomarker that can identify both, neoantigen and shared antigen reactive T cells.

CD137 is an important regulator of the immune response. The specific antigen-induced expression of CD137 provides an opportunity for the selection of tumor-reactive T cells.

Here, we report a simple, robust and fast method for the enrichment of tumor-reactive T cells for therapy. We describe for the first time a methodology that was designed to address clinical needs and limitations. Every step was validated and optimized. The clinical feasibility of the method was assessed. All reagents used for this process were GMP compliant and approved by the local IRB.

Moreover, the novelty and importance of this study lies in its most detailed and optimized strategy to obtain a T cell infusion product with superior antitumor reactivity, which can be easily embedded in the clinical cell production. CD137 was previously used to identify T cell reactivity against neo-peptides when pulsed on APC, but not as a selection tool for mutation-specific TIL.

Clinical aspects, such as the fact that the number of autologous tumor cells is often limited, the absence of tumor cells in the infusion product or the up scaling of the coculture assay to 200 × 10e6 TIL, were taken into account.

In short, we propose the following protocol (**Figure 7**): (1) surgical resection of a tumor lesion; (2) establishment of TIL and autologous tumor cultures (yielding at least 200 × 0e6 TIL). This step requires typically 18 days but may vary between 2 and 4 weeks; (3) transfer of tumor cells to 24-well plates, to allow their adhesion to the plates overnight and addition of TIL at an E:T of 8:1 for 6–8 h; (4) magnetic bead separation with CD137 CliniMACS reagents and MACS ART columns, all CE approved; (5) standard rapid expansion of the $CD137^+$ fraction with irradiated feeder cells, anti-CD3 antibody and IL-2, resulting in an approximately 1,000-fold expansion within 14 days; and (6) infusion. Steps 1,

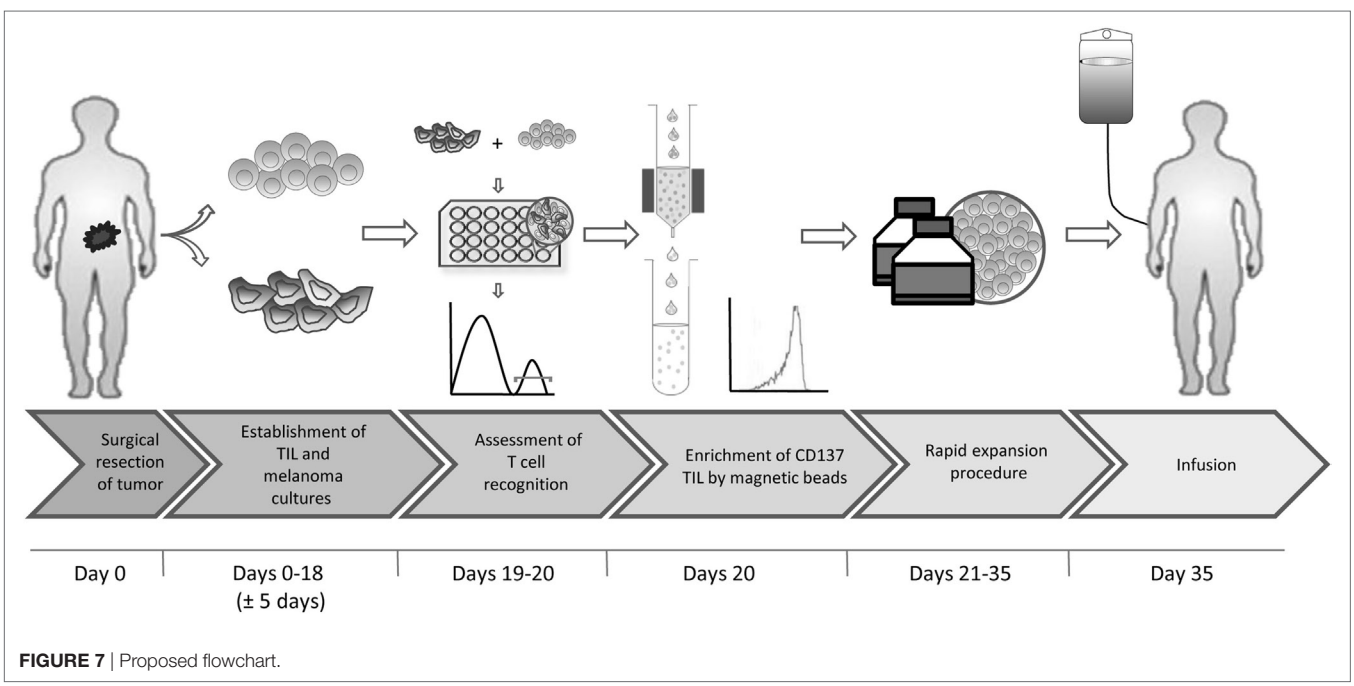


**FIGURE 7** | Proposed flowchart.

2, 5, and 6 are part of the standard TIL production protocol and require altogether 4–6 weeks. The coculture and magnetic bead separation (steps 3 and 4) just add another 2 days to this procedure, resulting in a total of about 35 days for the entire process.

It is the CD137 selection process itself, which enables the enrichment for tumor-specific T cells. As shown in **Table 1**, only 30.1 ± 25.9% of the TIL express CD137 following coculture, but nearly all cells express CD137 after the selection. CD137 selection may be especially of importance for patients with a low percentage of CD137-expressing cells. For example, in patient #2 $CD137^+$ cells were enriched by over fivefold (from 17 to 88%) by the magnetic bead selection. The data shown here provide strong evidence that CD137-selected TIL demonstrated increased *in vitro* antitumor reactivity and contained a higher fraction of neoantigen and shared tumor antigen reactive T cells. Selection based on CD137 expression enables the identification of tumor-reactive T cells without the need to know the epitope specificity or the antigen type. Further analysis of CD137-expressing T cells may lead to new epitope discovery.

The direct implementation of the CD137 separation method may provide a simple way to improve the objective response rate of TIL therapy. Based on the results shown here, we plan to initiate a clinical trial with CD137-selected TIL in the near future.

## AUTHOR CONTRIBUTIONS

SS-O, EM-S, and MB designed the study. SS-O, EM-S, OI, and SY acquired the data. SS-O, EM-S, OI, SY, GM, SY, JS, and MB analyzed and interpreted the data. All the authors revised the work, approved the final version, and agreed to be accountable of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01211/full#supplementary-material.

**FIGURE S1 |** Gating strategy.

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REVIEW
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# Virus-Specific T Cells for the Immunocompromised Patient

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While progress has been made in the treatment of both hematologic cancers and solid tumors, chemorefractory or relapsed disease often portends a dismal prognosis, and salvage chemotherapy or radiation expose patients to intolerable toxicities and may not be effective. Hematopoietic stem cell transplant offers the promise of cure for many patients, and while mismatched, unrelated or haploidentical donors are increasingly available, the recipients are at higher risk of severe immunosuppression and immune dysregulation due to graft versus host disease. Viral infections remain a primary cause of severe morbidity and mortality in this patient population. Again, many therapeutic options for viral disease are toxic, may be ineffective or generate resistance, or fail to convey long-term protection. Adoptive cell therapy with virus-specific T cells (VSTs) is a targeted therapy that is efficacious and has minimal toxicity in immunocompromised patients with CMV and EBV infections in particular. Products have since been generated specific for multiple viral antigens (multi-VST), which are not only effective but also confer protection in 70–90% of recipients when used as prophylaxis. Notably, these products can be generated from either virus-naive or virus-experienced autologous or allogeneic sources, including partially matched HLA-matched third-party donors. Obstacles to effective VST treatment are donor availability and product generation time. Banking of third-party VST is an attractive way to overcome these constraints and provide products on an as-needed basis. Other developments include epitope discovery to broaden the number of viral antigens targets in a single product, the optimization of VST generation from naive donor sources, and the modification of VSTs to enhance persistence and efficacy *in vivo*.

**Keywords: cell therapy, immunotherapy, adoptive, virus-specific T cells, immunocompromised host, *ex vivo* expansion, posttransplant complications**

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## INTRODUCTION

While hematopoietic stem cell transplant (HSCT) offers a chance of cure for patients with many high risk cancers or primary immunodeficiency syndromes, transplant recipients remain vulnerable to infectious complications due to prolonged and profound immunosuppression (1–4). These risks are modified by preparative regimen, transplant type, and duration of myelosuppression (1–4). With advances in conditioning regimens and improved posttransplant management, an increasing number of patients are eligible to receive mismatched, unrelated, or haploidentical donor HSCT. While there have been great improvements in outcome for patients with severe or otherwise untreatable disease, the immunosuppression required for engraftment and, when indicated, to treat graft versus host disease (GVHD), opens the door for infection. In particular,

viral infections cause significant morbidity and mortality, and the risk increases when T cell immune reconstitution is delayed (1–3). The relationship between immunosuppression, immune reconstitution, and the effects of GVHD, and infection are complicated and intertwined (5). Pharmacologic treatment and prophylactic options for viral infections remain limited and often ineffective, with associated morbidities notably from acute kidney injury and myelosuppression. Treatment may also generate resistance, and does not confer extended protection leaving patients at risk for viral reactivation (4). Given the correlation between delay in T cell immune recovery and viral disease, adoptive cell therapy is a logical alternative to pharmacologic therapy. Unmanipulated lymphocyte infusions from seropositive donors have been infused in patients with life-threatening disease such as EBV-associated lymphoma, demonstrating clinical efficacy with risks primarily associated with GVHD (6). This strategy has evolved over the past two decades, and donor lymphocyte products have been successful in reconstituting viral immunity in the host as a treatment for viral disease (including reactivation, new exposure, and lymphoma) and as prophylaxis (7). Following these initial studies, virus-specific T cell (VST) selection and/or expansion has been refined to maximize viral cytotoxicity and minimize alloreactivity to reduce and largely eliminate the risk of GVHD. In the current studies, VSTs offer targeted therapy and have demonstrated a very good safety profile to date (8–11). This review will detail developments in the manufacturing process, describe clinical success of VSTs and discuss future directions, including the use of naive donor sources and third-party banks.

# MATERIALS AND METHODS

## Antigen Selection

To successfully generate and expand VSTs, specific immunogenic epitopes need to be defined for each pathogen. It is well established that some viruses, notably CMV and EBV, are known to have certain antigens expressed at various stages of disease (12–14). Using available tools, epitope mapping has allowed identification of immunogenic antigens for other viruses, including adenovirus, human herpes virus 6 (HHV6), and BK virus (15–18). For many of these viruses, the immunodominant and subdominant antigens have been characterized, as well as antigens which promote enhanced T cell proliferation and immune protection *in vivo* (19). Several methods have been used to expand and select VSTs. Most recently, antigen-presenting cells (APCs) exposed to peptide mixtures consisting of overlapping, 15mer peptide libraries have proved highly successful for direct stimulation of $CD4^+$ and $CD8^+$ T cells (17–19). Alternative approaches use APC exposed to whole virus, viral lysates, whole proteins, or viral vectors (7, 9, 15, 20–24).

## Antigen Presentation

Once an appropriate antigen has been identified as an immune target, it must be effectively presented by APC to T cells in conjunction with costimulatory signals to promote T cell activation and proliferation. The APC type impacts production time, cell numbers, and product phenotype. Examples include dendritic cells, monocytes, B cells, and various artificial APCs. **Table 1** summarizes antigen and APC options. While dendritic cells are very effective APCs, they are limited by low numbers; thus, repeat stimulations require increasing amounts of donor cells. Whole virus has also been used to create potent APCs. For example, EBV lymphoblastoid cell lines (LCLs) infected with the B95-8 EBV strain, are effective and safe APC for generation of clinically useful products (9). However, this strategy is limited by lengthy incubation time with a potential risk of infection. Donor PHA blasts are potent, polyclonal stimulators of T cells and require only low cell numbers for generation (19). Artificial K-562 cells are another potential option for APC, and may be especially helpful to provide costimulation for generating VSTs from seronegative donors. This complementary costimulation in the presence of artificial APCs creates an effective antigen-presenting complex to promote VST stimulation and expansion (25).

## T Cell Expansion

Initial attempts at adoptive immunotherapy used unmanipulated donor lymphocyte infusions to transfer cytotoxic and memory T cells specific for certain viral infections. While an effective antiviral strategy, a major complication was GVHD, a natural consequence from infusing alloreactive T cells (6, 27). Furthermore this strategy was essentially limited to EBV and CMV where high frequencies of VSTs circulate in the donor (28). Modifications in the generation and *ex vivo* expansion of these T cell products have minimized GVHD to an almost negligible risk (28, 29). These processes have evolved over time to select and expand VSTs while minimizing alloreactive or naive T cells in the final product, with the emphasis currently on reducing production times and maximizing product functionality.

**TABLE 1** | Antigen selection and presentation.

| Antigen/APC | Advantage | Disadvantage |
|---|---|---|
| Whole virus/viral lysate | Potent antigen | Live virus, lengthy production time |
| Whole proteins | Readily available | Less potent antigen |
| Viral vectors | Reproducible | Lengthy production time |
| Peptide/peptide mixtures | Reproducible, standardized, readily available | Need identified immunodominant epitopes |
| Dendritic cells | Potent stimulators | Limited cell numbers, difficult to isolate |
| Monocytes | Easily isolated | Reduced potency |
| B cells | More robust numbers | Reduced generation of $T_{mem}$ (26), increased production time |
| PHA blasts | Reduced production time, easily expanded | Moderate potency |
| Artificial APC | Easily expanded and maintained; effective costimulation | Varying efficacy |

### Reduction of Alloreactive Cells

Several strategies have been aimed at inactivating or removing alloreactive T cells from donor products. One strategy evaluated blockade *via* monoclonal antibodies to the B7:CD28 costimulatory complex to produce an anergic response to recipient cells (30). This was successful, although time-consuming and not completely effective at preventing GVHD. Other strategies have employed selective depletion of alloreactive cells, either *ex vivo* or *in vivo*. *Ex vivo* methods use cells stimulated by recipient APCs followed by targeting alloreactive T cells through CD25, CD69, or CD95 and eliminating them by magnetic-coupled monoclonal antibodies or immununotoxins, photodynamic depletion, or apoptosis activation (31–34). These methods have seen some success *in vivo*, but results have been unreliable in terms of conveying viral protection and preventing GVHD. *In vivo* strategies employ a safety or suicide switch to deplete alloreactive T cells, to induce apoptosis in response to a specific signal. A thymidine kinase gene from herpes simplex virus I, acting as a trigger for cell elimination *via* ganciclovir exposure, was found to be effective but hampered by increased immunogenicity and a delay of several days to clinical effect after ganciclovir administration (35). Newer studies have transduced cells with the suicide gene-inducible caspase 9 (iC9), which triggers apoptosis after exposure to a dimerizing drug (36, 37). In this case, VSTs conveyed viral protection, and patients showed clinical improvement of GVHD symptoms soon after administration of the "safety switch" dimerizing drug.

### Approaches for Selection and Expansion of VSTs

Isolation of VSTs with or without *ex vivo* expansion offers an alternative means of eliminating alloreactive cells. More recent methods have simplified this process to reduce production times and simplify manufacturing strategies.

#### *Selection of VSTs*

Virus-specific T cells may be isolated directly from donor peripheral blood with the use of peptide-HLA multimers to facilitate the identification and purification of antigen-specific T cells. This process was originally hindered by irreversible binding and significant changes in T cell phenotype. The use of streptamers greatly improved this method, acting as multimers that use an HLA-peptide complex to reversibly bind the desired T cells without altering T cell phenotype or functional status. While a major benefit of this method is the rapid availability of an antigen-specific T cell product, the selected T cells are limited by HLA-restriction imposed by the streptamer (38–40). This process also requires knowledge of defined Class I HLA-restricted viral epitopes for effective isolation, and it selects for a limited repertoire of $CD8^+$ cells rather than the entire polyclonal, polyfunctional population of $CD4^+$ and $CD8^+$ T cells recognizing the full spectrum of available viral antigens. Despite noted limitations, investigators have been successful using VSTs isolated in this manner in the adoptive therapy for diseases with higher numbers of circulating VSTs, such as CMV and EBV. Such infused VSTs have also demonstrated expansion *in vivo* following transfer of these selected cells (41).

Interferon-γ (IFN-γ) capture also directly selects circulating VSTs from peripheral blood. Peripheral blood mononuclear cells (PBMCs) are stimulated with antigens specific for targeted virus and incubated over 4–16 h, inducing IFN-γ production in stimulated cells. A monoclonal antibody to IFN-γ coupled to a leukocyte-specific antibody (anti-CD45) then captures the IFN-γ producing cells, which are then selected *via* magnetic beads. This also allows rapid selection of VSTs free of HLA-restriction with the added benefit of stimulating and capturing a polyclonal population of $CD4^+$ and $CD8^+$ cells. This is clinically relevant, as the presence of $CD4^+$ cells enhances the memory and effector response and supports persistence and expansion of the cytotoxic T cells (42, 43). It also allows for selection of VSTs responding to multiple viral epitopes and has been successful in generating clinically functional VSTs targeted to various viruses (44–47).

Both these capture methods allow for rapid and precise selection of circulating VSTs, with obvious benefits for timely treatment of patients with active disease. However, they require VSTs to be circulating at a detectable level and leukapheresis is often needed to collect clinically relevant cell numbers. These methods are thus not useful for naive donors and ineffective if numbers of circulating VSTs are too low to generate a useful product.

#### *Expansion of VSTs*

The process of *ex vivo* T cell selection and expansion has been refined over the past two decades, with an emphasis on decreasing production times and complexity and optimizing *in vivo* function. The first techniques using EBV-LCL lines required at least 3 months to generate the APC and make a product. Despite still taking at least 10 days, current VST culture expansion systems generate polyclonal and polyfunctional products, properties which enhance *in vivo* expansion, function, and persistence. Clinical trials using *ex vivo* stimulated and expanded VSTs show that infused T cells persist long-term, detectible by gene-marking studies for as long as 9 years (48, 49). *Ex vivo* stimulation and expansion requires only a small volume of blood to establish the culture, eliminating the need for costly, time-consuming, and invasive leukapheresis. Lastly, expansion cultures make possible the generation of VSTs from low levels of circulating VSTs and naive donor sources (19–21, 50). Expanded VSTs infused in post-HSCT recipients carries a potential risk of causing GVHD. While some studies have shown cross reactivity of these VSTs with recipient targets *in vitro*, no increase in either acute or chronic GVHD has been reported (51). In fact, even when mild cross-reactivity of expanded VSTs with HLA-mismatched targets has been demonstrated *in vitro*, it has not correlated with increased risk of GVHD *in vivo* (52). Further refinements in this process continue to evolve as these procedures become standardized, including the use of overlapping peptide pools and alternative APCs to improve reliability and reproducibility of products.

## RESULTS OF VSTs IN CLINICAL USE

## CMV

Human cytomegalovirus is a pervasive β-herpes virus with prevalence rates of 50–100% in the general population. While it may manifest as mild self-limiting disease in the

immunocompetent host, CMV can cause severe life-threatening disease in the immunocompromised host. Because CMV persists in the latent form after acute infection, CMV-specific $CD4^+$ and $CD8^+$ T cells are necessary to maintain viral quiescence. In post-HSCT patients, in the absence of donor immunity and in other immunodeficient states, CMV may reactivate in the form of retinitis, pneumonitis, hepatitis, or enterocolitis (53). The adoptive transfer of CMV-specific T cells is a logical strategy for treating and preventing CMV reactivation in such individuals, and numerous clinical trials confirm the overall excellent efficacy of VST (10, 41, 46, 54–60). CMV-specific VSTs generated from naive T cells in umbilical cord blood (UCB) have also proved effective. These VSTs show specificity for atypical epitopes while maintaining functionality (21). Naive donor sources such as UCB are being explored as a source for other VSTs for generating third-party banks for on-demand use as well (see Other Viruses and Third-Party VST Products) (21).

## EBV

EBV is a ubiquitous, highly immunogenic γ-herpesvirus that can cause unique complications following transplant. Over 90% of the general population have been infected and retain lifelong seropositivity. Manifestations of primary EBV infection vary widely from asymptomatic infection to a debilitating viral illness (61). Thereafter in most cases, EBV remains latent lifelong in a B cell and mucosal epithelial reservoir under continuous T cell immune surveillance. In these healthy individuals, up to 2% of circulating T cells are EBV specific. In the period of immune deficiency after HSCT, EBV reactivation may cause viremia and life-threatening posttransplant lymphoproliferative disease (PTLD). While the monoclonal antibody rituximab successfully treats severe EBV disease in many patients by eliminating B cells in which the EBV virus resides, it results in long-term reduction in antibody production and is not always successful at controlling PTLD (61). Adoptive T cell therapy for PTLD is facilitated by the high probability of finding healthy EBV-exposed donors with measurable frequencies of circulating EBV-specific T cells. First attempts using donor lymphocyte infusions to treat EBV-PTLD were complicated by high rates of GVHD (6). Subsequent *ex vivo* strategies to select and expand EBV-specific T cells show broad efficacy and safety of EBV VST in numerous clinical trials for prevention and treatment of viremia and PTLD (47–49, 62–68).

## Adenovirus

Adenovirus infection can range from mild upper respiratory tract infections to a spectrum of life-threatening pneumonia, gastrointestinal, hepatic, renal, and neurologic complications. Following infection, latency is maintained in the lymphoid tissues, but the virus can reactivate during periods of prolonged absence of T cell immunity (69). Adenovirus causes potentially lethal viral complication in post-HSCT recipients. Antiviral drugs such as ribavirin are largely ineffective. However, adenovirus-specific T cells generated from healthy donors have proven effective at treating even advanced disease (45, 70, 71). For this reason adenovirus antigens are often incorporated in the generation of multivirus-specific T cell products (see below).

## Other Viruses

The BK and JC polyomaviruses, normally latent in healthy tissues of most adult individuals, reactivate after HSCT and in immunodeficient individuals (72). BK virus may manifest as nephropathy and life-threatening hemorrhagic cystitis (HC). Rarely, the closely associated JC virus causes fatal brain damage from progressive multifocal leukoencephalopathy (73). Polyoma-specific VST are being developed to combat these viruses. A single case report describes the successful use of BK VSTs, after which the patient had complete resolution of HC without bystander organ toxicity, GVHD, or graft rejection (74). It is now clear that the platforms developed for *ex vivo* selected and expanded VSTs are readily adaptable to many other viruses that complicate immune deficient states, and future developments include developing VST to target an array of viruses including VZV, HHV, and even HIV (16, 75–79).

## VSTs Targeting Multiple Viruses

Given the success in prophylaxis and treatment of individual viral infections with single-virus-specific VST, the targeting of multiple viruses in a single product is a logical extension for managing the post-HSCT patient at risk from multiple viral infections. Several groups have successfully manufactured multivirus-specific T cells for the more common viruses (11, 76, 80). Challenges, as with single virus-specific products, include production time, labor, and cost. Several groups have increased manufacturing efficiency through use of viral plasmids, standardized pepmixes, alternative APCs, and alternative donor sources such as UCB to produce polyclonal, clinically efficacious VSTs (19, 81, 82). A potential obstacle for multivirus pepmixes is the risk that the most immunodominant antigens will outcompete other T cell expansions and dilute the final product of clonal diversity. Various ways to maintain multiviral specificity are being explored (80, 83). To broaden the applicability of multivirus VST, Hanley et al used virus naive donors and UCB sources to generate tri-virus-specific T cells with success (20, 84). The viral repertoire of multi VST products is continually being extended and there is no apparent limit to the number of viruses that could be targeted in a single product. As an example, Gerdemann et al established a Good Manufacturing Practices (GMP) grade method for generating VST targeting seven different viruses: CMV, EBV, adenovirus, BK virus, HHV6, RSV, influenza (19). More recently, the group at Children's National has established a rapid, reproducible method in GMP-compliance for generating VSTs to CMV, EBV, Adenovirus, and BK virus from naive (cord blood) donor sources (82), paving the way for establishing third-party VST banks for "off the shelf" distribution.

## Third-Party VST Products

One of the more exciting developments in VST therapy is the generation of third-party VST banks. The development of a bank of efficacious, clinical-grade cell therapy products which pass all release testing requires an initial outlay in time, labor, and cost. However, immediate product availability avoids any risky delay in treatment of life threatening viral disease. Several groups have created third party VST banks for "off the shelf" administration.

Since these products are derived from unmatched donors and not autologous or HLA-matched sources, they carry an increased risk of GVHD. Nevertheless, with attempts to match at least one HLA molecule with the recipient, third-party VST products have been successful in several clinical trials (**Table 2**) (85, 86). Predictably higher number of HLA matches between the VST product and

**TABLE 2** | VSTs in clinical trials.

| Target | *N* | Method of T cell selection | Antigen presentation | GVHD occurrences | CMV status | Reference/ institution |
|---|---|---|---|---|---|---|
| CMV | 18 | IFN-γ capture | Peptide mixes of pp65 | 3 patients with grade I aGVHD; 3 patients with grade II/III aGVHD; 3 patients with cGVHD | 11 developed CMV reactivation, all responded to antivirals or repeat infusion of T cells | (46)/UCL[a] |
| | 7 | *Ex vivo* expansion | CMV lysate and peptide mixes of pp65 | No GVHD | Only 1 patient had persistent CMV viremia, one reactivation after steroids; CMV-specific T cell expansion in 6 patients | (54)/MKP[b] |
| | 14 | *Ex vivo* expansion | Dendritic cells with CMV-infected fibroblasts; only CD8 clonal population infused | 3 patients developed grade I/II aGVHD, all responding to steroids | No CMV disease, CMV immunity restored | (55)/FHCRC[c] |
| | 16 | *Ex vivo* expansion | Dendritic cells with CMV-infected fibroblasts | 3 patients with grade I aGVHD only | 8 patients also required ganciclovir but subsequently cleared viremia; 2 patients developed CMV reactivation postinfusion; CMV immunity restored | (10)/UCL |
| | 25 | *Ex vivo* expansion | CMV antigen; only CD4 clonal population infused | 1 case of GVHD | 7 patients with CMV reactivation; 5 patients with clinical disease; 2 patient deaths from CMV | (56)/U of Perugia[d] |
| | 18 | IFN-γ capture | pp65 protein | 1 case of GVHD | 4 patients died of CMV-related disease; 15 patients with *in vivo* expansion | (57)/UCH[e] |
| | 7 | *Ex vivo* expansion | Dendritic cells with peptide mixes (pp65, IE1) | No GVHD | 4 patients cleared CMV; 2 with reactivation (1 associated with high dose steroids), 1 with transient increase in CMV PCR | (58)/PSHCH[f] |
| | 9 | *Ex vivo* expansion | Dendritic cells with peptide mix (pp65) | 3 patients with grade III aGVHD, with one associated death; 2 patients with cGVHD | 2 patients with reactivation not requiring treatment | (59)/U of Sydney[g] |
| | 16 | *Ex vivo* expansion | Dendritic cells with peptide mix (pp65) | No GVHD | 14 patients cleared CMV | (60)/MSKCC[h] |
| | 2 | Streptamer-selection | PBMCs with pp65-HLA beads | No GVHD | Both cleared CMV with CMV-specific expansion | (41)/U of Ulm[i] |
| EBV | 39 | *Ex vivo* expansion | PBMCs with LCLs | No aGVHD or new cases of GVHD | EBV-specific immunity restored, clearance of viremia, no PTLD | (49)/SJCRH[j] |
| | 10 | IFN-γ capture | EBNA1 overlapping peptide mixtures | 1 patient with Grade I/II aGVHD | Expansion of EBV-specific T cells in 8 patients and clinical/virologic response in 7 patients | (47)/UCH |
| | 6 | IFN-γ capture | EBV peptide mix | No GVHD | Resolution of PTLD in 3 patients; progression of PTLD in 3 patients (all late stage at time of transfer) | (63)/HZM[k] |
| | 114 | *Ex vivo* expansion | PBMCs with LCLs | No *de novo* GVHD; 8 patients with reactivation of Grade I/II GVHD; 11 patients with limited cGVHD; 2 patients with extensive cGVHD | No PTLD development; remission of preexisting PTLD in 11 of 13 patients | (48)/BCM[l] |
| | 19 | *Ex vivo* expansion | T cells with LCLs | No GVHD | Resolution of PTLD in 13 patients; 2 patients with PD received DLI and 1 achieved CR | (64)/MSKCC |
| | 36 | *Ex vivo* expansion | T cells with LCLs | No aGVHD, 4 patients with limited cGVHD | No PTLD development | (65)/SJCRH |
| | 42 | *Ex vivo* expansion | T cells with LCLs | No GVHD | No PTLD development, reconstitution of EBV-specific immunity | (66)/SJCRH |
| | 4 | *Ex vivo* expansion | PBMCs with LCLs | No GVHD | Clearance of PTLD or EBV viremia | (67)/U of Pavia[m] |

(*Continued*)

**TABLE 2** | Continued

| Target | *N* | Method of T cell selection | Antigen presentation | GVHD occurrences | CMV status | Reference/ institution |
|---|---|---|---|---|---|---|
| Adenovirus | 9 | IFN-γ capture | Adenovirus antigen C | Exacerbation of preexisting skin GVHD | 5 patients responded with expansion of adenovirus-specific T cells in 5 patients | (70)/UCH |
| | 30 | IFN-γ capture | Hexon protein | 2 grade I GVHD; overall decrease in patients with GVHD | 21 patients responded | (45)/UCH |
| | 1 | IFN-γ capture | Hexon protein | No GVHD | Complete response | (71)/BGCH[n] |
| BK virus | 1 | IFN-γ capture | Large-T, VP1 | No GVHD | Complete response | (74)/HH[o] |
| **Multivirus specific** | | | | | | |
| EBV-CMV-Adeno | 10 | *Ex vivo* expansion | Dendritic cells nucleofected with viral plasmids: EBV (LMP1, LMP2, bzlf), CMV (IE1, pp65), adenovirus (hexon, penton) | 1 grade I/II GVHD | 8 patients with CR; 1 patient with stable EBV disease without PTLD | (81)/BCM |
| EBV-Adeno | 12 | *Ex vivo* expansion | PBMCs with Ad5f35 vector and LCLs | No GVHD | Expansion of virus-specific immunity, resolution or prevention of clinical disease | (11)/BCM |
| EBV-CMV-Adeno | 11 | *Ex vivo* expansion | PBMCs with LCLs transformed with Ad5f35-CMVpp65 vector | No GVHD | Expansion of EBV- and CMV-specific immunity in all patients, adenovirus-specific immunity in patients with clinical disease; clearance of all clinical disease | (80)/BCM |
| EBV-CMV-Adeno-VZV | 10 | *Ex vivo* expansion | PBMCs with Ad5F35-pp65, Ad5F35-EBNA1/LMP, VZV vaccine | 1 grade II GVHD, 1 grade III GVHD | 6 patients with CMV reactivation, only one receiving antiviral therapy; no EBV, adenovirus, or VZV reactivation | (76)/U of Sydney |
| EBV-CMV-Adeno-BKV-HHV6 | 11 | *Ex vivo* expansion | PBMCs with pepmixes (LMP2, BZLF, EBNA1, penton, hexon, pp65, IE-1, VP1, large T, U11, U14, U90) | 1 grade II aGVHD | No viral reactivation in 3 patients infused prophylactically; EBV—5 patients with CR, including PTLD; CMV—2 patients with CR, 1 PR; adenovirus—1CR; BKV—5 patients with CR, 1 PR, 1 NR; HHV6—2 patients with CR | (75)/BCM |
| **Third party** | | | | | | |
| EBV | 8 | *Ex vivo* expansion | PBMCs with LCLs | No GVHD | 3 patients with CR; 1 patient with PR, subsequently refused treatment; 2 patients with no response; 2 patients passed away before evaluation (unrelated to VSTs) | (85)/U of Edinburgh[p] |
| EBV | 33 | *Ex vivo* expansion | PBMCs with LCLs | No GVHD | 21 patients with CR or PR; 6 month OS 79% | (86)/U of Edinburgh |
| EBV-CMV-Adeno | 50 | *Ex vivo* expansion | PBMCs with LCLs transformed with Ad5f35-CMVpp65 vector | 6 with grade I GVHD; 1 with grade II GVHD, 1 with grade III GVHD | 17 of 23 with PR/CR for CMV; 14 of 18 PR/CR for adenovirus; 6 of 9 PR/CR for EBV | (22)/BCM |
| EBV | 2 | *Ex vivo* expansion | PBMCs with LCLs | No GVHD | Both with CR | (87)/MSKCC |

*N = number of patients in study.*
[a]*University College London, London, England.*
[b] *Medizinische Klinik und Poliklinik, Tübingen, Germany.*
[c]*Fred Hutchinson Cancer Research Center, Seattle, WA, USA.*
[d]*University of Perugia, Perugia, Italy.*
[e]*University Children's Hospital, Tübingen, Germany.*
[f]*Penn State Hershey Children's Hospital, Hershey, PA, USA.*
[g]*University of Sydney, Sydney, NSW, Australia.*
[h]*Memorial Sloan-Kettering Cancer Center, New York, NY, USA.*
[i]*University of Ulm, Ulm, Germany.*
[j]*St. Jude Children's Research Hospital, Memphis, TN, USA.*
[k]*Helmholtz Zentrum München and Ludwig-Maximilians-Universität, Munich, Germany.*
[l]*Baylor College of Medicine, Houston, TX, USA.*
[m]*University of Pavia, Pavia, Italy.*
[n]*Bambino Gesù Children's Hospital, Rome, Italy.*
[o]*Hammersmith Hospital, London, UK.*
[p]*University of Edinburgh, Edinburgh, UK.*

the recipient (particularly HLA class I) correlate with better *in vivo* proliferation and superior efficacy (86). However, even less closely matched products can be effective despite limited persistence of these mismatched T cell products. In one study, third-party cells were identified up to 12 weeks after infusion and approximately 70% of VST recipients benefited (22). Reassuringly there is no indication of enhanced alloreactivity from the VST as measured by GVHD or graft rejection. Third-party banks are thus emerging as a promising option for treating refractory post-transplant viral infections.

## DISCUSSION AND FUTURE DIRECTIONS

Over the past two decades, VST treatment has evolved from first proof of principle to a broadening acceptance that these cell products are a valuable, low-risk and effective tool to treat viral infections in immunocompromised individuals. Response rates reach approximately 90% for patients post-HSCT receiving VST from the matched transplant donor, and are around 70% for patients receiving third-party VST products (**Table 2**). VSTs appear promising as prophylaxis for high risk patients, conferring a high probability of protection against reactivation. A recent review of VSTs given to 36 patients with primary immunodeficiency syndromes over the past 10 years reported excellent responses to VSTs both for treatment (response rates 76–100% depending on the virus) and prophylaxis (81% of patients protected from viral reactivation) (24). Improved technology, including standardized pepmixes and alternative APCs, has improved the speed and efficiency of the manufacturing process. In parallel, the successes with VST from naive donor sources, multivirus-specific products, and generation of third-party banks have widened the scope of VST applicability. Ongoing studies are evaluating the safety and feasibility of increasing the number of viruses in a single product and extending the size of third-party banks for rapid use. Given the success seen in viral infections, the expansion of antigen-specific adoptive cell therapy to other complicated diseases, including HIV, fungal disease, and malignancies is increasingly within reach.

The studies included in **Table 2** demonstrate the safety of VSTs in various settings. The risk of GVHD, a primary concern in initial trials using unmanipulated donor products, has been decreased by improved methods of selecting and expanding VSTs. Current GVHD rates after VST do not exceed those expected for patients post-HSCT. Of studies with particularly high rates of GVHD, nearly all of the patients who developed GVHD (both acute and chronic) had prior risk factors that would explain these outcomes, including history of or active GVHD, subtherapeutic immunosuppression, or recipients of T cell-replete grafts (46, 59). Critical, taking into consideration the patient-specific risk factors, no correlation has been identified between GVHD development and the method of VST generation, product phenotype, or duration of *in vivo* activity of infused VSTs.

Through the multitude of clinical trials utilizing VSTs, we have gained some important information regarding predictors of response. Most methods of generating antigen-specific T cells yield a very heterogenous population of $CD4^+$ (typically about 30%) and $CD8^+$ T cells, unless they are generated against a single $CD8^+$-restricted epitope. While we know this polyclonal phenotype supports persistence of VSTs in vivo (43), it is not clear whether differing proportions of $CD4^+/CD8^+$ T cells are associated with increased or decreased clinical efficacy. One special situation is the use of third-party products, where it appears critical to ensure that there is shared antiviral activity through a shared HLA allele when selecting the "right" product. This can be either class I or class II for most cases, although the endogenous immune response is HLA-specific for certain viruses and must be matched accordingly. For example, the response to adenovirus is mediated through HLA class II, thus products for patients with adenoviral disease should be matched through HLA class II, whereas for CMV class I matching is typically preferred.

Immune reconstitution studies in patients following infusion of VSTs have also lead to increased knowledge about the *in vivo* activity of different VST products. For latent viral infections (e.g., CMV and EBV), enhanced detection of circulating antiviral T cells has been correlated with a better response (10); however, this is not always the case for viruses that are not latent (e.g., adenovirus) (80). While the gene marking studies by Heslop and Rooney suggest that adoptively transferred EBV-specific T cells can persist for a decade, there is also the suggestion that adoptive transfer of VSTs can stimulate endogenous anti-viral immunity. Additionally, epitope spreading is another marker of improved immune response as illustrated in the EBV-associated lymphoma setting (88).

For all the successes observed with VST products over the years, some patients still fail to respond to therapy with no identifiable mechanism. Hence, an important area of ongoing research is evaluating the mechanisms underlying VST resistance. For example, tumor (or virus)-secreted TGFβ inactivates antigen-specific T cells. To overcome this obstacle, gene manipulation of the TGFβ receptor on the antigen-specific T cells to render them resistant to the effects of TGFβ is being explored (89, 90). Other groups have found success infusing galunisertib, a small molecule inhibitor of TGFβ, abrogating the anti-inflammatory effect (91). Targets may also evade the immune system by upregulating expression of immunomodulators such as programmed death-1 ligand, which binds PD-1 on T cells in response to IFNγ. Checkpoint inhibitors, such as pembrolizumab targeted to PD-1, have increasing applications to many malignancies or in combination therapies and may also enhance VSTs especially in the HIV setting (92). Such modifications may therefore increase the *in vivo* efficacy of adoptively transferred VSTs in patients post-HSCT or for virus-associated diseases.

Other challenges of treatment are related to the severe immune dysregulation in the majority of patients at risk of viral disease. Steroid treatment, often used in high doses to treat GVHD, can reactivate dormant viruses and also render VST infusion futile through inactivation of the product. To this end, VST have been gene manipulated to inactivate the glucocorticoid receptor, allowing them to maintain cytotoxicity in the presence of steroids (93). T cells can also be genetically manipulated to be resistant to calcineurin inhibitors (cyclosporine A and tacrolimus, commonly used in post-HSCT setting), which inhibit T cell activation *in vivo* (94, 95). Such transduced VSTs proliferate, lack alloreactivity, and maintain cytotoxicity.

Planned advanced phase clinical trials will focus on many of these crucial points, including delineating the importance of VST product phenotype for efficacy *in vivo*, overcoming challenges such as unfavorable microenvironment, and studying immune reconstitution markers following infusion. The application of adoptive cell therapy has become increasingly broad, now extending to other infections as well as hematologic malignancies and solid tumors. As we gain more information about the interplay between the host immune system and the disease, modifications to T cells or combination therapy approach may be increasingly used to maximize efficacy while maintaining safety. Current results and these future developments ensure that VSTs will have an increasingly successful and everwidening role in the management of the immunocompromised patient.

## AUTHOR CONTRIBUTIONS

All authors have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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# The More, The Better: "Do the Right Thing" For Natural Killer Immunotherapy in Acute Myeloid Leukemia

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Natural killer (NK) cells are circulating $CD3^-$ lymphocytes, which express CD56 or CD16 and an array of inhibitory receptors, called killer-immunoglobulin-like receptors (KIRs). Alloreactive KIR-ligand mismatched NK cells crucially mediate the innate immune response and have a well-recognized antitumor activity. Adoptive immunotherapy with alloreactive NK cells determined promising clinical results in terms of response in acute myeloid leukemia (AML) patients and several data demonstrated that response can be influenced by the composition of NK graft. Several data show that there is a correlation between NK alloreactivity and clinical outcome: in a cohort of AML patients who received NK infusion with active disease, more alloreactive NK cell clones were found in the donor repertoire of responders than in non-responders. These findings demonstrate that the frequency of alloreactive NK cell clones influence clinical response in AML patients undergoing NK cell immunotherapy. In this work, we will review the most recent preclinical and clinical data about the impact of alloreactive NK cells features other than frequency of alloreactive clones and cytokine network status on their anti-leukemic activity. A better knowledge of these aspects is critical to maximize the effects of this therapy in AML patients.

**Keywords: natural killer cells, acute myeloid leukemia, immunotherapy, biomarkers, cell dose**

## INTRODUCTION

In the last years, major advances have been achieved in the understanding of acute myeloid leukemia (AML) biology (1). However, these preclinical results have weakly impacted on the clinical outcome of AML patients, whose prognosis is overall largely unsatisfactory (1–4). A significant improvement in AML outcome is provided by allogeneic stem cell transplantation (SCT), where alloreactive T cells and natural killer (NK) cells mediate the graft-versus-leukemia (GvL) effect, contributing to leukemia eradication and relapse prevention. However, for many patients, the cellular immunotherapy within SCT is hampered by the high risk of treatment-related morbidity and mortality. To expand the option of cellular immunotherapy besides the SCT setting, adoptive transfer of effector immune cells is a promising approach (5, 6).

Natural killer cells are circulating $CD3^-$ lymphocytes, which express CD56 or CD16. They account for 5–15% of circulating lymphocytes in humans and can be categorized into subpopulations with different maturation status and functional specificities. $CD56^{lo}CD16^+$ NK cells with high cytotoxic potential are predominant in human blood, while the immunomodulatory $CD56^{hi}CD16^-$ subset is more predominant in lymph nodes (7). NK cells mediate innate immune response and cancer immunological control independently from major histocompatibility complex (MHC)-based antigen recognition (8). A net effect between inhibitory receptors, such as killer-Immunoglobulin-like receptors (KIRs) and C-type lectin receptor (NKG2A), and activating receptors, including natural cytotoxic receptors (NCR), NKp46, NKp30, NKp44, and NKG2D, drives NK cell cytotoxic activity (9–11). Inhibitory KIRs are the most clinically relevant and interact with self and not self-HLA class I ligands (HLA-Bw4, HLA-C1, and HLA-C2) (12). During their maturation process, NK cells acquire cytolytic activity by upregulating cytotoxic receptors and expressing perforin under the stimulation of the T-cell derived cytokine interleukin (IL)-2. The subsequent "licensing" or "education" process is crucial for acquiring self-tolerance, avoiding NK-mediated self-damage (13). Several cytokines, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines, such as CCL3, CCL4, and CCL5, are produced by stimulated NK cells, which, then, acquire the capacity of killing target cells, including tumor cells, through the perforin/granzyme pathway (14). As for AML, the first evidence of NK-mediated anti-leukemia effect was reported in the haploidentical T-cell depleted SCT setting, where it was demonstrated that a KIR-ligand mismatch between donor and recipient protects patients from leukemia relapse (15, 16).

## NK IMMUNOTHERAPY FOR AML: THE STATE-OF-ART OF CLINICAL TRIALS

Acute myeloid leukemia cells are more sensitive to NK-mediated cytotoxicity than solid tumors (7). For this reason, several groups have been prompted to exploit in AML the potential of NK cell adoptive immunotherapy (17). High risk patients with myeloid malignancies were safely infused with highly purified NK cells to consolidate engraftment after haploidentical SCT (18), thus providing the rationale for exploring this strategy as a means of increasing GvL without concurrent GVHD effects (19). Besides the transplantation setting, adoptive immunotherapy with haploidentical NK cells in AML was exploited in adults and in childhood patients (20–23). Altogether, these studies demonstrated that NK cell-based adoptive immunotherapy is safe and feasible in AML patients. Of note, donor NK cells expanded after infusion and were *in vivo* alloreactive against recipient's leukemic cells with some promising clinical responses. To increase the number of NK cells to be infused, *ex vivo* NK cell expansion is under preclinical and early clinical investigation. Co-culture of NK cells with feeder cells plus cytokines is effective in generating large number of NK cells with high antitumor effect and long survival (24). Clinically, expanded NK cells have proven to be safe and feasible (25). Very recently, a first-in-man study investigated the role of memory-like NK cells, obtained after *in vitro* differentiation from human NK cells with IL-12, IL-15, and IL-18 (26).

## IN SEARCH FOR BIOMARKERS PREDICTIVE OF RESPONSE TO NK IMMUNOTHERAPY FOR AML

Within clinical trials, "biology-driven approaches" have the potential to identify an array of biomarkers, which may be used to predict clinical response to NK immunotherapy and/or to guide clinical decision process. Some of these biomarkers may derive from the donor repertoire, whereas some others are related to host modifications after NK cell infusion (**Figure 1**).

### KIR–KIR-L Mismatch

The NK cells cytolytic activity is regulated by inhibitory KIRs, which mediate self-tolerance by engaging HLA class I antigens. Four different inhibitory KIRs seem to be primarily involved in inducing NK alloreactivity because of a stronger recognition of HLA class I ligands: KIR2DL2/2DL3 recognize HLA-C1 ligands (HLA-Cw), KIR3DL1 recognizes Bw4 haplotypes, and KIR3DL2 targets HLA-A3 and HLA-A11 ligands. NK alloreactivity can be predicted according to four models. The "missing-self" or "KIR-ligand mismatch" or "ligand incompability model" is based on the absence in the recipient of inhibitory HLA-I molecules that are present in the donor. According to the "receptor–ligand" model, donor NK cells are activated through inhibitory KIRs, which do not bind their HLA ligands in the recipient. The "missing-ligand" model predicts alloreactivity when at least one HLA-ligand is missing. The last of the models mentioned above states that activating KIRs on donor cells are needed in order to achieve NK cell alloreactivity (27). In haploidentical SCT, KIR-L mismatch dramatically impacts on the efficacy of NK cells in enhancing anti-leukemia effect (15, 16, 23). Whether these findings may be directly translated into the immunotherapy field has not been fully elucidated. In their pioneering study of adoptive NK immunotherapy, Miller et al. showed that KIR-L mismatch between donor and recipient had a predictive value in terms of clinical response (20). These data have not been confirmed in the subsequent study on a wider number of patients (28). Moreover, KIR-L mismatch between recipient and donor was not enough to translate into a significant clinical benefit for previously selected KIR-L-mismatched donor–recipient pairs (20). These results may indicate that a deeper characterization of KIR–KIR-L interaction is probably needed in the setting of NK immunotherapy. Donors with group B KIR haplotypes have been shown to improve survival of AML patients undergoing unrelated SCT (29). Very recently, based on specific HLA/KIR subtype combinations, a predictive algorithm for donor selection has been developed in a cohort of 1,328 patients with AML who received HLA-matched SCT transplant (30). Then, the selection of donors with favorable KIR genes of the B haplotype and/or with specific HLA/KIR subtype combinations may be used to increase the potential benefit of KIR–KIR-L mismatch between donor and recipient.

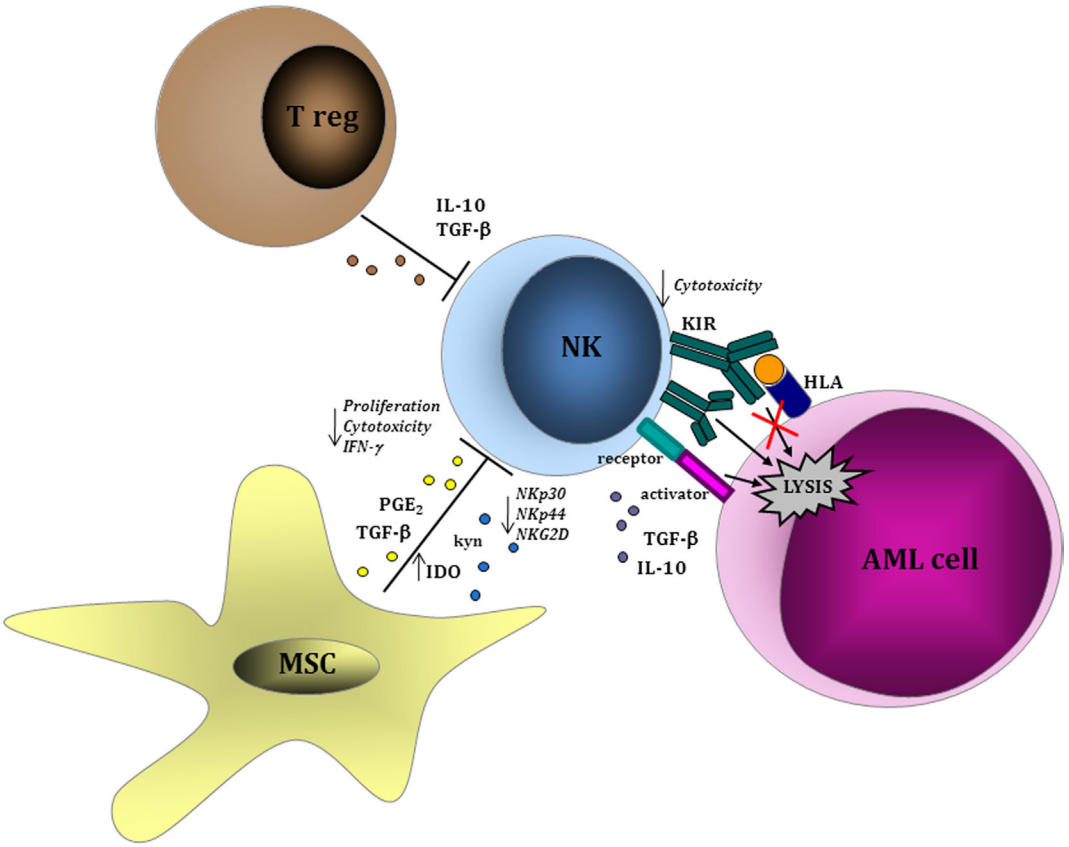


**FIGURE 1** | Leukemia-cell intrinsic and extrinsic factors influencing natural killer (NK) cell function. NK cells are able to recognize acute myeloid leukemia (AML) blasts in a major histocompatibility complex-unrestricted manner inducing cell lysis through perforin/granzyme pathway. Within tumor microenvironment, regulatory T lymphocytes and MSCs play a role in inhibiting NK cell-mediated proliferation and cytotoxic function *via* the production of soluble factors, such as interleukin (IL)-10, transforming growth factor (TGF)-β, and indoleamine 2,3-dioxygenase (IDO), thus resulting in the downregulation of activating receptors (NKp30, NK944, and NKG2D) and in the upregulation of inhibitory receptors. Similar evasion mechanisms have been described as a consequence of a direct effect from AML cells on NK cells.

## Host Regulatory T Cells

Regulatory T lymphocytes (Tregs) are distinct CD4, CD25, and Foxp3 positive immunosuppressive lymphocytes, which induce tolerance by inhibiting immune responses, including NK cell-mediated cytotoxicity. During the development of malignancies, Tregs proliferate under the stimulation of transforming growth factor (TGF)-β secreted by myeloid dendritic cells (DCs) and accumulate in blood, draining lymph nodes, and tumor microenvironment (14). Many studies demonstrated that Tregs are able to influence NK cell activity (14, 28, 31). In particular, NK cell inhibition induced by Tregs is mediated by soluble and Tregs-bound TGF-β, both resulting in downregulating of NKG2D on NK cells (14). Accordingly, in mice Treg depletion with anti-CD25 monoclonal antibody abrogated tumor growth and allowed the proliferation of NK cells (32). The influence of Tregs in NK adoptive immunotherapy has been recently addressed by Miller et al. in a large group of patients infused with NK cells. In this study, 57 patients with relapsed or refractory AML who received lymphodepleting chemotherapy with fludarabine and cyclophosphamide followed by NK cell infusion were analyzed. Patients were divided into three subgroups on the basis of the methods of NK cell product preparation: cohort 1 received NK cells after CD3 depletion alone, cohort 2 received NK cells after CD3 depletion followed by CD56 positive selection, and cohort 3 received NK cells after a single-step CD3/CD19 depletion. Patients included in Cohort 3 received the highest NK cell dose and after NK infusion were treated with IL-2 diphteria toxin (IL-2DT), a cytotoxic fusion protein able to selectively deplete high affinity IL-2 receptors (CD25)-expressing cells, including Tregs. A clear association between NK expansion and clinical responses was documented and such correlation was partly dependent on host Tregs. These data suggest that Tregs frequency in patients infused with NK cells may be a biomarker predictive of NK cell expansion and activity and may have clinical implications for the choice and schedule of cytokine-based therapy after NK cell infusion. In particular, the potent effect of IL-2, commonly used in NK-based immunotherapy protocols, on NK cell expansion and activity may be counterbalanced by its detrimental proliferative activity on Tregs. Clinical trials, including post-infusion administration of cytokines, such as IL-15, with no proliferative effect on Tregs are highly warranted.

## Leukemia-Cell Intrinsic and Extrinsic Factors

A large body of evidence support the interplay between AML cells and the different cell components of the immune system (33). Some reports indicate that NK cells may be defective in AML patients (34, 35). In particular, a deficient expression of NCRs in NK cells from AML patients and during leukemia evolution has been observed (36). Conversely, NK cells with an activated phenotype from AML patients are cytotoxic against primary AML cells (37). Leukemia-induced phenotypic and functional NK cell abnormalities have been described in AML patients and are predictive of remission-induction after chemotherapy (38). Soluble factors, including IL-10, TGF-β, or indoleamine 2,3-dioxygenase (IDO), have been correlated with AML-mediated impairment of NK function (39). Sera collected from human AML patients contain microvescicles enriched in TGF-β, which mediates the inhibition of NK cell activity (40). In support of a pathogenetic role of these mechanisms during AML development, the defects of NK cells were restored in patients achieving complete remission after chemotherapy (38, 40). Moreover, the expression by AML cells of the immunosuppressive surface proteins CD200 (41) and CD137 (42) has a direct suppressive effect on NK cell activity, thus contributing to leukemia escape from NK-mediated control. The role of leukemic bone marrow (BM) microenvironment is critical in mediating immunotolerance (43–45). An increasing body of evidence has shown that immune effector cells are dysfunctional as a consequence of inhibitory signals deriving from a wide variety of immune cell components of tumor microenvironment, such as tolerogenic DCs, macrophages, and myeloid-derived suppressor cells (MDSCs). In solid tumors, these immunosuppressive cell subsets are expanded in patients and their presence in the tumor microenvironment correlates with worse prognosis and a dysfunctional activity of NK cells (46–48). In AML, the role of these immunosuppressive cells is not well-understood yet, whereas the activity of mesenchymal stromal cells (MSCs) on NK cells in BM leukemic microenvironment has been widely investigated. In particular, MSCs can negatively interfere with the function of NK cells by reducing their cytotoxic activity and cytokine production (49). Similar to the effects derived from AML cells, MSC-driven impairment of NK cell function was related to a downregulation of the activating NK receptors NKp30, NKp44, and NKG2D and mediated by IDO and prostaglandin $E_2$. Whether these evasion mechanisms by AML cells and by other cell components of leukemic BM niche may be active during adoptive NK cell transfer has not been specifically addressed within clinical trials. Future immunotherapy trials are expected to take into accounts some of these parameters, which may be correlated with response to NK immunotherapy.

# NK IMMUNOTHERAPY IN AML: THE QUESTION OF DOSE

In SCT and cell therapy, how many cells should be infused to achieve a therapeutic effect represents a common and crucial question. Many efforts have been made, for example, to identify the minimum and optimal number of hematopoietic $CD34^+$ stem/progenitor cells capable of a durable engraftment in autologous SCT (50) or, in the setting of allogeneic SCT, the dose of $CD3^+$ T cells to be used as donor lymphocytes infusions (DLI) for relapsing leukemia (51, 52). For NK immunotherapy, whether the dose of infused NK cells really impacts on the clinical response is still a matter of debate. Although infused NK cells may eradicate AML cells in virtue of several factors other than their dose, preclinical studies have demonstrated that NK cell cytotoxicity is directly correlated to the ratio between NK and target cells (53–55). Then, to infuse an adequate number of NK cells is likely to be clinically relevant. Since NK cells account for only a small portion of circulating lymphocytes, several groups are exploring the option of increasing the number of infused NK cells by *ex vivo* expanding cytotoxic NK cells and different NK cell production protocols are under investigation (56). An overview of these strategies is out of the focus of the current review. Indeed, we consider a preliminary and fundamental step to address the question of the minimum number of effective NK cells to be infused as a means of adoptive immunotherapy. Under this viewpoint, there is no consensus on the parameters to be used for enumerating functionally active NK cells among infused cells, which increases variability among clinical studies and makes the comparison of clinical results extremely difficult, if not impossible (57). In a pediatric cohort of AML patients undergoing NK cell infusion as part of their post-remission strategy, mainly because of body weight discrepancy between donor and recipient, high numbers of total $CD56^+CD3^-$ NK cells/kg were infused (21). Of note, $CD56^+CD3^-$ NK cells were highly purified and the amount of contaminating cells, other than NK cells, was minimal. Interestingly, the clinical results were remarkable with 100% of patients achieving durable response. In our recent report on a group of elderly AML patients, who were infused with the same highly purified NK cell population as the pediatric study, a trend for better outcome in those patients who received higher numbers of NK cells was shown (23). In the largest study by Miller et al. (28), comparing the clinical results of 57 patients undergoing infusion of NK cells, total NK cell dose resulted as being not correlated with clinical response. Notably, in this study (28), three different methods for NK cell manufacturing were used and only in a minority of patients NK cells were highly purified as the studies above. Moreover, cell dose was not correlated with *in vivo* expansion and clinical response when purified NK cells were *in vitro* primed with IL-12, IL-15, and IL-18 (26). Although not conclusive, the results from these studies may suggest that the optimal NK cell dose may differ depending on the cell manufacturing system and that, in case $CD56^+CD3^-$ NK cells are highly purified and not manipulated, the simple count of total infused NK cells may be used as a useful parameter to predict clinical response.

The possibility of infusing a number of functionally active NK cells is likely to have greater impact on the efficacy of NK infusion than simply enumerating the number of total NK cells. Indeed, in our cohort of elderly AML patients, a better response was observed when at least $2 \times 10^5$ functionally alloreactive donor NK cells/kg were infused (**Figure 2**). Our results indicate a highly predictive threshold of NK cells to be defined at the functional level ("functional dose"). Although such dose may be used to

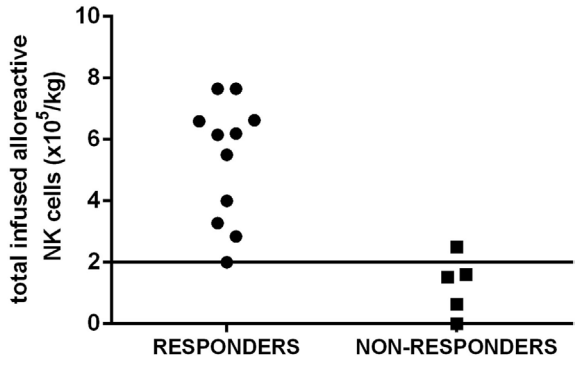


**FIGURE 2** | The "functional dose" of infused alloreactive natural killer (NK) cells is highly predictive of response in acute myeloid leukemia (AML) patients. A cohort of elderly AML patients in first complete remission received highly purified NK cells after lymphodepleting fludarabine and cyclophosphamide to prevent disease relapse. A better response in leukemia control was observed when at least $2 \times 10^5$ functionally alloreactive donor NK cells/kg were infused. Such threshold has been chosen as a "functional cell dose" to be used to select donor(s) and guide NK cell processing.

guide NK cell processing, the feasibility of such approach, which is based on a laborious and time-consuming alloreactive NK cell cloning, represents a major concern. Then, a major achievement will be to identify a functional dose of NK cells, predictive of response to NK cell infusion, by using alternative methods and tools. By moving from the results in the transplant setting (17), a new and predictive-of-response expression platform to be used in donor selection is likely to be a concrete possibility. The development of novel anti-KIR monoclonal antibodies that can distinguish inhibitory versus activating KIRs allows to identify alloreactive NK subpopulations in the majority of individuals at the phenotypical level. Although these new methods and tools require formal clinical testing and validation in large cohorts of patients undergoing NK immunotherapy, we may expect that the "functional dose" of NK cells to be infused will be identified without standard and cumbersome functional studies of NK cell cloning (58–60). Such approach will, then, allow to dissect donor NK cell repertoire at baseline, thus leading to definitively address the question of the correlation between NK cell dose and clinical response.

## CONCLUSION

Although several clinical reports have clearly paved the way for exploiting NK cell-based adoptive immunotherapy for AML patients, several crucial issues remain to be established and settled. In particular, the founding question of the therapeutic dose of infused NK cells potentially exerting *in vivo* anti-leukemia effect is still unanswered. Preliminary data from recent studies have shed light on this point, although the great heterogeneity of clinical strategies, which include freshly isolated *versus* expanded, non-manipulated *versus* cytokine-primed NK cells, makes the comparison among trials extremely difficult. Advancements of diagnostic tools, coupled with a "biology-driven" approach, are expected to provide an array of biomarkers, which may help in selecting the best donor for the best patient, thus making NK immunotherapy really beneficial for a specific subset of AML patients.

## AUTHOR CONTRIBUTIONS

SP wrote and revised the final manuscript; ML and DO provided figures and revised the final manuscript; VS, MC, DF, and GC contributed to manuscript preparation and editing; AC contributed to manuscript writing and final revision.

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REVIEW
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# Current Advances in γδ T Cell-Based Tumor Immunotherapy

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γδ T cells are a minor population (~5%) of CD3 T cells in the peripheral blood, but abound in other anatomic sites such as the intestine or the skin. There are two major subsets of γδ T cells: those that express Vδ1 gene, paired with different Vγ elements, abound in the intestine and the skin, and recognize the major histocompatibility complex (MHC) class I-related molecules such as MHC class I-related molecule A, MHC class I-related molecule B, and UL16-binding protein expressed on many stressed and tumor cells. Conversely, γδ T cells expressing the Vδ2 gene paired with the Vγ9 chain are the predominant (50–90%) γδ T cell population in the peripheral blood and recognize phosphoantigens (PAgs) derived from the mevalonate pathway of mammalian cells, which is highly active upon infection or tumor transformation. Aminobisphosphonates (n-BPs), which inhibit farnesyl pyrophosphate synthase, a downstream enzyme of the mevalonate pathway, cause accumulation of upstream PAgs and therefore promote γδ T cell activation. γδ T cells have distinctive features that justify their utilization in antitumor immunotherapy: they do not require MHC restriction and are less dependent that αβ T cells on co-stimulatory signals, produce cytokines with known antitumor effects as interferon-γ and tumor necrosis factor-α and display cytotoxic and antitumor activities *in vitro* and in mouse models *in vivo*. Thus, there is interest in the potential application of γδ T cells in tumor immunotherapy, and several small-sized clinical trials have been conducted of γδ T cell-based immunotherapy in different types of cancer after the application of PAgs or n-BPs plus interleukin-2 *in vivo* or after adoptive transfer of *ex vivo*-expanded γδ T cells, particularly the Vγ9Vδ2 subset. Results from clinical trials testing the efficacy of any of these two strategies have shown that γδ T cell-based therapy is safe, but long-term clinical results to date are inconsistent. In this review, we will discuss the major achievements and pitfalls of the γδ T cell-based immunotherapy of cancer.

**Keywords: γδ T cells, immunotherapy, adoptive transfer, Zoledronate, immunoevasion**

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**Abbreviations:** ADCC, antibody-dependent cell-mediated cytotoxicity; BTN, butyrophilin; BrHPP, bromohydrin pyrophosphate; CAR, chimeric antigen receptor; CSC, cancer stem cell; DR5, death receptor 5; Fc, fragment crystallizable; FcR, fragment crystallizable receptor; Fv, variable fragment; GMP, good manufacturing practice; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; IFN, interferon; IL, interleukin-; iPSC, inducible pluripotent stem cells; mAbs, monoclonal antibodies; MHC, major histocompatibility complex; MICA, MHC class I-related molecule A; MICB, MHC class I-related molecule B; MM, multiple myeloma; n-BP, aminobisphosphonate; NCR, natural cytotoxicity receptors; NHL, non-Hodgkin lymphoma; NKG2D, natural-killer group 2, member D; NSCLC, non-small cell lung cancer; PAg, phosphoantigen; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TCR, T cell receptor; TFH, follicular T helper; TGF, transforming growth factor; Th, T helper; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; Treg, T regulatory; ULBP, UL16-binding protein; VEGF, vascular endothelial growth factor.

## INTRODUCTION

T cells carrying the γδ T cell receptor (TCR) are a minor lymphocyte population that accounts for 2–5% of CD3 T cells in the peripheral blood, but predominate in several anatomic sites such as the intestine and the skin. There are two major γδ T cell subsets in humans which are distinguished based on the δ chain they use to make their TCR: T cells expressing the Vδ2 gene paired with the Vγ chain (Vγ9) are the great majority of the γδ T cell population in the peripheral blood and secondary lymphoid organs of healthy individuals. In contrast, γδ T cells expressing the Vδ1 gene, paired off with different Vγ elements, are the predominant γδ T cell subset in epithelia (skin and mucosa). Finally, a third subset of γδ T cells expressing the Vδ3 chain abound in the liver (1).

Vδ1 T cells have a largely private TCR repertoire with different clonotypes present in each individual, while the Vγ9Vδ2 repertoire has limited complexity with invariant Vγ9-JP sequences common to multiple individuals, and many CDR3δ2 sequences although are relatively private compared with TCRγ9 lengths, are shared between individuals (2, 3). Therefore, the Vγ9Vδ2 T cell population expresses a TCR with very limited variability, suggesting recognition of a limited set of antigens.

Antigen recognition by γδ T cells is a field of intense research. Vγ9Vδ2 T cells recognize non-peptidic phosphorylated intermediates of the non mevalonate pathway of isoprenoid biosynthesis called phosphoantigens (PAgs), in the absence of processing, presentation, and major histocompatibility complex (MHC) restriction (4). PAgs are synthesized in mammalian cells through the mevalonate pathway (5), but PAg concentrations required for Vγ9Vδ2 T cell activation are not achieved in physiological conditions, but only after infections or tumor transformation (6). Therefore, from this point of view, the Vγ9Vδ2 TCR works in a similar way to a pattern-recognition receptor, which senses metabolic changes found in transformed or infected cells.

Intracellular PAg levels can be modulated by drugs. Thus, aminobisphosphonates (n-BPs) such as Zoledronate, widely used in the clinic for the treatment of osteoporosis and bone metastasis, inhibit farnesyl pyrophosphate synthase (FPPS), a downstream enzyme of the mevalonate pathway, thereby causing accumulation of upstream PAgs and thus favoring Vγ9Vδ2 T cell activation (7, 8). Conversely, statins inhibit hydroxy-methylglutaryl-CoA reductase (HMGCR), the upstream enzyme of the mevalonate pathway, and significantly reduce PAgs production and Vγ9Vδ2 T cell activation (9).

Vδ1 T cells recognize MHC class I-related molecule A (MICA), MHC class I-related molecule B (MICB), and UL16-binding proteins (ULBPs) molecules, a group of proteins expressed on stressed and tumor cells (10, 11), and the MHC-related class Ib molecules CD1c and CD1d, which are typically involved in glycolipid presentation (12, 13). However, as Vδ1 T cells constitutively express natural-killer group 2, member D (NKG2D), the "true" receptor of MICA and MICB, it is still to be determined if Vδ1 T cell recognition of MICA and MICB is mediated by the TCR or by NKG2D. Moreover, Vδ1 T cells can also be activated by engagement of natural cytotoxicity receptors (NCRs, such as NKp30 and NKp44) by yet unidentified ligands (14). Similar to Vδ1 T cells, Vδ3 T cell ligands are poorly unknown and there is only one study showing that these cells are activated by CD1d possibly bound to a yet unidentified glycolipid (15).

Phosphoantigens recognition by Vγ9Vδ2 T cells requires butyrophilin (BTN) 3A1 (also called CD277) (16), but how PAgs interact with BTN3A1 and how the PAg/BTN3A1 complex in turn interacts with the Vγ9Vδ2 TCR is a matter of debate. Initial studies by Vavassori et al. (17) found a PAg-binding site located in the extracellular domain of BTN3A1, but a subsequent study by Adams and coworkers (18) found that PAgs bind to the intracellular domain of BTN3A1, leading to the possibility that intracellular PAgs provoke a conformational change of BTN3A1, which allows its extracellular domains to interact with the reactive Vγ9Vδ2 TCR.

Vγ9Vδ2 T cells express several cell surface molecules correlated with distinct functional differentiation phenotypes. The combined use of CD27 and CD45RA permits identification of "naive" and "central memory" subsets of Vγ9Vδ2 T cells ($T_{Naive}$, $CD45RA^+CD27^+$; $T_{CM}$, $CD45RA^-CD27^+$) that circulate between the blood and secondary lymphoid organs, but are excluded from peripheral tissues and lack effector function; and "effector memory" ($T_{EM}$, $CD45RA^-CD27^-$) and "terminally differentiated" ($T_{EMRA}$, $CD45RA^+CD27^-$) subsets that circulate between the blood and peripheral tissues, are recruited to sites of inflammation and immediately perform effector function (19).

While $T_{Naive}$ and $T_{CM}$ cells readily respond to PAg stimulation, $T_{EM}$ and $T_{EMRA}$ respond to homeostatic cytokines as interleukin (IL)-15 (20) and may acquire highly diverse effector functions in the presence of polarizing cytokines (21). In general, circulating Vγ9Vδ2 T cells have a Th1 pattern of cytokine production (21), but under certain conditions they polarize to Th2 (22, 23), Th17 (24–26), follicular T helper (27, 28), Th9 (29), and T regulatory (Treg) cells (30). Such a flexibility emphasizes the capacity of Vγ9Vδ2 T cells to efficiently participate to immune responses to different antigen challenges.

## RATIONALE FOR HARNESSING γδT CELLS IN CANCER IMMUNOTHERAPY

In the following section, we will briefly summarize the rationale for harnessing γδ T cells in cancer immunotherapies.

(1) The major objective of immunotherapy is the generation of a long-lasting efficient antitumor response, particularly mediated CD8 cytotoxic T cells, but also by CD4 T cells (31, 32). Nonetheless, despite efforts, durable responses are only rarely achieved and moreover tumors often develop strategies to escape immune responses (33). In contrast to CD4 or CD8 T cells, γδ T cells have unique features which make them good candidates for effective tumor immunotherapy: they do not require MHC restriction and co-stimulation and they recognize antigens shared by a variety of stressed and tumor cells, making it possible for a single γδ T cell to target a vast array of tumor cells. Hence, recognition of commonly shared tumor antigens in the absence of MHC restriction provides the rationale for application of γδ T cell-based

therapy to a wide range of tumors and in patients with different MHC molecules (34).

(2) A distinctive feature of T lymphocytes equipped with antitumor potential is their ability to secrete appropriate cytokines. Typically, activated γδ T cells secrete interferon (IFN)-γ and tumor necrosis factor (TNF)-α, two cytokines with cytotoxic and antitumor activities (35–37).

(3) A large body of studies have demonstrated that γδ T cells kill *in vitro* a broad array of tumor cells, while sparing normal cells (34), and display antitumor activity in mouse models *in vivo* (34). The cytotoxic activity of γδ T cells against tumor cells is strictly dependent on augmented production of PAgs (38), which partly relies on increased expression of HMGCR (38). Moreover, intracellular PAgs levels can be substantially increased by n-BPs (13–15, 38), thereby promoting activation of Vγ9Vδ2 T cells (38). Killing may also be reinforced by the tumor cell expression of NCRs (39) and/or NKG2D ligands (such as MICA, MICB, and ULBPs) (40–42) or by antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by CD16 interacting with antibody-coated tumor cells (43) (**Figure 1**).

Whatever the mechanism of γδ T cell recognition of tumor target cells, killing involves the perforin/granzyme (44) and TNF-related apoptosis-inducing ligand (TRAIL) (45) pathways, and Fas/FasL interaction (46). The choice of the mechanism is mostly dictated by the nature of the target cell itself (47). For instance, we previously found that colon cancer stem cells (CSCs), which are typically resistant to γδ T cell-mediated cytotoxicity, are efficiently killed upon sensitization with Zoledronate (48). Killing of

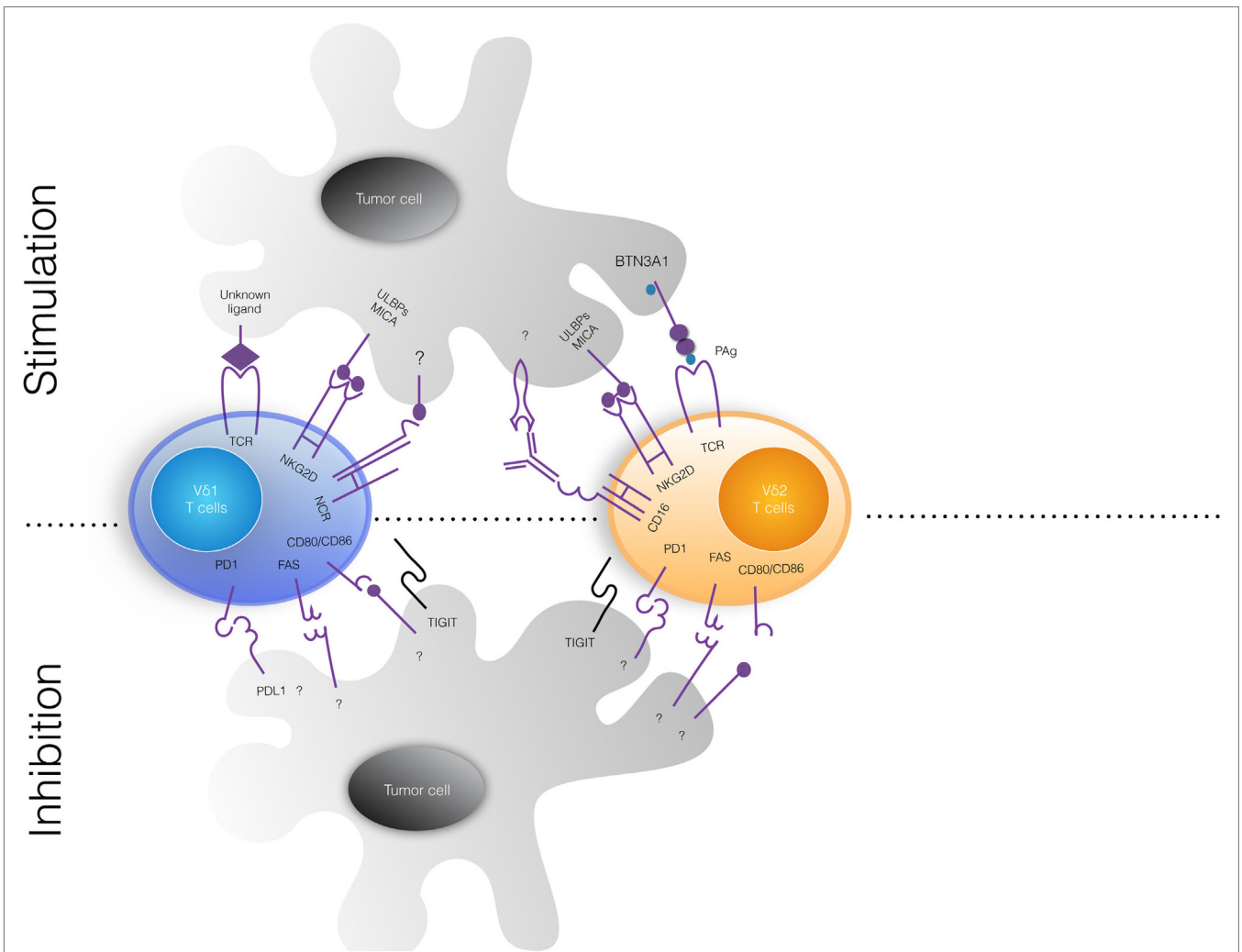


**FIGURE 1** | Tumor cell ligands recognized by human γδ T cells. The upper and lower panels show stimulatory and inhibitor signals delivered by tumor cells to Vδ1 (left) and Vδ2 (right) γδ T cell subsets. Vγ9Vδ2 T cells recognize *via* their TCR non-peptidic phosphoantigens (PAgs) and BTN3A1, while Vδ1 T cell receptor (TCR) ligands are not defined yet. Both γδ T cell subsets constitutively express surface natural cytotoxicity cell receptors (NCRs) that bind MICA/MICB and ULBPs, frequently expressed on tumor cells. Upon activation, Vγ9vδ2 T cells express fragment crystallizable receptor for IgG (FcγRIII; also known as CD16) that can bind therapeutic antibodies and mediate antibody-dependent cell-mediated cytotoxicity phenomena. Inhibitor signals delivered by tumor cells have not been well characterized. MICA/B, MHC class I-related chain A/B; ULBP, UL16-binding protein; BTN3A1, butyrophilin 3A1.

Zoledronate-treated colon CSCs was abrogated by anti-CD3 or anti-γδ TCR monoclonal antibodies (mAbs), or mevastatin, which inhibits HMGCR and prevents PAg accumulation, and by Concanamycin A that blocks degranulation, indicating that Vγ9Vδ2 T cells recognize Zoledronate-treated colon CSCs by the TCR interacting with PAgs and utilize the perforin pathway to kill them (48). The colon CSCs are usually resistant also to chemotherapy, but we unexpectedly found that pretreatment with 5-Fluorouracil and Doxorubicin sensitizes colon CSCs to killing by Vγ9Vδ2 T cells. However, killing of chemotherapy-sensitized colon CSCs by Vγ9Vδ2 T cells was inhibited by anti-NKG2D mAb and by blocking TRAIL interaction with its death receptor 5 (DR5), indicating that Vγ9Vδ2 T cells recognize chemotherapy-treated colon CSCs by NKG2D interaction with MICA/B or ULBPs and kill them through mechanisms involving TRAIL interaction with DR5 (49).

(4) In order for T lymphocytes to interact with tumor cells they should be capable to infiltrate tumors. Tumor-infiltrating leukocytes are found in a several different solid tumors (50) and include both myeloid (granulocytes, macrophages, and myeloid-derived suppressor cells) and lymphoid (T, B, and NK) cells, each of which impacts differently on tumor prognosis (51). Tumor-infiltrating Vγ9Vδ2 T lymphocytes have been detected in several types of cancer (52), but their clinical relevance has remained long obscure because of inconsistent results. However, analysis of expression signatures from ~18,000 human tumors with overall survival outcomes across 39 malignancies identified tumor-infiltrating γδ T cells as the most significant favorable cancer-wide prognostic signature (53). Similarly, our own results of data mining transcriptomes and clinical files from a large cohort of colorectal cancer samples ($n = 585$), revealed that the 5-year disease-free survival probability was significantly higher in patients with high number of tumor-infiltrating γδ T cells (54).

(5) Two synthetic drugs, the PAg bromohydrin pyrophosphate (BrHPP) and the n-BP Zoledronate, activate human Vγ9Vδ2 T lymphocytes *in vitro* and in clinical trials *in vivo*. BrHPP is produced as good manufacturing practice grade for use in humans under the name Phosphostim (55). Zoledronate, a third generation n-BP used to treat osteoporosis and bone metastasis, inhibits FPPS and causes accumulation of endogenous PAgs which thus reach the threshold required for Vγ9Vδ2 T cell activation (56). Second generation n-BPs, such as Pamidronate, Alendronate, and Risedronate, have similar activities of Zoledronate but at higher concentrations (55). Of note, *in vitro* and *in vivo* expansion of Vγ9Vδ2 T cells by either PAgs or n-BPs requires exogenous IL-2.

Overall, the above functional aspects of γδ T cell biology, have led to their utilization in cancer immunotherapy, and two strategies have been developed: (1) *in vivo* administration of PAgs or n-BPs that activate Vγ9Vδ2 T cells and (2) adoptive transfer of *ex vivo*-expanded Vγ9Vδ2 T cells. Several small-sized phase I clinical trials have assessed the safety and efficacy of these two strategies in patients with various tumor types, and available data suggest that Vγ9Vδ2 T cell-based immunotherapy is well tolerated and may give some clinical benefit to patients, thus providing a proof of principle for its utilization in addition to conventional therapies (57).

In the following sections, we will review the major achievements and pitfalls of the Vγ9Vδ2 T cell-based immunotherapy.

## RESULTS FROM CLINICAL TRIALS BASED ON *IN VIVO* ACTIVATION OF γδ T CELLS

A survey of clinical trials based on *in vivo* activation of γδ T cells in different types of cancer is shown in **Table 1**.

Since B-cell type non-Hodgkin lymphoma (NHL) and multiple myeloma (MM) are highly sensitive to lysis by Vγ9Vδ2 T cells *in vitro*, a pioneering study by Wilhelm and colleagues (58) analyzed *in vivo* the toxicity, Vγ9Vδ2 T cell activation and anti-lymphoma activity of Pamidronate and IL-2 in 19 patients with NHL or MM. Ten patients received Pamidronate followed by IL-2, but neither Vγ9Vδ2 T cell activation nor response to treatment were observed. Therefore, a second group of nine patients was selected for *in vitro* Vγ9Vδ2 T cell response to Pamidronate and IL-2 and was treated with Pamidronate followed by increasing doses of IL-2. Significant *in vivo* expansion of Vγ9Vδ2 T cells was detected in this group, and three patients achieved objective responses. This was the first study demonstrating activation of Vγ9Vδ2 T cells in patients with B-cell lymphomas by Pamidronate and low-dose IL-2 was well tolerated and induced a clinical response; moreover, the immunologic and clinical outcome could be nicely predicted by Vγ9Vδ2 T cell proliferation *in vitro*.

At the same time as the aforementioned study, we performed an observational study in nine cancer patients with bone metastases to determine if Zoledronate affected activation and maturation of circulating Vγ9Vδ2 T cells *in vivo* (8). The results of that study showed that Zoledronate-induced the *in vivo* differentiation of Vγ9Vδ2 T cells to the $T_{EM}$ subset producing IFN-γ. Therefore, and based on this, we then conducted a phase I clinical trial in 18 patients with metastatic hormone-refractory prostate cancer (59). Patients were randomized into two groups, one receiving Zoledronate alone and the other receiving Zoledronate together with low-dose IL-2 subcutaneously (s.c.).

**TABLE 1** | Survey of clinical trials based on *in vivo* activation of γδ cells.

| Author | Year | Tumor | Treatment | Reference |
|---|---|---|---|---|
| Wilhelm et al. | 2003 | MM, NHL | Pamidronate + IL-2 | (58) |
| Dieli et al. | 2003 | Prostate, breast | Zoledronate | (8) |
| Dieli et al. | 2007 | Prostate | Zoledronate/ Zoledronate + IL-2 | (59) |
| Meraviglia et al. | 2010 | Breast | Zoledronate + IL-2 | (60) |
| Bennouna et al. | 2010 | Solid tumors | BrHPP + IL-2 | (61) |
| Gertner-Dardenne et al. | 2010 | FBCL | Rituximab + BrHPP + IL-2 | (62) |
| Lang et al. | 2011 | RCC | Zoledronate + IL-2 | (63) |
| Kunzmann et al. | 2012 | RCC, MM, AML | Zoledronate + IL-2 | (64) |
| Pressey et al. | 2016 | Neuroblastoma | Zoledronate + IL-2 | (65) |

*MM, multiple myeloma; NHL, non-Hodgkin lymphoma; FBCL, follicular B-cell lymphoma; RCC, renal cell cancer; AML, acute myeloid leukemia; BrHPP, bromohydrin pyrophosphate; IL, interleukin.*

The treatments were well tolerated and a significant clinical response was observed in the group receiving Zoledronate and IL-2 during the 1-year follow-up, which correlated with sustained elevated numbers of blood Vγ9Vδ2 $T_{EM}$ cells producing IFN-γ and TRAIL.

We also conducted a phase I trial in 10 advanced metastatic breast cancer patients, using the same Zoledronate and IL-2 regimen as in the above study (60), and found that 3 patients who sustained Vγ9Vδ2 T cell numbers achieved either disease stabilization (2 patients) or partial remission (1 patient).

While the above studies by the Wilhelm's group and our group have used n-BPs and IL-2, Bennouna and colleagues (61) conducted a phase I trial using the synthetic PAg BrHPP with low doses of IL-2 in 28 patients with solid tumors. Patients first received BrHPP alone intravenously (i.v.) and then were treated with BrHPP i.v. in combination with IL-2 s.c. at weekly intervals. The BrHPP and IL-2 treatment was well tolerated and induced *in vivo* dose-dependent Vγ9Vδ2 T cell amplification. Based on these findings and the results from a preclinical study in macaques (62), Bennouna and colleagues conducted a multicentric phase II trial with BrHPP and IL-2 in 45 patients with follicular B-cell lymphoma who had been previously treated with the anti-CD20 mAb Rituximab. The treatment provoked expansion of Vγ9Vδ2 T lymphocytes in 39 out of the 45 patients, which peaked 1 week after the first injection of BrHPP, but declined upon subsequent injections. However, Vγ9Vδ2 T cells acquired the capability to produce IFN-γ and TNF-α and expressed FcγRIII (CD16) which promoted ADCC activity after the second and third injections of BrHPP. Clinical results from 38 patients consisted of 10 instances of complete remission (CR) and 17 overall response rate. Therefore, administration of BrHPP, IL-2 and Rituximab produced very promising results, with limited side effects, overall supporting the potential of combining Vγ9Vδ2 T cell-based therapies with mAbs.

In contrast with these extremely promising results, two other phase I trials have confirmed that the Vγ9Vδ2 T cell-based therapy is well tolerated, but have not shown evidence of antitumor effects. Lang and colleagues (63) have conducted a phase I trial with Zoledronate and IL-2 in 12 patients with metastatic renal cell carcinoma. All patients experienced low grade adverse events, but no clinical response was observed. Rather, the treatment induced a significant decrease of the *in vitro* Vγ9Vδ2 T cell proliferative response in the majority of the patients.

In another study, Kunzmann and coworkers (64) conducted a prospective phase I study with Zoledronate and IL-2 in 21 patients with different advanced malignancies. The regimen was well tolerated and caused a marked *in vivo* activation and IFN-γ production of Vγ9Vδ2 T cells in all evaluable patients, but objective responses (partial remission) were observed only in two patients with acute myeloid leukemia. Interestingly, the lack of clinical response was associated with elevated pretreatment levels of serum vascular endothelial growth factor, which were even increased upon injection of Zoledronate and IL-2.

Finally, a recent prospective, non-randomized Phase I trial, has been conducted in nine young patients with refractory neuroblastoma, which has demonstrated that *in vivo* administration of Zoledronate and IL-2 s.c. can safely expand *in vivo* circulating Vγ9Vδ2 T cells, suggesting that intentional *in vivo* activation of Vγ9Vδ2 T cells might represent a strategy for the treatment of neuroblastoma (65).

## RESULTS FROM CLINICAL TRIALS BASED ON ADOPTIVE TRANSFER OF *EX VIVO*-EXPANDED γδ T CELLS

Phase I clinical trials using adoptive transfer of *ex vivo*-expanded γδ T cells have yielded somewhat conflicting results. A survey of these studies in different types of cancer is shown in **Table 2**.

Five studies have given results suggesting an antitumor effect of the therapy. Two trials were carried out by Kobayashi's group in patients with advanced renal cell carcinomas; in one study (66), seven patients received Zoledronate-expanded Vγ9Vδ2 T cells and IL-2 i.v. All patients had mild adverse events, four patients showed a significant *in vivo* expansion and IFN-γ production by Vγ9Vδ2 T cells, but the clinical benefit was moderate, as only three out of seven patients showed delayed tumor doubling time (66). In the second trial from the same group, all 11 patients receiving Zoledronate-expanded Vγ9Vδ2 T cells and IL-2 showed prolonged tumor doubling time (67).

In another trial Nicol and coworkers (68) evaluated the safety and feasibility of the adoptive transfer of Vγ9Vδ2 T cells expanded *ex vivo* with Zoledronate and IL-2, in combination with Zoledronate given i.v. to 18 patients with advanced solid tumors who continued their previously ineffective chemotherapy. No toxicity was reported, and 3 out of the 18 patients had clinical responses (68). Interestingly, authors tracked Vγ9Vδ2 T cells labeled with $^{111}$In in three patients. The cells localized to the lungs and remained there for 4–7 h after injection and then migrated to the liver and spleen. In one patient with a large metastasis in the left adrenal gland, the cells accumulated in the metastatic site 1 h after injection and remained there until 48 h.

In a fourth trial, four patients with advanced hematological malignancies received haploidentical transplants (69) highly enriched for Vγ9Vδ2 T cells, followed by *in vivo* administration of Zoledronate and IL-2. Three patients showed CR during the

**TABLE 2** | Survey of clinical trials based on adoptive transfer of *ex vivo*-expanded γδ cells.

| Author | Year | Tumor | Treatment | Reference |
|---|---|---|---|---|
| Wada et al. | 2014 | Gastric cancer | Vγ9Vδ2 + Zoledronate | (70) |
| Abe et al. | 2009 | MM | Vγ9Vδ2 + Zoledronate + IL-2 | (71) |
| Kobayashi et al. | 2007, 2011 | RCC | Vγ9Vδ2 + Zoledronate + IL-2 | (66, 67) |
| Nicol et al. | 2011 | Solid tumors | Vγ9Vδ2 + Zoledronate | (68) |
| Bennouna et al. | 2008 | RCC | Vγ9Vδ2 + BrHPP + IL-2 | (72) |
| Wilhelm et al. | 2014 | | Vγ9Vδ2 + Zoledronate + IL-2 | (69) |
| Nakajima et al. | 2010 | NSCLC | Vγ9Vδ2 + Zoledronate + IL-2 | (73) |
| Sakamoto et al. | 2011 | NSCLC | Vγ9Vδ2 + Zoledronate + IL-2 | (74) |

*MM, multiple myeloma; RCC, renal cell cancer; NSCLC, non-small cell lung cancer; BrHPP, bromohydrin pyrophosphate; IL, interleukin.*

6-month follow-up, while one patient died of an infection 6 weeks after the cell transfusion.

Most recently, Wada and coworkers have conducted a pilot study in seven patients with neoplastic ascites caused by gastric cancer with Vγ9Vδ2 T cells expanded *ex vivo* with Zoledronate and IL-2, administered together with Zoledronate i.v. Weekly Intraperitoneal injection of Vγ9Vδ2 T cells had no severe adverse events and caused a significant reduction of the number of tumor cells in the ascites, which was evident soon after the first cycle of therapy and sustained over time. CT scan also revealed a significant reduction in volume of ascites in two out of the seven patients. Authors conclude that injection of Vγ9Vδ2 T cells can result in the control of malignant ascites in patients for whom no standard therapy is available (70).

In contrast to the above successful studies, several other phase I trials, while showing that Vγ9Vδ2 T cell adoptive therapy is well tolerated, failed to providing evidence of antitumor effects.

Abe et al. (71) conducted a trial in six subjects with MM who received Zoledronate-expanded Vγ9Vδ2 T cells in combination with Zoledronate and IL-2. The treatment was safe but clinical efficacy, as assessed by M-protein serum levels remained at baseline in four patients and increased in two patients, in the absence of between the number of Vγ9Vδ2 T cells injected and clinical outcome.

Bennouna et al. (72) conducted a phase I trial using *ex vivo*-expanded Vγ9Vδ2 T cells in combination with BrHPP and IL-2, in 10 patients with metastatic renal cell carcinoma. Overall, the therapy was well tolerated with only one severe effect, 6 out of 10 patients showed stable disease, but there was no significant antitumor effect.

Finally, in 2 studies of non-small cell lung cancer (NSCLC) involving 10 and 15 patients, respectively (73, 74), who received *ex vivo*-expanded Vγ9Vδ2 T cells and IL-2, there were no objective clinical responses although about one-third to one-half of the patients showed stable disease after therapy. In one study, Nakajima and coworkers (73) treated 10 patients with NSCLC with Vγ9Vδ2 T cells expanded *ex vivo* with Zoledronate and IL-2. The treatment was safe, three patients showed stable disease and five patients showed a progression of the disease 4 weeks after the last treatment. In the other study, Sakamoto and coworkers (74) injected *ex vivo*-expanded γδ T cells in patients with advanced NSCLC. Fifteen patients undergoing treatment with these γδ T cells did not have severe adverse events, all patients remained alive during the study period, but there were no objective responses.

## WHAT DO THE γδ T CELL-BASED CLINICAL TRIALS TEACH US?

Clinical trials exploiting γδ T cells in cancer have been conducted over the past decade, with a good safety profile but variable efficacy. What is clear from these studies is that there is enormous variation in the types of cancer treated, combined with heterogeneity in the protocols used to expand γδ T cells *in vivo* or *ex vivo* for cellular immunotherapy, or in how the immunotherapy was delivered (e.g., PAgs or Zoledronate with or without IL-2, or in combination with other drugs, γδ T cells alone or in combination with activating drugs such as IL-2 and Zoledronate). In addition, several factors may influence the success of γδ T cell-based immunotherapy, which will be discussed in this section.

Immunotherapy strategy based on intentional activation of Vγ9Vδ2 T cells *in vivo* by administration of PAgs or n-BPs and IL-2 has been effective in activating circulating Vγ9Vδ2 T cells, but there is no evidence that this approach reaches tissue-resident γδ T cells or even promotes their recruitment at the tumor site, where they should in fact exert their antitumor activities.

Moreover, patients with several types of tumors have low numbers and unresponsive γδ T cells (75), even if more recent evidences indicate that reductions of γδ T cell numbers and functions might be associated with age and sex and not with the presence of the tumor (76–78).

In addition, a decreased number of circulating Vγ9Vδ2 T cells have been observed as injections of PAgs or Zoledronate and IL-2 progressed, which was accompanied by a lower response of peripheral blood Vγ9Vδ2 T cells to PAgs.

The precise mechanism underlying this phenomenon remains unknown and further investigations are thus necessary. Among the possibilities, activation-induced Vγ9Vδ2 T cell anergy has been frequently reported (75), possibly due to inadequate signals delivered during activation, exposure to suboptimal PAgs concentration or from Vγ9Vδ2 T cell intrinsic features.

A recent clinical trial of Zoledronate given i.v. to cancer-free patients showed that the inflammatory-type side effect of Zoledronate (flu-like syndrome) could be easily predicted by analyzing *in vitro* production of IFN-γ by Zoledronate-stimulated peripheral blood mononuclear cells (79). In agreement with these data, we and others have shown that repeated i.v. injections of Zoledronate was accompanied by decrease of circulating Vγ9Vδ2 $T_{CM}$ cells and reduction of their proliferative responses *in vitro* (79, 80). Circulating neutrophils may also contribute as they take up Zoledronate and produce hydrogen peroxide that inhibits T cell proliferation (81). Finally, repeated stimulation of Vγ9Vδ2 T cells may also cause terminal differentiation and exhaustion (82–84).

Immunoevasion strategies can be exploited by cancer cells to escape recognition and attack by Vγ9Vδ2 T cells. Indeed, several evidences demonstrate that cancer cells acquire the capability to inhibit immunological checkpoints using several different strategies. However, a very recent study has shown that Vγ9Vδ2 T cells express very low programmed death-1 (PD-1) compared with conventional αβ CD8 and CD4 T cells, which was markedly up-regulated over the first 4 days of exposure to Zoledronate and IL-2 *in vitro* but by day 7 dropped nearly to baseline (85). While these results suggest that Vγ9Vδ2 T cells may circumvent the PD-1/PD-1L checkpoint *in vivo*, Hayday and coworkers found that Vγ9Vδ2 T cells express another negative checkpoint receptor, TIGIT, upon *in vitro* activation, thus providing an additional opportunity to cancer cells to escape Vγ9Vδ2 T cell-mediated elimination (Hayday, unpublished results). Evasion strategies that specifically impair Vγ9Vδ2 T cell functions can involve diverse immunosuppressive mediators produced in the tumor microenvironment, as, for example,

transforming growth factor-β, prostaglandins, kynurenins, or potassium (86–89).

All of the above pitfalls may be partly overcome by utilization of the adoptive cell transfer of *ex vivo*-expanded Vγ9Vδ2 T cells, which thus seems to be a more effective procedure. However, the problem appears to be how to sustain the levels and functions of the transferred Vγ9Vδ2 T cells. In metastatic renal cell carcinoma, two groups reported superior efficacy when Vγ9Vδ2 T cells were administered with Zoledronate and/or IL-2, as compared to Vγ9Vδ2 T cells administered alone (67, 90).

While the aforementioned trials utilized patients' autologous peripheral blood-derived Vγ9Vδ2 T cells, a recent study by Wilhelm and colleagues (69) utilized Vγ9Vδ2 T cells from haploidentical donors; this treatment did not cause graft-versus-host disease and was clinically effective as three out of four patients achieved CR (69). Vγ9Vδ2 T cells from the haploidentical donor persisted for 28 days and expanded *in vivo* following injection of Zoledronate and IL-2.

Other studies have shown that it is possible to sustain injected Vγ9Vδ2 T cells without IL-2 supplementation, probably relying on IL-15 (91) or on IL-18 (92, 93) spontaneously produced by the host.

## CAN WE IMPROVE γδ T CELL-BASED TUMOR IMMUNOTHERAPY?

γδ T cells can be redirected to the cancer cell using antibodies (**Figure 2**). This can be achieved, for instance, by using bispecific antibodies, in which one binding site recognizes a tumor-specific cell surface molecule (for example, EpCAM or HER2/neu) and the other binding site targets CD3 or the Vγ9 chain of the Vγ9Vδ2 TCR; such bispecific antibodies have been demonstrated effective in preclinical models (94, 95).

As a variant of the bispecific antibody technology, Zheng et al. (96) prepared a chimeric molecule in which the variable portion derived from the extracellular domains of a Vγ9Vδ2 TCR (cloned from a Vγ9Vδ2 T cell infiltrating ovarian cancer) and the constant region was the fragment crystallizable (Fc) domain of human IgG1. This chimeric construct bound to several ovarian cancer cells, recognizing a yet unknown antigen and promoted the killing of the cells *via* ADCC mediated by binding of the Fc region of the chimeric construct to CD16.

Wesch and colleagues (97) developed recombinant immunoligands consisting of a CD20 single-chain variable fragment (scFv) linked to MICA or ULBP2 and found that both constructs

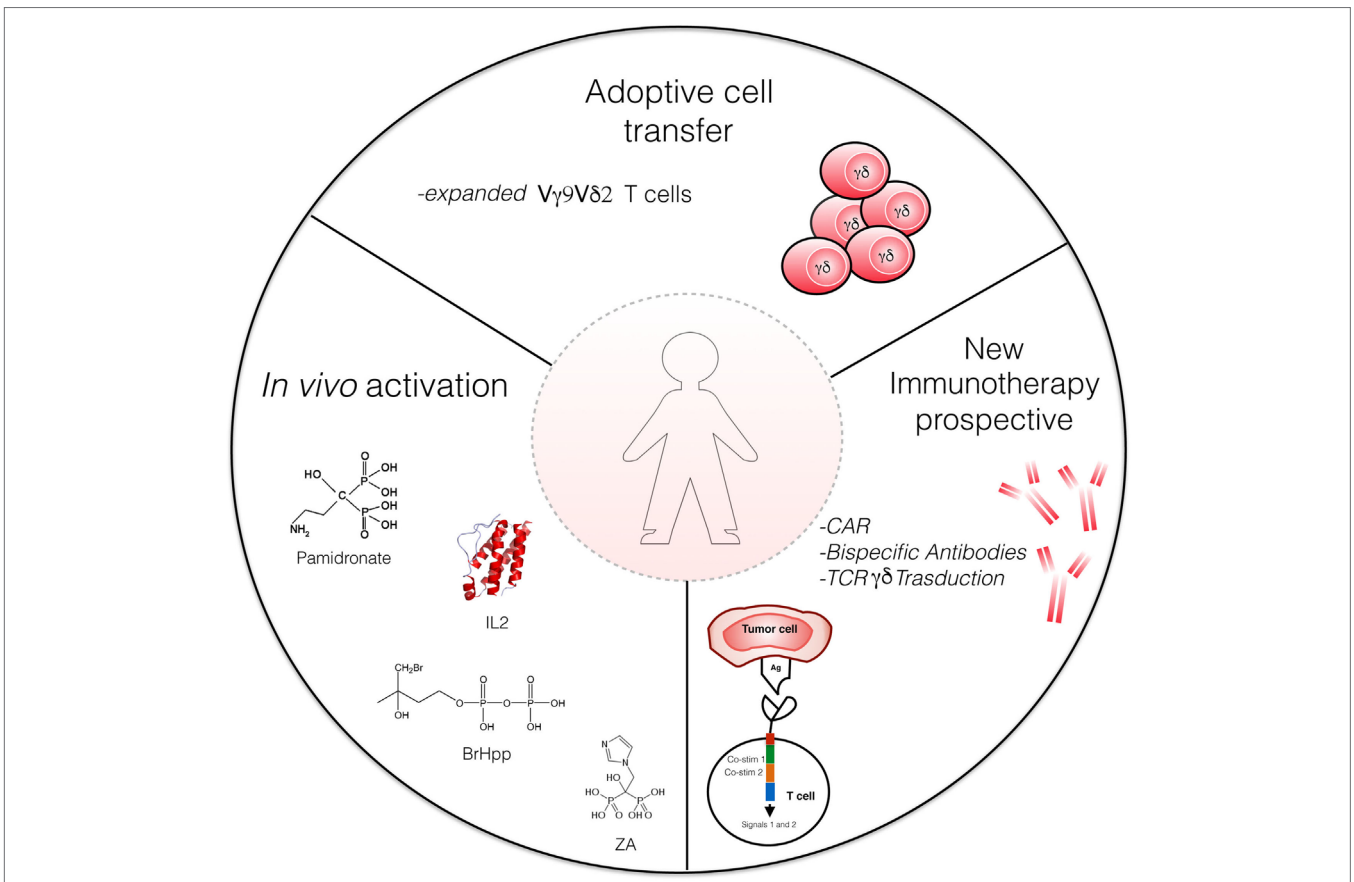


**FIGURE 2** | Strategies for γδ T cell-based immunotherapy. Actual strategies include adaptive cell transfer of γδ T cells expanded *in vitro* with Zoledronate and interleukin (IL)-2, and *in vivo* activation of Vγ9Vδ2 T cells by phosphoantigens [e.g., bromohydrin pyrophosphate (BrHPP)] or aminobisphosphonates (Zoledronate) and low-dose IL-2. Novel γδ T cell-based therapeutic strategies involve bispecific antibodies and CAR-T cells. ZA, Zoledronate acid; CAR, chimeric antigen receptors.

promoted the cytotoxic activity of *ex vivo*-expanded γδ T cells (containing both Vδ1 and Vδ2 T cells) against CD20-positive lymphoma cells. Importantly, these two immunoligands mediated the killing of chronic lymphocytic leukemia cells isolated from patients by γδ T cells, which was even enhanced by the PAg BrHPP. Thus, the utilization of recombinant immunoligands which engage NKG2D, with or without simultaneous TCR triggering, may represent an attractive strategy to enhance antitumor cytotoxicity of γδ T cells.

Another approach consists in lentiviral-mediated transduction of T cells with chimeric antigen receptors (CARs; **Figure 2**). CARs are usually derived from scFvs of antibodies specific for tumor antigens, thus enabling the CAR-transduced T cells to recognize tumor epitopes independently on their TCR [reviewed in Ref. (98)].

To date, most CAR utilize αβ T cells, but γδ T cells are also an appealing target, due to their antitumor effector functions.

Deniger et al. (99) have transduced polyclonal γδ T cells with a CD19-specific CAR which conferred the capability to efficiently kill $CD19^+$ leukemia cells. The CAR technology has been combined with the generation of induced pluripotent stem cells from human peripheral blood T cells (100). Such cells showed a very similar phenotype to γδ T cells and exerted antitumor activity.

T cells can be redirected to tumors by lentiviral-mediated transduction with an exogenous TCR of known anticancer specificity, following adoptive transfer into patients. Typically, the vast majority of studies have involved transduction of an αβ TCR of well known antitumor specificity into another αβ T cell (101). The major problem with this strategy is the risk of mispairing between the endogenous and exogenous TCR α and β chains, resulting in receptors with autoreactive specificities (102, 103). γδ T cells offer an attractive solution to this problem, in the sense that a given tumor-specific αβ TCR can be introduced into γδ T cells without the risk of mispairing (104, 105). Another advantage is that γδ T cells transduced with an αβ TCR retain the functionality of their original TCR, thereby responding rapidly upon antigen stimulation (106).

The main obstacle associated with the αβ TCR transfer, is that γδ T cells do not express CD4 or CD8 co-receptors, which are required for efficient recognition of peptide–MHC complexes on target cells. This implies that co-transduction with a co-receptor (107) or use of very high affinity TCRs (108) would be desirable to enhance antitumor activity of αβ-transduced γδ T cells. It is also possible to transduce peripheral lymphocytes (both γδ and αβ) with a specific γδ TCR, as successfully demonstrated by Zhao and coworkers (109, 110).

Finally all γδ T cell-based clinical trials in patients with hematologic and solid tumors have relied on the utilization of Vγ9Vδ2 T cells. Vδ1 T cells are typically less susceptible to activation-induced exhaustion and in theory could persist long after adoptive transfer, providing the host with a durable antitumor immune response (111). Moreover, as Vδ1 T cells express several NK receptors and possess a highly cytotoxic potential (8), they may constitute a potent therapeutic lymphocyte population that could be exploited in alternative to, or in addition to Vγ9Vδ2 T cells. Accordingly, Silva Santos and coworkers (112) have recently developed a clinical-grade method to selectively expand Vδ1 T cells. The expanded Vδ1 T cells efficiently inhibited tumor growth and prevented dissemination in xenograft models of leukemia, thus providing a preclinical proof of principle for application of Vδ1 T cells in adoptive immunotherapy of cancer.

## CONCLUSION

Overall, studies performed to date have clearly demonstrated that γδ T cell-based tumor immunotherapy is safe, but clinical performance has been inconsistent (31). Identification of the ligands recognized by $V\delta1^+$ and $V\delta2^+$ T cells, the antigen and cytokine requirements for their differentiation and survival, and the interactions they establish with tumor cells and other different components of the tumor microenvironment, will lead to a better understanding of how γδ T cells work and to properly harness these cells for effective and durable tumor immunotherapy.

## AUTHOR CONTRIBUTIONS

EG, GC, and GG provided clinical samples and patient's data. EP and GP analyzed data in the literature and prepared figures. FD and SM wrote the manuscript.

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# Herceptin Enhances the Antitumor Effect of Natural Killer Cells on Breast Cancer Cells Expressing Human Epidermal Growth Factor Receptor-2

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Optimal adoptive cell therapy (ACT) should contribute to effective cancer treatment. The unique ability of natural killer (NK) cells to kill cancer cells independent of major histocompatibility requirement makes them suitable as ACT tools. Herceptin, an antihuman epidermal growth factor receptor-2 (anti-HER2) monoclonal antibody, is used to treat HER2$^+$ breast cancer. However, it has limited effectiveness and possible severe cardiotoxicity. Given that Herceptin may increase the cytotoxicity of lymphocytes, we explored the possible augmentation of NK cell cytotoxicity against HER2$^+$ breast cancer cells by Herceptin. We demonstrated that Herceptin could interact with CD16 on NK cells to expand the cytotoxic NK (specifically, CD56$^{dim}$) cell population. Additionally, Herceptin increased NK cell migration and cytotoxicity against HER2$^+$ breast cancer cells. In a pilot study, Herceptin-treated NK cells shrunk lung nodular metastasis in a woman with HER2$^+$ breast cancer who could not tolerate the cardiotoxic side effects of Herceptin. Our findings support the therapeutic potential of Herceptin-treated NK cells in patients with HER2$^+$ and Herceptin-intolerant breast cancer.

**Keywords: natural killer cells, breast cancer, Herceptin, antibody-dependent cell-mediated cytotoxicity, CD16**

## INTRODUCTION

Clinical studies have revealed the promising effects of adoptive cell therapy (ACT) in cancer treatment (1). The success of ACT requires the therapeutic strategies to meet four critical criteria, including clinical efforts that should focus on obtaining sufficient numbers of immune effector cells, the ability of the immune cells to reach the tumor site and recognize the targets, efficient tumor

**Abbreviations:** ACT, adoptive cell therapy; ADCC, antibody-dependent cell-mediated cytotoxicity; CAR, chimeric antigen receptor; ELISA, enzyme-linked immunosorbent assay; FCM, flow cytometry; HER, human epidermal growth factor receptor; IFN, interferon; IL, interleukin; LDH, lactate dehydrogenase; NK, cell natural killer cell; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; siRNA, small-interfering RNA.

cell killing, and minimal or no harm caused to healthy cells. Increasing evidence has been reported to support the application of natural killer (NK) cells for ACT. NK cells, a component of innate immunity, are cytotoxic lymphocytes that play an important role in the host's early protective responses against transformed cells (2). It has been reported that $CD3^{-}CD56^{+}$ NK cells can exert a main histocompatibility complex-independent antitumor effect on both solid and hematological malignancies without affecting hematopoietic precursors or normal tissues (3, 4). Furthermore, the results from a clinical study support the therapeutic efficacy of an NK cell infusion in patients with colon carcinoma (5).

Natural killer cells, a heterogeneous population of lymphocytes, represent a small fraction of the peripheral blood mononuclear cells (PBMCs) (6). Since NK cells are significant effectors of the innate immune system and have a unique ability to directly lyse transformed or virus-infected cells without prior sensitization or MHC class restriction, they are regarded as promising candidates for immunotherapy of cancer (7–9). However, breast cancer patients in all clinical stages of disease had significantly decreased NK cell activity and that it was most profound in advanced stage of this disease (10, 11). Sufficient numbers of fully functional NK cells are usually generated *ex vivo* to achieve satisfactory therapeutic efficacy. Currently, several clinical studies have focused upon adoptive autologous NK cells infusion in an attempt to treat common malignancies, such as breast cancer, lymphoma, renal cell carcinoma and non-small cell lung cancer (6, 12–14). Cytokines, such as interleukin (IL)-2, IL-7, IL-10, IL-15, or IL-18 have been reported to amplify NK cells isolated from PBMCs (15–17). Previous studies from our laboratory have shown that the combination of IL-2 and IL-15 stimulated the expansion of NK cells, without affecting the cytotoxicity of NK cells (18).

Breast cancer is the most commonly diagnosed cancer and is the second highest cause of cancer death in women (19). In China, breast cancer is the most common cancer for females (20). Herceptin is a widely used human epidermal growth factor receptor-2 (HER2)-targeted therapy for treating metastatic breast cancer by down-regulating tumor cell proliferation. Herceptin is an anti-HER2 monoclonal antibody, which is engineered by inserting the complementary determinant regions of a murine antibody (clone 4D5) into the consensus framework of human IgG1 (21). In addition to common side effects, such as fever, rash and infection, a severe side effect, cardiotoxicity, limits the application of Herceptin in some patients. Nakagawa et al. found that Herceptin could increase the cytotoxicity of lymphocytes and Herceptin-activated lymphocytes could inhibit the growth of breast cancer cells *in vitro* (22). Therefore, the aim of the present study was to identify the effects of engaging NK cells with Herceptin on the activities of NK cells under IL-2 and IL-15 stimulation conditions. We found that Herceptin increased the NK cell proliferation, migration, and cytotoxicity against $HER2^{+}$ cancer cells. These results revealed a new function of Herceptin for increasing antitumor effects of NK cells, in addition to directly suppressing proliferation in $HER^{+}$ cancer cells and support the application of targeted antibodies against tumor cells to enhance the clinical efficacy of ACT.

# MATERIALS AND METHODS

## Generation and Characterization of NK Cells

This study was carried out in accordance with the recommendations of the ethical standards of the Institutional Review Committee on Human Research of the Tianjin Medical University Cancer Institute and Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Committee on Human Research of the Tianjin Medical University Cancer Institute and Hospital.

Peripheral blood mononuclear cells were obtained from female patients who were pathologically diagnosed with breast cancer. The method of NK cell expansion was previously reported by our group (18). The day before Day 0, T75 flasks were separately treated with Herceptin at 1 mg/ml (Roche, Swiss), IgG1 at 1 mg/ml (Abcam, USA), or same volume of washed twice with phosphate-buffered saline (PBS). At Day 0, we pour out the coating liquid and washed flasks twice with PBS. On Day 0, PBMCs were isolated from enriched peripheral blood by Ficoll-Hypaque density gradient centrifugation, washed twice with PBS, and cultured in GT551-H3 serum free medium (TaKaRa Biomedical Technology, Japan) supplemented with 10% fetal bovine serum plus IL-2 (10 ng/ml) and IL-15 (50 ng/ml, Peprotech Inc., USA), in the presence or absence of pretreatment T75 flasks. The culture condition was a temperature of 37°C in the humidified atmosphere of a $CO_2$ incubator. Cells cultured in the PBS-treated flasks were served as controls. The medium was changed every 3 days with the addition of GT551-H3 serum free medium supplemented with 10% fetal bovine serum plus IL-2 (10 ng/ml) and IL-15 (50 ng/ml). Expanded NK cells were transferred to T125 flasks at a density of $1.0 \times 10^6$ cells/ml on Day 5. On Day 10, the cells were transferred to cell culture bags at $1.0 \times 10^6$ cells/ml. Cells were harvested at Day 15 and enriched using a MACS® human NK cell negative-selection isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. Briefly, non-target cells were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against lineage-specific antigens and a cocktail of MicroBeads. The magnetically labeled non-target cells were depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled NK cells passed through the column.

## Breast Cancer Cell Culture

A breast cancer cell line, SK-BR-3 cells, was cultured in RPMI 1640 (Takara Biomedical Technology, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), penicillin (100 IU/ml; Sigma, USA), and streptomycin (100 μg/ml; Sigma, USA).

## Small-Interfering RNA (siRNA) Transfection

The siRNA technique was employed to silence the expression of the HER2 gene of SK-BR-3 cells. Double-stranded siRNAs

were transfected into SK-BR-3 cells using Lipofectamine 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The siRNA sequences used to silence the HER2 gene were 5′-GCAGUUACCAGUGCCAAUATT-3′ (sense) and 5′-UAUUGGCACUGGUAACUGCTT-3′ (antisense). The non-targeting sequences, 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense), were used as the negative control.

## Flow Cytometry (FCM) Analysis

Cells from cultures for 0, 5, 10, and 15 days were labeled using following PE-, FITC-, APC-, or PerCP-conjugated mouse anti-human antibodies in fluorescence activated cell sorter buffer (BD Biosciences, USA) for 30 min at room temperature; the antibodies included anti-CD16 (3G8), anti-NKp30 (P30-15), anti-NKG2D (1D11), anti-DNAM-1 (11A8), and anti-CD107a (H4A3) (Biolegend, USA) as well as CD3-FITC and CD56-PE (BD Biosciences). APC- and FITC-conjugated and purified antimouse IgG1 (Biolegend) were used as isotype controls. Goat antihuman IgG (Thermo Fisher Scientific) and the matched isotype control antibody were used to stain cells and to identify cells binding with Herceptin and IgG. Cells were analyzed using FACS Calibur and FACS CantoII instruments (BD Biosciences, USA) and FlowJo software to determine the percentage of positive cells in $CD3^{-}CD56^{+}$ NK cell populations.

## Cytotoxicity Assay

The cytotoxicity of NK cells was measured using the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, USA) according to the manufacturer's instructions. Briefly, enriched NK cells ($4 \times 10^6$/ml) were cultured with the same volume of SK-BR-3 or SK-BR-3/HER2si cells ($0.25 \times 10^5$/ml, $0.5 \times 10^5$/ml, and $1 \times 10^5$/ml) in a 96-well plate for 4 h at 37°C in a humidified $CO_2$ incubator prior to removal of the supernatant for lactate dehydrogenase (LDH) release measurements. LDH release is previously standardized to represent the percentage of cytotoxicity using this formula (23, 24):

$$\%\text{Cytotoxicity} = (\text{Experimental-Effector spontaneous-Target spontaneous}) / (\text{Target maximum-Target spontaneous}) \times 100.$$

## CD107a Degranulation Assay

Enriched NK cells ($4 \times 10^6$/ml) were cultured with the same volume of SK-BR-3 or SK-BR-3/HER2si cells ($1 \times 10^5$/ml) for 4 h at 37°C in a humidified $CO_2$ incubator. Monensin (Sigma, USA) was added at a final concentration of 2 µl/ml after 1 h of incubation. Cells were then stained for CD107a and CD3/CD56/CD45. The percentage of CD107a-positive cells was estimated using FCM by gating for $CD3^{-}CD56^{+}$ NK cells. Spontaneous CD107a expression was detected in the absence of target cells.

## Enzyme-Linked Immunosorbent Assay (ELISA)

Enriched NK cells ($8 \times 10^6$/ml) were cultured with the same volume of SK-BR-3 or SK-BR-3/HER2si cells ($2 \times 10^5$/ml) for 24 h at 37°C in a humidified $CO_2$ incubator. The concentrations of interferon (IFN)-γ in cell culture supernatants obtained from three independent experiments were detected using commercial ELISA kits (DAKEWE, China), according to the manufacturer's instructions. The human IFN-γ kit sensitivity was 5 pg/ml.

## Migration and Adhesion of NK Cells to Target Cells

Target cells, SK-BR-3 cells, were labeled with carboxyfluorescein succinimidyl amino ester using a Cell Labeling Kit (Abcam) according to the manufacturer's instructions. Effector cells (NK cells) and labeled target cells were mixed at an effector: target ratio of 40:1 in a final volume of 500 µl of GT551-H3 medium without IL-2 in a 35 mm glass-bottom cell culture dish. The cells were allowed to settle at the bottom of the dish for 2 min. Images were immediately captured every 30 s for up to 2 h using a 40× magnification objective on an Andor Revolution living-cell imaging station (Olympus) while cells were maintained at 37°C. The images in the figures were excised from live-cell imaging videos at different time points. Finally, twenty target cells from each group were randomly chosen and the cumulative number of adherent NK cells was from 20-min periods up to 2 h. The average number of adherent NK cells in each group was determined.

## Statistical Analysis

The statistical significance of the differences between two groups was determined with a two-tailed, paired Student's *t*-test. Differences among multiple groups were analyzed with one-way analysis of variance using Prism 6 software (GraphPad). Values are expressed as the medians or means ± SD, as indicated, and differences were considered statistically significant at $p < 0.05$.

# RESULTS

## Herceptin Increased NK Cell Proliferation

We first investigated the effects of Herceptin on NK cell proliferation by evaluating the percentage and number of NK cells treated with IL-2 plus IL-15 (blank group), as well as IL-2/IL-15 in combination with IgG1 (IgG1-treated group) and Herceptin (Herceptin-treated group) at various time periods. During 15 days culture, we measured cell number of three groups every 5 days. The final product was a 40-fold enrichment (range, 16–139-fold) after culture for 15 days in Herceptin-treated group, which was similar to that in the blank and IgG1-treated group (**Figure 1A**). The gating strategy used to determine the percentages of $CD56^{dim}$ and $CD56^{bright}$ NK cells is shown in Figure S1 in Supplementary Material. The typical changes in the percentage of NK cells in these 3 groups by using NK cells from 20 patients were evaluated (**Figure 1B**). At Day 0, we observed 2 groups of primary NK cells, $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells. Based on these findings, Herceptin, IgG1, and cytokines amplified the $CD56^{bright}$ NK cells. Cells in IgG1 and blank groups were maintained as 2 populations until Day 15. Notably, in the Herceptin-treated group, most of the amplified NK cells observed on Day 15 were $CD56^{dim}$ cells (57.80 ± 7.60%)

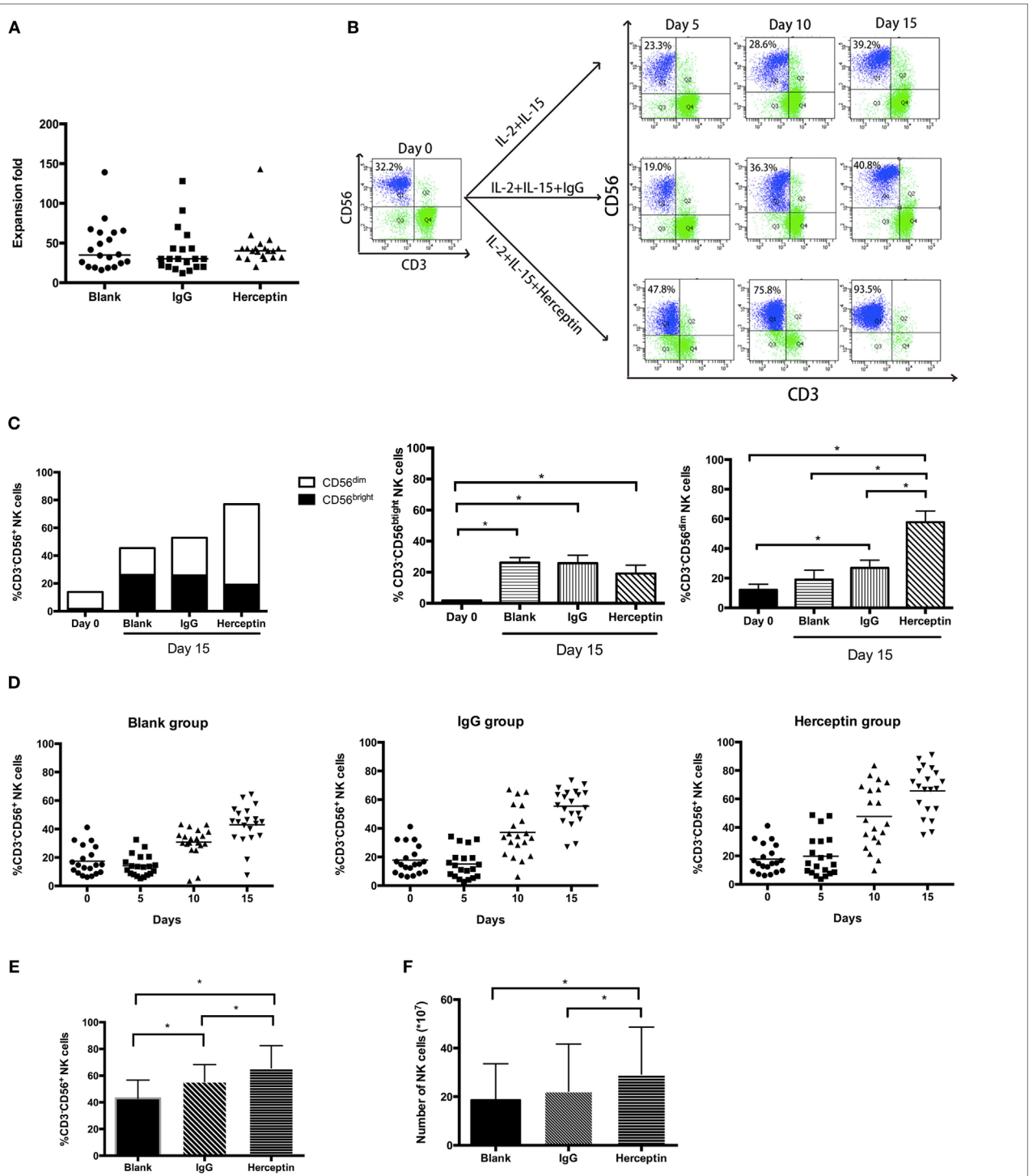


**FIGURE 1** | The expansion of *ex vivo* expanded human natural killer (NK) cells in the presence of interleukin (IL)-2 and IL-15. Freshly prepared peripheral blood mononuclear cells (PBMCs) were cultured in flasks coated with IgG1 or Herceptin for 15 days. **(A)** Expansion fold of the final *ex vivo* expanded products. We counted the cell number on Day 15 and determined the expansion fold. The results are presented as the median values for 20 patients. **(B)** A typical change in NK cell expansion observed among 20 samples under different culture conditions. The percentage of NK cells ($CD56^+CD3^-$) in the expanded PBMCs culture was determined by flow cytometry (FCM). The left panel represents the primary PBMCs. Rows of the right nine graphs from top to bottom represent the blank group, the IgG1 group and the Herceptin group; columns from left to right represent the expansion products obtained after 5, 10, and 15 days. **(C)** The percentages of $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells. We evaluated the NK cell subsets in three samples from the three groups on Days 0 and 15. **(D)** Changes in the percentage of NK cells over 15 days under different culture conditions. The graphs from left to right show the blank group, the IgG1 group and the Herceptin group. **(E)** Percentages of NK cells in the final expansion products of three groups. **(F)** The number of NK cells in the three groups observed on Day 15. The results shown in **(E,F)** are presented as the means of 20 patients. The results are presented as the means of independent experiments, and the error bars represent the SD. $^*p < 0.05$.

compared with Day 0; however, but in the Blank-NK group, most of the amplified NK cells were $CD56^{bright}$ cells (26.23 ± 3.25%) (**Figure 1C**). The overall trends in the changes in the percentages of NK cells are shown in **Figure 1D**. All three groups showed increases in the percentages of NK cells after 15 days, but Herceptin-treated NK cells had the greatest proliferation during this period (from 17.68 ± 9.94 to 65.71 ± 16.72%) (**Figure 1E**). When we calculated the number (the number of final cells multiplied by the percentage of NK cells) of NK cells at Day 15, we found that Herceptin efficiently increased the number of NK cells ($29.26 \pm 19.38 \times 10^7$), compared with the IgG1 group ($22.22 \pm 19.47 \times 10^7$) and blank group ($18.57 \pm 14.99 \times 10^7$) (**Figure 1F**).

Therefore, Herceptin increases the percentage of NK cells in the final expansion products.

## Herceptin Increased the Cytotoxicity of NK Cells against $HER2^+$ Breast Cancer Cells

Next, we evaluated the cytotoxicity of NK cells using three approaches, including LDH release, target cell degranulation measured using CD107a expression, and IFN-γ secretion. Herceptin-treated NK cells had higher levels of cytotoxicity toward $HER2^+$ cells compared to blank and IgG1 treated groups when the E:T ratio was 40:1 or 20:1; however, Herceptin-treated NK cells did not display higher cytotoxicity than IgG1 treated and blank groups for $HER2^-$ cells (**Figure 2A**). Herceptin-treated NK cells also increased the expression level of CD107a on the cell surface in $HER2^+$ cells, but not $HER2^-$ cells, suggesting that degranulation is increased when target cells are engaged with $HER2^+$ cells (**Figure 2B**). Consistent with the results obtained from the LDH and CD107a release assays, Herceptin-treated NK cells induced a higher level of IFN-γ secretion when cocultured with $HER2^+$ cells but not with $HER2^-$ cells (**Figure 2C**). These results indicate that Herceptin treatment increases the NK cell cytotoxicity on $HER2^+$ breast cancer cells. This effect may be caused by increasing NK cell activity toward cancer cells and direct effects of Herceptin on cancer cells.

## Herceptin Improved the Migration and Adhesion of NK Cells to $HER2^+$ Breast Cancer Cells

To examine the effects of Herceptin treatment on NK cell migration and adhesion to breast cancer cells, NK cells were cocultured with $HER2^+$ and $HER2^-$ cells in the live-cell imaging station for 2 h. A greater frequency of Herceptin-treated NK cells migrated faster toward SK-BR-3 cells (Video S1 in Supplementary Material), and NK cells in the blank group were not changed (Video S2 in Supplementary Material) (**Figure 3A**). In contrast, Herceptin had no effect on NK cell migration when cocultured with $HER2^-$ cells (data not shown). Interestingly, NK cells in the blank group had a round shape during the 2-h observation in contrast to the Herceptin-treated group, and they were continuously transforming while moving toward the target cells (**Figure 3A**). By analyzing the number of NK cells that adhered to breast cancer cells, we found that Herceptin enhanced the adhesion of Herceptin-treated NK cells with $HER2^+$ cells but not $HER2^-$ cells, within 20 min of coculture (**Figure 3B**). The migration and adhesion of IgG1-stimulated NK cells were increased compared with the blank group and reduced compared with Herceptin-treated cells (Video S3 in Supplementary Material). When cultured alone, NK cells exhibited little change (Video S4 in Supplementary Material). Therefore, Herceptin significantly enhanced the migration and adhesion of NK cells toward $HER2^+$ breast cancer cells.

## Herceptin Does Not Affect NKp30, NKG2D, and DNAM-1 Surface Receptors on NK Cells

To understand how Herceptin stimulates the activity of NK cells, including proliferation, cytotoxicity, and migration, we evaluated the effects of Herceptin on cytotoxicity-associated activated receptors NKp30, NKG2D, and DNAM-1. Every 5 days, the same number of cells from three groups was analyzed using FCM to examine the expression levels of these receptors. The percentage of receptors means the number of receptors every one hundred NK cells expressing. The number of NK cells expressing these surface activating receptors changed in a similar manner among the blank, IgG1, and Herceptin groups during the 15-day culture period (**Figure 4A**). The numbers of NK cells expressing these surface activating receptors were increased in all three groups 15 days after culture, but no difference was observed among these three groups (**Figure 4B**). In addition, the mean fluorescent intensity values, which evaluate the number of receptors on one NK cells, showed the same trend as the frequency in these three groups (data not shown). Therefore, neither IgG1 nor Herceptin influenced the activation of these surface receptors in NK cells.

## Herceptin Interacts with CD16 for Increasing NK Cell Activity

Ligation of CD16 leads to activation of this receptor and activation of CD16 regulates NK cell activity. Therefore, the unbinding-CD16 expression level represents the activation status after CD16 ligation. Next, we determined the effects of Herceptin on CD16 activation in NK cells by detecting unbinding-CD16 expression level in NK cells. We examined the percentage of CD16 positively stained cells in $CD3^-CD56^+$ NK cells by using FCM analysis (representative FCM plots are shown in Figure S2 in Supplementary Material). Although both Herceptin and IgG1 induced decreases in the number of NK cells expressing unbinding-CD16 starting at 5 days after culture, compared to the blank group, Herceptin treatment had a more potent effect (**Figures 5A,B**).

We also examined the interaction between antibodies and NK cells during short-term culture. There was a more rapid reduction in binding of anti CD16 antibody after Herceptin incubation compared to control IgG1 (Figure S3 in Supplementary Material, representative FCM plots were shown in Figure S4 in

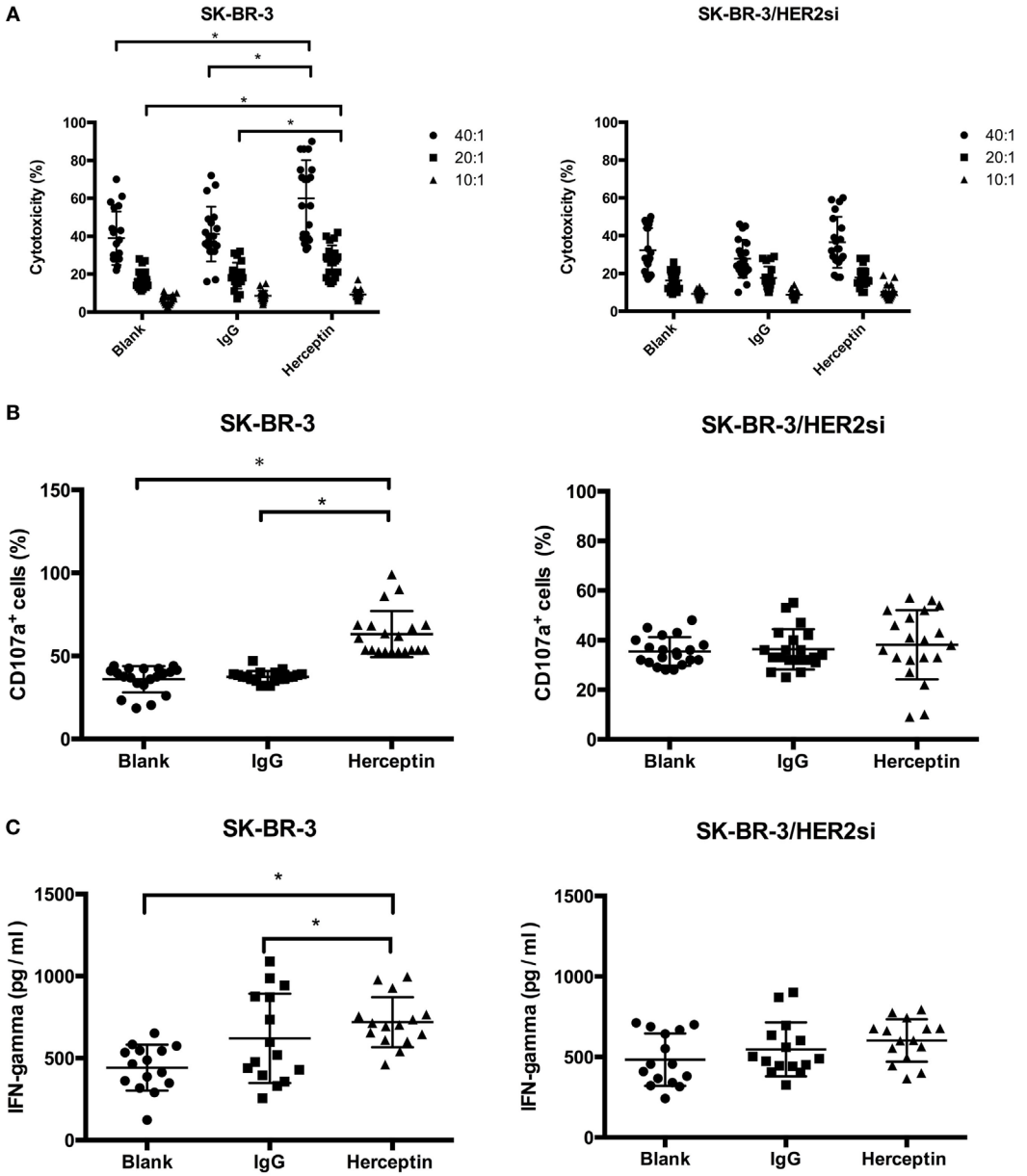


**FIGURE 2** | Cytotoxicity of expanded natural killer (NK) cells toward breast cancer cells. For the cytotoxicity assay, NK cells were enriched prior to the assay. **(A)** The cytotoxicity of expanded NK cells toward SK-BR-3 and SK-BR-3/HER2si cells was measured using the lactate dehydrogenase (LDH) release assay at 40:1, 20:1, 10:1 effector-to-target (E:T) ratios for 4 h. **(B)** NK cell degranulation after coculture with cancer cells. The cytotoxicity of expanded NK cells toward SK-BR-3 and SK-BR-3/HER2si cells was measured using a CD107a release assay at 40:1 effector-to-target (E:T) ratios. Differences in the frequency of $CD107a^+$ cells among NK cells in the Herceptin, IgG1 and blank groups were analyzed for statistical significance. **(C)** The level of interferon (IFN)-γ secretion after coculture with cancer cells. NK cells were stimulated with the indicated targets, SK-BR-3 and SK-BR-3/HER2si cells. After 3 days, supernatants were collected and IFN-γ levels were measured. The results are presented as the means of 15 patients and error bars represent the SD. $^*p < 0.05$.

Supplementary Material). The result thus suggested that CD16 bind more efficiently to Herceptin than to control IgG1 and was in agreement with the results in **Figure 5B**.

We further examined the amount of IgG1 and Herceptin binding to CD16 on the cell surface of NK cells by staining using an anti-IgG antibody and FCM analysis. Herceptin was present on the NK cells and occupied CD16 sites (73.80 ± 3.24%), which were higher than that in the IgG1-treated group (51.60 ± 3.99%) at 15 days after culture (**Figure 5C**).

Our data suggested that Herceptin induced more CD16 occupation, as compared to IgG1, indicating that Herceptin has a higher effect on CD16 activation. Compared to IgG1, the interaction between Herceptin and CD16 might induce stronger CD16 activation, which may help activate a series of signaling

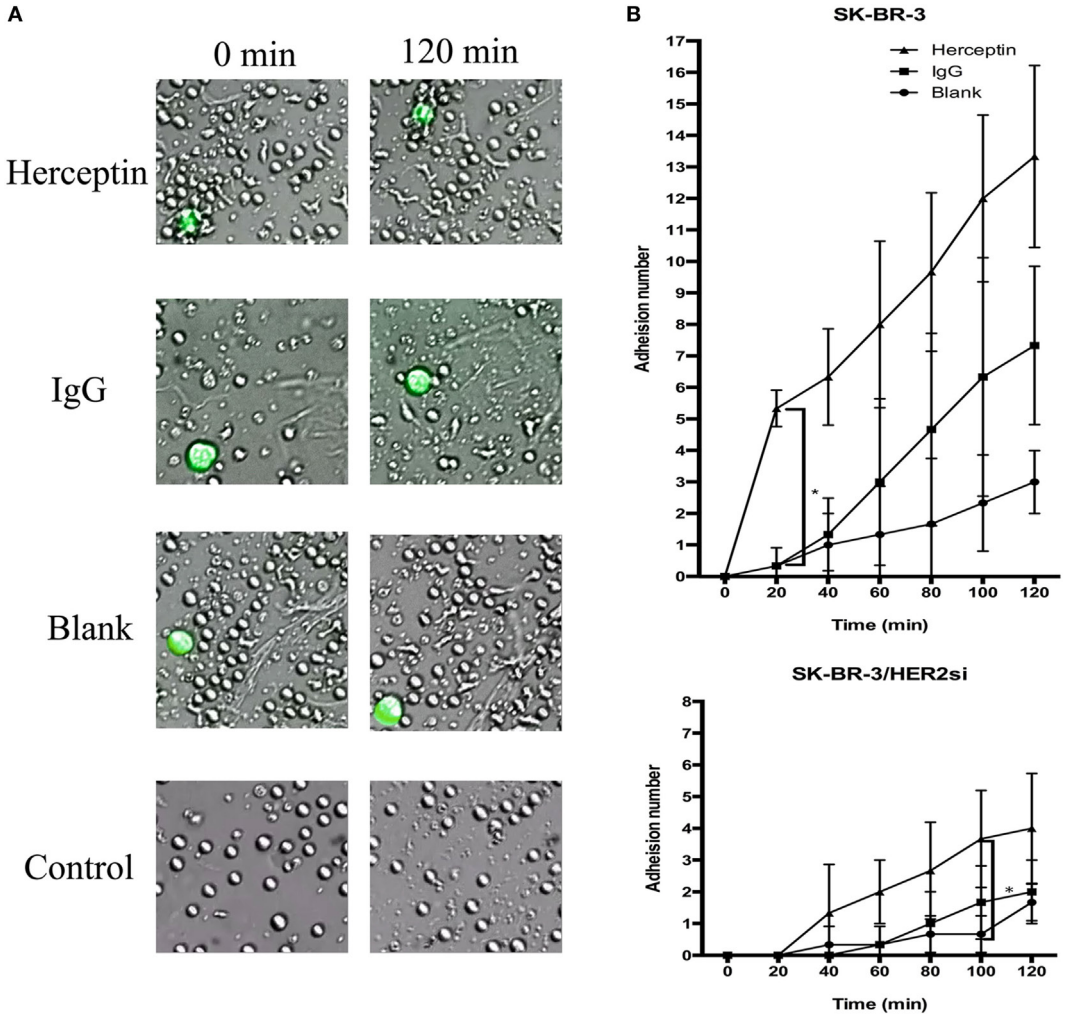


**FIGURE 3** | The migration and adhesion of expanded natural killer (NK) cells. The larger green cells in the photos are target cells and the smaller cells are NK cells. **(A)** Images of expanded NK cells cocultured with SK-BR-3 at 0 min (left row) and 120 min (right row). **(B)** Statistical analysis of the calculated number of adherent NK cells. The results are presented the means of three independent experiments and error bars represent the SD. *$p < 0.05$.

pathways downstream from CD16 to stimulate cell cytotoxicity and migration.

## The Clinical Efficacy of Herceptin-Treated NK Cells for a Patient with Breast Cancer Expressing HER2$^+$—A Pilot Trial

A 67-year-old female patient was diagnosed with HER2-expressing breast cancer 13 years prior. She underwent right radical mastectomy and partial left mastectomy followed by chemotherapy. She did not receive complete Herceptin treatment because of severe cardiotoxicity. She received a combination treatment, including chemotherapy, radiotherapy, endocrine therapy, and biotherapy during the previous 10 years. Six months prior to the Herceptin-treated NK cell treatment, the patient had a pulmonary metastasis based on B-mode ultrasound and computed tomography examination and then received Lapatinib-based chemotherapy treatment. Unfortunately, none of the therapy could stop the cancer progression. The shadows and nodules continued to grow. The Herceptin-NK cell treatment was approved by the ethical committee of Tianjin Medical University Cancer Hospital with written consent. All the procedures of NK cell expansion were operated in strictly controlled GMP lab and all reagents used in the procedure met the requirement of China Food and Drug Administration. No FCS was used for the preparation of adoptively transferred cells. Before the treatment, the biological safety and efficiency was guaranteed by a series QC steps of bacterial detection, toxin detection, immunotyping, and cytotoxicity examination. The patient underwent four cycles of Herceptin-treated NK cell treatment at intervals of 2 months. $5.9 \times 10^9$, $3.9 \times 10^9$, $8.1 \times 10^9$, and $6.5 \times 10^9$ Herceptin-treated NK cells were administered at each occasion. The treatment was well tolerated, with no treatment-related morbidity, life-threatening complications, or side effects. The diameter of the foci before the first treatment was approximately 36.0 mm and decreased to 12.0 mm after four

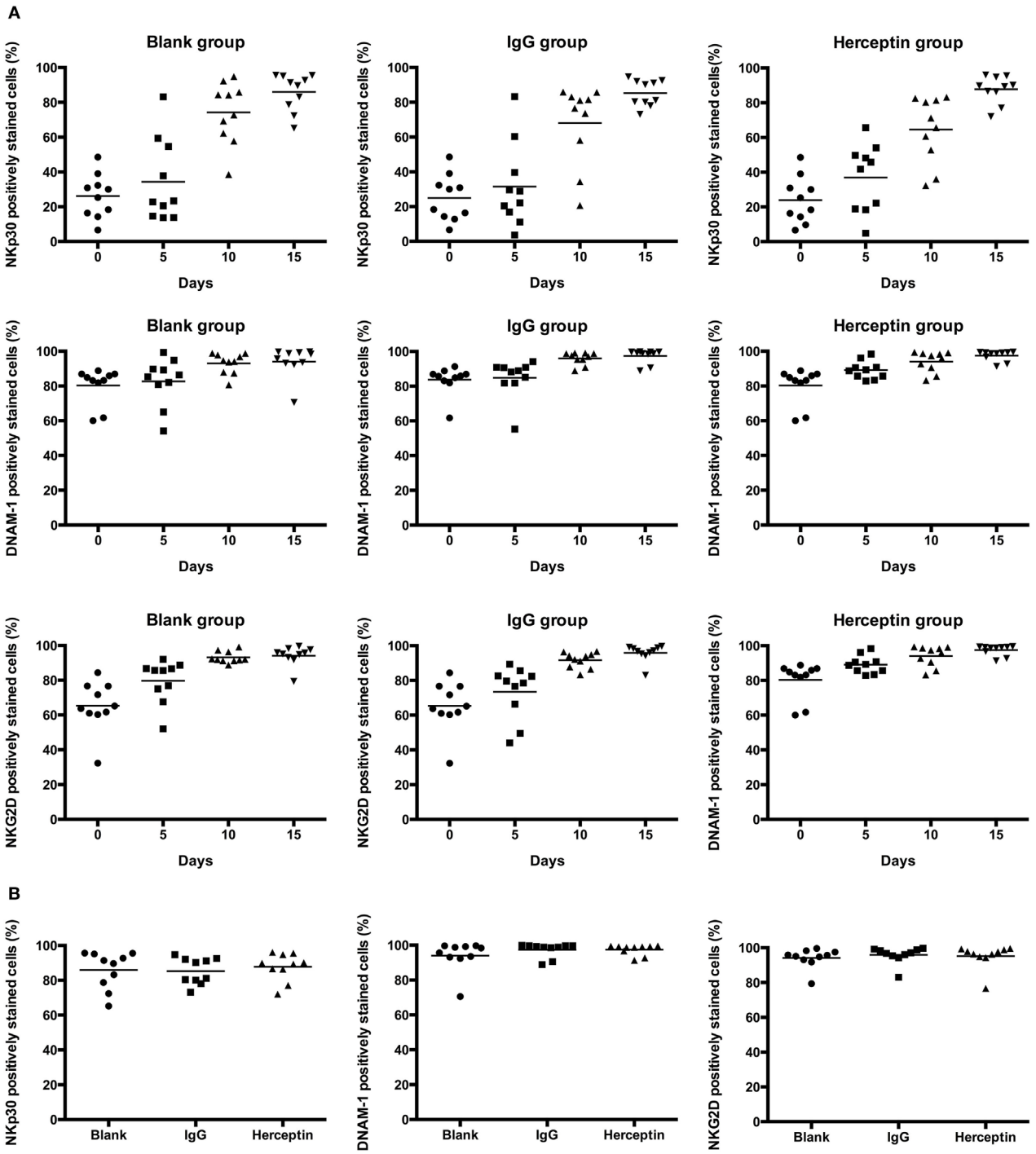


**FIGURE 4** | Expression of the cytotoxicity-associated activated receptors, including NKp30, NKG2D, and DNAM-1, in NK cell subsets among the final expansion products cultured in three different environments. Expression in electronically gated $CD3^{-}CD56^{+}$ NK cells was analyzed by FCM. **(A)** Changes in the expression of each receptor over 15 days. **(B)** The expression of each receptor on the final expanded NK cells among three groups is shown. The results are presented as the means of 10 patients and error bars represent the SD.

cycles of Herceptin-treated NK cell treatment (**Figure 6**), which was considered a partial response. This evidence supports the application of Herceptin-treated NK cells as a potential clinical treatment.

## DISCUSSION

The functional status of the host immune system affects the efficiency of chemotherapy and radiotherapy (25). ACT improves the

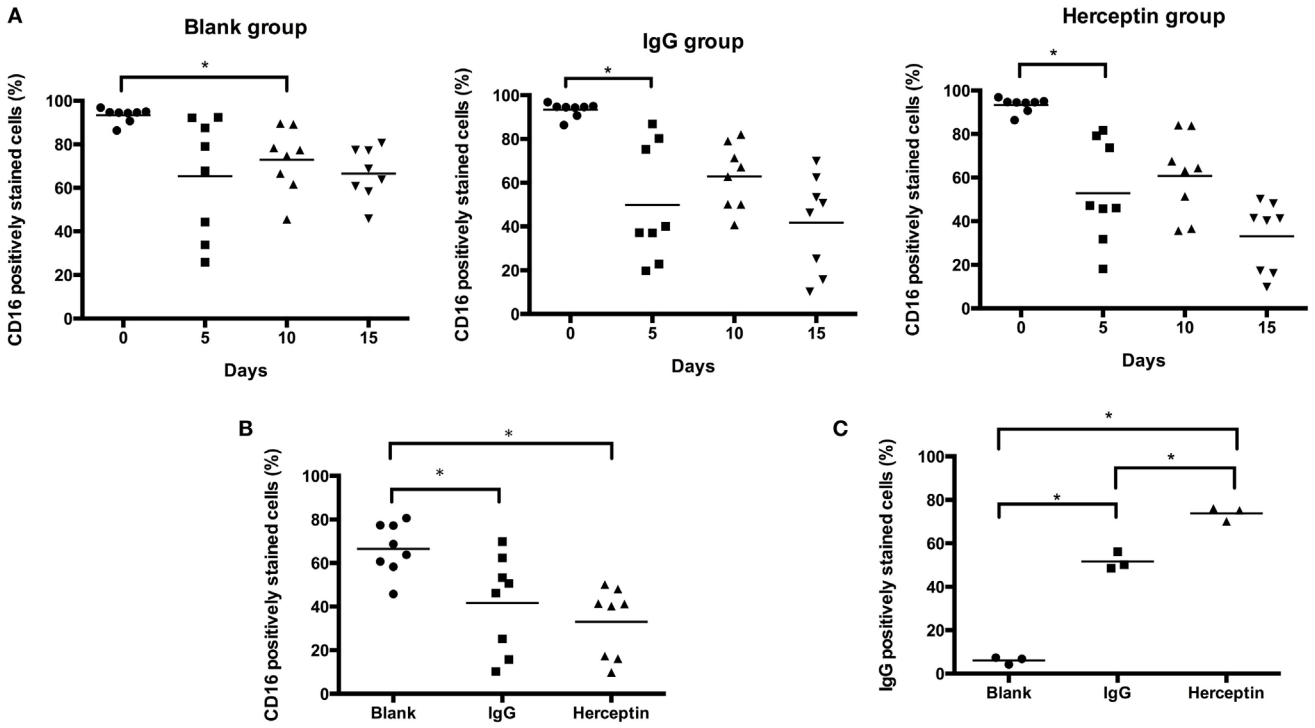


**FIGURE 5** | CD16 and Herceptin levels on expanded natural killer (NK) cells among the final expansion products cultured under three different conditions. Expression in gated $CD3^{-}CD56^{+}$ NK cells was analyzed by flow cytometry (FCM). **(A)** Changes in the expression of CD16 at 0, 5, 10, and 15 days. **(B)** Comparison of CD16 expression at 15 days between the final expanded NK cells in each of the three groups. **(C)** Amounts of Herceptin and IgG1 remaining on the NK cell surface at Day 15. We used a secondary antibody to detect Herceptin and IgG1 on the NK cells in the final products using FCM. The results shown in **(A,B)** are presented as the means of eight patients and the results shown in **(C)** are presented as the means of three patients. Error bars represent the SD. $^{*}p < 0.05$.

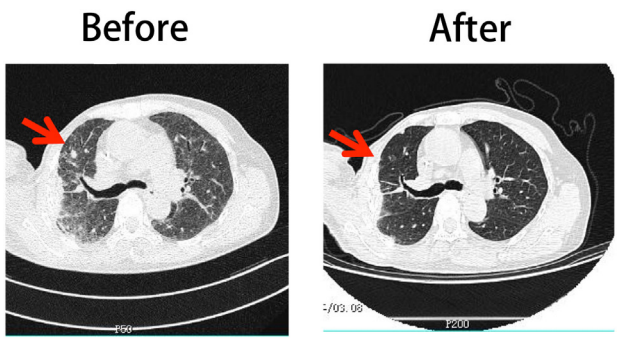


**FIGURE 6** | Comparison of the patient's CT scan images before and after Herceptin-treated natural killer (NK) cell therapy. The left column shows the image obtained before treatment and the right column shows the image obtained after four cycles of NK cell therapy.

host immune functions to efficiently treat cancer. Several types of immune cells have been used to enhance the immune activities, such as NK cells, cytokine-induced killer cells, lymphokine activated killer cells, γδT cells, αβT cells, cytotoxic T cells, dendritic cells, and chimeric antigen receptor (CAR)-redirected T cells. NK cells are characterized by direct and efficient main histocompatibility complex-unrestricted antitumor activity. This advantage makes NK cells a proper candidate for ACT. However, NK cell function is reported to be impaired in cancer patients (26). Therefore, we developed an *ex vivo* NK cell expansion approach to acquire fully functional NK cells for NK cell-based ACT.

Compared with CAR-redirected T cells, the main disadvantage of NK cells is that their cytotoxicity is not specific for target cells. The generation of CAR-modified T cells that specifically kill the targets involves genetic manipulation of donor lymphocytes, requires specialized facilities for generation and is expensive compared with NK cell expansion. CAR-modified NK cell are a new tool for the rapid development of adoptive immunotherapy for tumors that has been reported in recent years (27). CAR-modified NK cells have targeted cytotoxic activity, overcome the tumor immunosuppressive microenvironment and disrupt the host immune tolerance. However, CAR-NK cells do not expand in response to an applied stimulus that is in contrast to current CAR-T cells (28). Therefore, the CAR-driven expansion observed for CAR-T cells seems to be unlikely to occur in NK cells, limiting their long-term antitumor effects. Using the low-cost method reported in the present study, NK cells with specific targeting were easily amplified for use in ACT.

Herceptin has limited effectiveness when used alone (3). The percentage of HER2 expressing patients who responded to Herceptin was reported to reach merely 30% (29). On the other hand, the majority of patients who achieve an initial response to Herceptin-based regimens generally acquire resistance within

one year (30). Herceptin is also known to exert specific side effects on the heart when used in combination with chemotherapy (31). In the present study, using antibody-dependent cell-mediated cytotoxicity (ADCC) with Herceptin, NK cells had targeting ability. Therefore, the aforementioned limitations of Herceptin treatment were overcome.

Human NK cells have two distinct subsets identified by the cell surface density of CD56; approximately 90% are $CD56^{dim}$ cells and 10% are $CD56^{bright}$ cells (32). $CD56^{dim}$ NK cells exhibit a marked cytotoxicity potential, while $CD56^{bright}$ cells have a greater capacity to secrete cytokines (33). Peripheral blood $CD56^{bright}$ NK cells lack CD16 expression (34), and $CD56^{dim}$ NK cells have a higher level of CD16. Our results showed that cytokines amplified the number of $CD56^{bright}$ NK cells; however, Herceptin could expand the proportion of $CD56^{dim}$ NK cells. CD16 expression level was lower in the Herceptin-treated NK cells that have a higher level of CD16, indicating that more CD16 on NK cells was ligated and occupied by Herceptin in Herceptin group and Herceptin might induce NK cells to exert a high level of cytotoxicity.

NKp30, NKG2D, and DNAM-1 are important surface activated receptors involved in NK cell cytotoxicity. Almost all mature NK cells express NKp30 (35). NKG2D combined with specific ligands transmits signals required for cytotoxicity through DAP10 and DAP12 (36). DNAM-1 combined with the specific ligand would cause tighter adhesion between NK cells and target cells (37). In this experiment, we compared the expression level of the receptors in two different aspects. We compared the receptor expression level on Days 0, 5, 10, and 15 in each group. In all three groups, receptors expression levels were increased during 15-day culture with enhanced adhesion and cytotoxicity. We inferred that increased NK activity during culture period was due to the cytokines induced the increasing expression of activation receptors that is in accordance with previous study. It was reported that cytokines, such as IL-2 and IL-15, could increase the expression level of NK cells surface activated receptors during *ex vivo* expression (38). Moreover, in a clinic trial, the IL-2 treatment resulted in expansion of NK cells in the blood along with increased NK cell expression of NKp30 (39). On the other hand, when compared receptor expression levels among three groups on Day 15, we found that there was no significant difference among three groups. But the expansion, adhesion and cytotoxicity ability among three groups on Day 15, we found significant increase in Herceptin group and IgG1 group than control group. Therefore, we speculated that increased NK activity on Day 15 among three groups is not related to the increasing expression level of activation receptors and Herceptin did not significantly increase the number of activation receptors in relation to the control.

Most clinical studies have used *ex vivo* activated NK cells instead of *in vivo* activated NK cells (40). The *ex vivo* activated NK cells express low or undetectable levels of CD16; therefore, they are not a choice for ADCC (40). However, the interaction between Herceptin and CD16 induced longer and stronger CD16 occupation than IgG1 in the present study, and Herceptin-treated NK cells were only effective against $HER2^{+}$ breast cancer cells. The hypothesis that ligation of CD16 by Herceptin induces NK cell hyper-responsiveness is strengthened by the finding that IgG1 had no effect on the cytotoxicity toward target cells. The Herceptin-occupied CD16 sites remaining on the NK cells maintained the activity of NK cells against target cells. The Fc region of Herceptin was ligated with CD16 on NK cells and Fab regions related to HER2 on target cells, while the Fab regions of IgG1 did not recognize HER2 on the target cells to mediate ADCC. We hypothesized that other monoantibodies, such as Cetuximab, may have the same effect on colorectal cancer cells.

CD16, occupied by antibodies, is coupled to ITAM-containing signaling adaptors (41) and then activates a downstream signaling pathway. The important relevant proteins in the downstream pathways include PI3-K, VAV1, and RAC (42). RAC induces granule secretion and cytotoxicity (43). PI3-K correlates with cell proliferation (44). VAV1 is an important factor involved in cell transformation and migration (45). These proteins were associated with proliferation, migration, and cytotoxicity. More research will be performed to study the mechanism underlying the correlations between these signaling pathways and CD16 to further explain our hypotheses. Notably, CD16 internalization may affect the decrease in CD16 accessibility. However, in this article, we mainly considered the interaction between Herceptin and accessible CD16 on NK cells. We will evaluate the level of CD16 internalization and how the internalized CD16-Herceptin complex transduces or regulates signaling in NK cells.

In previous studies, Herceptin was used as a target therapy to directly inhibit the growth of HER2-positive cancers by blocking the transduction of HER2-mediated growth signal in cancer cells. Few reports were focused on the antitumor effects of Herceptin-mediated immunotherapy. Until now we have not find similar *in vitro* method to observe the effect of Herceptin on NK cells. Though ADCC effect was reported to induce tumor inhibition both *in vitro* and *in vivo*, no relationship between Herceptin and lymphocytes activation has been identified except ref.20. It is a clinic dilemma that high-dose Herceptin could induce resistance with high probability but low-dose Herceptin could not get the ideal clinic benefits. In this study, we proposed a novel Herceptin-mediated NK activation method to amplify NK cells with considerably lower dose of Herceptin pretreatment *in vitro* which displayed significantly enhanced cytotoxicity against HER2-positive breast cancer cells and proposed an alternative solution for cancer patients resistant to conventional Herceptin treatment. Furthermore, different from previous study, we observed that Herceptin could increase NK cells amplification and activation at the expansion stage rather than effect stage, which indicated that an alternative mechanism except ADCC effect has involved in Herceptin-mediated NK activation. During expansion stage, CD16 are occupied which was bound by Herceptin to activate downstream signaling pathways, such as RAC, VAV, and ERK. CD16-mediated activation of the downstream signaling pathways is related to enhanced adhesion and cytotoxicity of NK cells at the expansion stage while the ADCC effect further increases the cytotoxicity of NK cells at the effect stage when target cells expressed specific HER2 protein on the surface. Therefore, this research proposed an alternative Herceptin-mediated immunotherapy for HER2-positive cancer and optimized the method of NK cell expansion for future clinical application.

Since Herceptin's irreversible cardiotoxicity, this study found a new alternative way to use Herceptin in HER2-positive patient who has poor cardiac function. For patients with Herceptin resistance, using Herceptin-induced NK cell therapy might provide an alternative treatment to overcome the problem of drug resistance. In this study, we specifically described and analyzed the Herceptin's effect on NK cell expansion, activation, adhesion, and cytotoxicity and confirmed that Herceptin-mediated NK activation is feasible and repeatable among different cancer patients no matter the disparity of age, gender, clinical stage, and response to conventional therapy. Therefore, we developed an easy, stable, accurate method with high repeatability in this study and could be feasible in clinic application. Since less amount of Herceptin was used in NK cells amplification, the culture cost of Herceptin-NK cells is comparably lower than standard Herceptin therapy and would be an economic way in breast cancer treatment. However, since no animal models were established in this study, it is difficult to evaluate the ability of adoptively transferred cells to traffic to tumor sites and to enter into cancer tissues. We would like to improve our study in the future.

Herceptin and the isotype antibody belong to the same allotype. One interesting finding from our study is that Herceptin has a higher ability to bind to CD16 on NK cells and exerts more potent effects on NK cell proliferation compared to IgG1 treatment. Glycans have many structural and functional roles in antibodies, and the effector function can be altered by different glycoforms in antibodies (46). It has been shown that the immunogenicity, pharmacokinetics, distribution, solubility, and stability of the antibodies could be affected by glycosylation in the Fc fragment (47). Glycosylation on the IgG Fc fragment mediates the enhancement of the functions of Fc receptors (48). One of the important glycan patterns in the Fc fragment is *N*-acetylglucosamine. Studies have shown that the Fc fragment with the low level of *N*-acetylglucosamine stimulates a higher level of activity compared to that with bisected *N*-acetylglucosamine (49). Another glycan pattern is fucosylation. It was reported that a low level of Fc fragment core-fucosylation enhances several biological functions of the Fc fragment (50).

IgG1 and Herceptin have the same N-linked glycosylation site at the position of Asn297 in the Fc region of each heavy chain (51). It has been reported that the cell culture condition, fermentation method, and other factors can alter the glycosylation of proteins (52). Herceptin was expressed in CHO cells and IgG1 used in this study was purified from human plasma. Since control IgG1 is of different origin, as compared to Herceptin, they might bind and cross-link Fc receptors with a significantly lower effectiveness. It is possible that the glycosylation status between Herceptin and IgG1 differs. We presumed that the differences in glycosylation pattern on the Fc fragment might contribute to differences in the binding capacity to CD16 in NK cells by Herceptin and IgG1. Therefore, Herceptin and IgG1 have different effects on NK cells. As shown in **Figure 5C**, NK cells are largely Herceptin positive at the end of the incubation period, thus suggesting that, maybe, half-life of control IgG1 is also significantly shorter. In addition, the IgG1 used for this study was purchased as a lyophilized protein. IgG1 may become multimeric aggregated and lose functions under the reconstituted condition (53). Therefore, reconstituted IgG1 might not be as active as Herceptin for stimulating CD16 in NK cells. In this experiment, we inferred that Herceptin occupying CD16 (Fc receptor) on NK cells increase their effects. In the further experiments, we could focus on CD16 and Fc region to evaluate the CD16 functions on NK cells.

For patients, Herceptin is a heavy economic burden. Combining Herceptin and NK cells together is an economic way in breast cancer treatment. Even though considering the cost of culture medium, the total expense is very low compared to directly use Herceptin as targeted medicine administrated passively. However, we just observed the effect of Herceptin on NK cells and we will ran more animal experiments to evaluate the ability of Herceptin-treated NK cells transferring to tumor sites *in vitro* next step.

## CONCLUSION

Our results demonstrated that Herceptin promoted NK cell proliferation, cytotoxicity, and migration by occupying CD16 to enhance the antitumor effects of NK cells on $HER2^+$ breast cancer cells. We initially developed an easy, stable, accurate method with high repeatability and could be feasible in clinic application. This transitional research uses Herceptin to optimize the original way of NK cell expansion. The clinical efficacy of Herceptin-treated NK cells for a patient with $HER2^+$ and Herceptin-intolerant breast cancer was identified in a pilot case. Therefore, the use of Herceptin-treated NK cells might serve as a promising alternative treatment for patients with $HER2^+$ and Herceptin-intolerant breast cancer.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ethical standards of the Institutional Review Committee on Human Research of the Tianjin Medical University Cancer Institute and Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Committee on Human Research of the Tianjin Medical University Cancer Institute and Hospital.

## AUTHOR CONTRIBUTIONS

Conception and design; drafting of the manuscript: XT, JY, and XR. Execution of experiments: XT, LW, WY, and NZ. Acquisition of data: XT, WY, and LW. Analysis and interpretation of data: XT, FW, JY, and XR. Pilot trial: YH and XZ. Obtained funding: XZ. Critical revision of the manuscript; final approval of the version to be published, study supervision; and agreement to be accountable for all aspects of the work: JY and XR.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01426/full#supplementary-material.

**VIDEO S1** | Herceptin-treated NK cells co-cultured with SK-BR-3 cells.

**VIDEO S2** | NK cells in Blank group co-cultured with SK-BR-3 cells.

**VIDEO S3** | IgG1-treated NK cells co-cultured with SK-BR-3 cells.

**VIDEO S4** | Herceptin treated NK cells cultured alone.

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# The State of Cellular Adoptive Immunotherapy for Neuroblastoma and Other Pediatric Solid Tumors

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Research on adult cancer immunotherapy is proceeding at a rapid pace resulting in an impressive success rate exemplified by a few high profile cases. However, this momentum is not readily extended to pediatric immunotherapy, and it is not for lack of trying. Though reasons for the slower advance are not apparent, some issues can be raised. Pediatric cancer patients represent a distinct demographic group whose immune system is inherently different from that of mature adults. Treating pediatric patients with immunotherapy designed for adults may not yield objective clinical responses. Here, we will present an update on adoptive T-cell and natural killer-cell therapies for neuroblastoma and other childhood solid tumors. Additionally, we will delineate key differences between human fetal/neonatal and adult immune systems. We hope this will generate interests leading to the discussion of potential future directions for improving adoptive cancer immunotherapy for children.

**Keywords: pediatric solid tumors, recurrent/refractory/relapsed neuroblastoma, adoptive T-cell therapy, immune cell-based therapy, natural killer cells, *Cbx3*/HP1γ, CD4$^+$ regulatory T cells, effector CD8$^+$ T cells**

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## INTRODUCTION

In the past few decades, pivotal studies have yielded invaluable information on pediatric oncology leading to the formulation of standard therapies still being performed today (1–9). However, it is now evident that the majority of resistant, metastatic, recurrent/refractory tumors are non-responsive to conventional therapies (10–20). In addition, current approaches often rely on non-specific, cytotoxic chemotherapy and/or radiotherapy that result in long lasting, debilitating toxicities, and in some instance morbidity (21–26). Therefore, there is a need to explore new avenues to eradicate pediatric cancers.

Two seminal reports, published by the surgeon and cancer researcher William Bradley Coley in the late 19th century, show sarcoma tumor regression in patients repeatedly immunized with live or killed streptococcus bacteria (27, 28). His observations suggest an active function for the immune system to control tumor growth, thus laying the foundation for modern cancer immunotherapy. Today, harnessing the immune system to control cancer is proven effective and garnering momentum. Currently, immunotherapy is largely classified into two functional treatment groups: (1) those that amplify/reactivate host existing innate and adaptive tumor immunity including check point inhibitors, dendritic cell (DC) vaccines and cytokines; (2) those that involve the adoptive transfer of genetically manipulated immune cells to target tumor cells *in vivo* such as chimeric antigen receptor (CAR) T cells, genetically enhanced effector T cells and natural killer (NK) cells. We will focus primarily on T- and NK-cell adoptive immunotherapy in this perspective.

## ADOPTIVE T-CELL THERAPY (ACT)

Adoptive T-cell therapy represents an attractive viable option for the control of solid tumor growth for the following reasons: T cells can systemically home to tumor sites through the entire body and can cross the blood–brain barrier (BBB). By contrast, antibodies such as checkpoint inhibitors cannot effectively cross the BBB and are not consistently or adequately distributed deep inside solid tumors. To date, ACT with CAR T cells is the prevalent type of immunotherapy to treat solid tumors.

### Neuroblastoma (NB)

Neuroblastoma is the most common extracranial solid tumor of childhood and the third most common cause of pediatric cancer death (29–32). Despite conventional multimodal therapy, patients with high-risk NB have a poor prognosis due to high relapse rate (33). Since 1999, tremendous efforts and funds have been dedicated to discovering and testing various forms of immunotherapy to control refractory/recurrent NB (**Table 1**). To date the most studied NB-associated antigen (NBAA) identified is the ganglioside GD2 expressed on a subset of NB tumor cells (34). This discovery heralds in the era of GD2-based immunotherapy to treat human NB. The current standard of care for refractory/recurrent NB in human is anti-GD2 therapy, which recognizes and binds GD2 (34–39). However, because GD2 is also expressed on pain fibers, this therapy has side effects that include severe pain requiring continuous opiate infusions (34). Because of the paucity of identified NBAAs, all CAR clinical trials are GD2-based or variations thereof (38, 40–45) (**Table 1**). It is still early to know whether these GD2-dependent CAR trials will yield objective clinical responses.

The antigen 4Ig-B7-H3 (CD276), a member of the B7 family of immune regulators such as CD80, CD86, PD-L1, PD-L2 and ICOSL, is expressed in a subset of NB tumors (46–49). In mouse and human, 4Ig-B7-H3 is expressed on many normal tissues such as spleen, lymph nodes, thymus and fetal liver as well as other tumors. However, its expression is induced on DCs and macrophages by inflammatory cytokines. 4Ig-B7-H3 binds to a yet to be identified cognate receptor induced on activated T and NK cells, and blockade of this interaction results in reduced interferon γ production and loss of cytotoxic activity of these cells. In mice, deficiency or blockade of B7-H3 leads to improved antitumor immunity suggesting that B7-H3 checkpoint may serve as a novel target for immunotherapy against cancers (50). Indeed, a mouse mAb anti-B7-H3 conjugated to iodine 131 (131I-burtomab) has been designed to bind and directly kill NB cells. 131I-burtomab is recently designated a breakthrough drug to treat metastatic NB by the Food and Drug Administration and a clinical trial has been filed (NCT03275402). The immune status of NB tumors treated with 131I-burtomab has not been published. It would be interesting

**TABLE 1** | Present and past adoptive immunotherapy trials for refractory/recurrent/relapsed neuroblastoma.

| Approach | Status | NCT# | Sponsor | Year |
|---|---|---|---|---|
| Adoptive therapy of donor lymphocytes | Completed/no results | 00003887 | Fred Hutchinson Cancer Research Center | 1999–2011 |
| Adoptive therapy of autologous lymphocytes | Completed/no results | 00006480 | Fred Hutchinson Cancer Research Center | 2000–2010 |
| Adoptive therapy of autologous lymphocytes with EBV-lymphoblastoid vaccine | Unknown/no results | 00101309 | Milton S. Hershey Medical Center | 2005–2007 |
| Adoptive therapy of donor-derived tri-virus specific cytotoxic T cells | Completed/no results | 01460901 | Children's Mercy Hospital Kansas City | 2011–2015 |
| Adoptive therapy of activated autologous chimeric GD2-iCas9 CAR T cells | Ongoing/no results | 01822652 | Baylor College of Medicine | 2013–present |
| Adoptive therapy of CAR T cells expressing anti-GD2 | Completed/no results | 02107963 | National Cancer Institute | 2014–2017 |
| Adoptive therapy of CAR T cells expressing anti-CD171 | Recruiting | 02311621 | Seattle children's Hospital | 2014–present |
| Adoptive therapy of activated bispecific GD2 CAR T cells | Recruiting | 02173093 | Barbara Ann Karmanos Cancer Institute | 2014–present |
| Infusion of haploidentical NK cells | Terminated/no results | 00698009 | MD Anderson Cancer Center | 2008–2012 |
| Infusion of allogeneic NK cells, humanized anti-GD2, and standard chemotherapy | Ongoing/no results | 00877110 | Memorial Sloan Kettering Cancer Center | 2009–present |
| Infusion of allogeneic NK cells, humanized anti-GD2, and standard chemotherapy | Ongoing/no results | 01576692 | St. Jude Children's Research Hospital | 2012–present |
| Infusion of *in vitro*-activated/expanded NK cells | Completed/no results | 01875601 | National Cancer Institute (NCI) | 2013–2016 |
| Infusion of allogeneic NK cells from an acceptable parent | Recruiting | 01857934 | St. Jude Children's Research Hospital | 2013–present |
| Infusion of donor NK cells following haploidentical hematopoietic cell transplant | Recruiting | 02100891 | Monica Thakar Medical College of Wisconsin | 2014–present |
| Infusion of $CD133^+$ autologous stem cells followed by haploidentical NK cells | Recruiting | 02130869 | St. Jude Children's Research Hospital | 2014–present |
| Infusion of autologous expanded NK cells and anti-GD2 | Not yet recruiting | 02573896 | New Approaches to Neuroblastoma Therapy Consortium | 2015–present |
| Infusion of allogeneic NK cells and humanized anti-GD2 | Recruiting | 02650648 | Memorial Sloan Kettering Cancer Center | 2016–present |
| Infusion of *in vitro*-activated/expanded haploidentical NK cells and anti-GD2-IL2 | Not yet recruiting | 03209869 | University of Wisconsin, Madison | 2017 |

*EBV, Epstein–Barr virus; CAR, chimeric antigen receptor; GD2, glanglioside GD2; NK, natural killer; iCas9, inducible caspase 9.*

to know whether the antitumor activity of burtomab results from direct killing of tumor cells or activated DCs thus reactivating existing anti-NB immunity. Although no published data are available to show the feasibility of B7-H3 CAR T cells for the treatment of human NB, two patents have been filed for such an invention (US Application No. 14/779,586; US Application No. 61/805,001; PCT Application No. PCT/US2014/031543 and PCT Application No. PCT/US2016/050887; US Application No. 62/216,447). It remains to be determined whether B7-H3 CAR T cells can inhibit NB tumor growth and whether the potential antitumor activity of B7-H3 CAR T cells is due to killing of intratumoral suppressive DCs/macrophages or tumor cells or both.

The anaplastic lymphoma kinase (ALK or CD246) is a receptor protein tyrosine kinase predominantly expressed in the central nervous system (CNS) and peripheral nervous system in mouse and human suggesting its role in normal brain development and function (51). A series of studies show that *Alk* is frequently mutated (mainly $ALK^{R1275Q}$ and $ALK^{F1174L}$) and duplicated in high-risk NB tumors (52–58). The $ALK^{R1275Q}$ mutation results in a constitutively active kinase suggesting a role for ALK in NB development. However, mice harboring human $ALK^{R1275Q}$ or $ALK^{F1174L}$ alone do not develop aggressive NB irrespective of genetic background (53, 57). In the contrary, animals having both MYCN amplification and $ALK^{R1275Q}$ or $ALK^{F1174L}$ mutation succumb to NB at a higher rate (53, 57). These findings suggest that mutations in *Alk* are necessary but not sufficient to drive aggressive NB development. Because ALK is a cell surface kinase, developing CAR T cells targeting ALK has been suggested. Indeed, in a xenogeneic NSG mouse model for NB, human ALK CAR T cells can eradicate ALK-positive tumors; both tumor antigen and receptor density governs the efficacy of these CAR T cells (59). Clinical trials have not been initiated.

Although CAR T-cell therapy is being propelled to the forefront, problems exist that need further investigation. Production of CAR T cells requires the identification of tumor-associated antigen (TAA), generation of an antibody or T-cell receptor (TCR) capable of recognizing the TAA, cloning of genes encoding the antibody or TCR to be introduced into isolated tumor-infiltrating lymphocytes or haploidentical T cells. For most pediatric solid tumors, the identity of TAAs is still unknown and neoantigen load is low thus limiting the use of CAR T cells for this group. In patients with solid tumors for which TAAs have been identified, the use of CAR T cells has proven less effective than in patients with fluid tumors. Recent data are showing a previously unpredicted phenomenon observed in patients treated with CAR T cells: the emergence of tumor cells that have lost expression of the TAA targeted by CAR T cells, undoubtedly due to negative selection imposed by CAR therapy (60–62). New evidence demonstrates that CAR T cells once in the tumor microenvironment (TME) may suffer from exhaustion caused by suppressor cells including myeloid-derived suppressor cells or $CD4^+$ regulatory T (Treg) cells present in the TME (63–65). Perhaps a more dangerous issue arisen is the development of cytokine release syndrome (CRS) (66, 67) and neurologic toxicity observed in patients undergoing CAR therapy (68).

To circumvent problems posed by CAR T cells, we propose that perhaps the most effective strategy to control solid tumor growth is one that does not require identifying TAAs and corresponding tumor-reactive $CD8^+$ T cells, can enhance effector activity of $CD8^+$ T cells, and can simultaneously eliminate immune suppression within the TME. Our recent studies suggest that such an approach is attainable. We show that by targeting the histone reader *Cbx3*/HP1γ, we can enhance the tumor killing capacity of effector $CD8^+$ T cells (69, 70). As a result, adoptive transfer of *Cbx3*/HP1γ-deficient $CD8^+$ effector T cells alone into wild type (wt) tumor-bearing mice greatly reduces NB growth. Within the NB TME of *Cbx3*/HP1γ-deficient mice or wt mice treated with *Cbx3*/HP1γ-deficient $CD8^+$ T cells, we detect an increase of *Klrk1*/NKG2D$^+$ infiltrating $CD8^+$ effector T cells and a decrease in $CD4^+$ Treg cells. PD-1 and *Pdl1* expression is not altered in $CD8^+$ T cells or NB tumors, respectively. Moreover, *Cbx3*/HP1γ-deficient $CD8^+$ T cells appear to have overcome exhaustion. These findings suggest that targeting *Cbx3*/HP1γ can represent an alternative and rational therapeutic approach to control NB as well as other solid tumors.

## Other Pediatric Solid Tumors (CNS Tumors, Sarcomas, and Nasopharyngeal Sarcomas)

As for NB, there is a dearth of identified TAAs available for the formulation of CAR therapy to treat most pediatric solid tumors (71–73). Tumor immunity against pediatric solid tumors is not completely understood. The human epidermal growth factor receptor 2 (HER2) is expressed on pediatric as well as adult glioblastoma, glioma, and medulloblastoma tumors, and overexpression of HER2 has been associated with poorer prognosis. In animal models, HER2 CAR T cells efficiently cause the regression of CNS tumors. These preclinical studies have paved the way for a few HER2-based CAR clinical trials (74–77) (**Table 2**); results of these trials are not yet available. It would be crucial to determine whether HER2-targeted CAR therapy will induce the emergence of HER2-negative tumors as has been shown in animal models and in patients receiving CD19 targeted CAR therapy (60, 61) or will cause CRS as in adults (67).

Pediatric nasopharyngeal carcinoma patients with local-regional bulky and metastatic disease have a poor prognosis (78). It is a rare tumor that is almost always associated with Epstein–Barr virus (EBV) (78, 79), and EBV-specific cytotoxic T lymphocytes (CTLs) can be found in individuals infected with this ubiquitous virus. These findings lead to the design of EBV-based T-cell therapy. In adults, ACT with EBV-specific CTLs is more effective in patients with low disease burden while results for pediatric trials are not available (80–83) (**Table 2**).

Prognosis for pediatric patients with recurrent/refractory sarcomas is poor, the survival rate ranges from 10 to 30% (73). For this group of children, few immunotherapy clinical trials are being tested, and past trials using autologous T cells (NCT00001566 and NCT 00001564) have not yielded much information to advance the field (**Table 2**).

**TABLE 2** | Present and past adoptive immunotherapy for pediatric central nervous system tumors, sarcomas, and nasopharyngeal carcinomas.

| Approach | Status | NCT# | Sponsor | Year |
|---|---|---|---|---|
| Adoptive therapy with autologous T cells following tumor vaccine | Completed (Ref) | 00001566 | National Cancer Institute (NCI) | 1999–2012 |
| Adoptive therapy with autologous T cells followed by DC vaccine | Completed/no results | 00001564 | National Cancer Institute (NCI) | 1999–2014 |
| Adoptive therapy with allogeneic EBV-specific T cells | Recruiting | 00002663 | Atara Biotherapeutics | 1999–present |
| Adoptive therapy with autologous EBV-specific CTLs | Completed/no results | 00516087 | Baylor College of Medicine | 2007–2017 |
| Adoptive therapy with EBV-specific CTLs expressing HER2 CAR | Ongoing | 00889954 | Baylor College of Medicine | 2009–present |
| Adoptive therapy with HER2/CD28 CAR T cells | Recruiting | 00902044 | Baylor College of Medicine | 2009–present |
| Adoptive therapy with autologous EBV-specific CTLs | Completed/no results | 00953420 | Baylor College of Medicine | 2009–2017 |
| Adoptive therapy with CMV-specific CTLs expressing HER2 CAR | Ongoing | 01109095 | Baylor College of Medicine | 2010–present |
| Adoptive therapy with haploidentical EBV-specific CTLs | Ongoing | 01447056 | Baylor College of Medicine | 2011–present |
| Adoptive therapy with CD22 CAR T cells | Recruiting | 02315612 | National Cancer Institute (NCI) | 2014–present |
| Adoptive therapy with glycan 3-specific autologous CAR T cells | Not yet recruiting | 02932956 | Baylor College of Medicine | 2016–present |
| Adoptive therapy with NY-ESO-1 TCR transduced PBMC and NY-ESO-1 DC vaccine | Recruiting | 02775292 | Jonsson Comprehensive Cancer Center | 2016–present |
| Infusion of autologous NK cells following peripheral blood stem cell transplant | Ongoing/no results | 01287104 | National Cancer Institute (NCI) | 2011–present |
| Infusion of *in vitro*-activated/expanded NK cells | Completed/no results | 01875601 | National Cancer Institute (NCI) | 2013–2016 |
| Infusion of *in vitro*-activated/expanded NK cells | Completed/no results | 01875601 | National Cancer Institute (NCI) | 2013–2016 |
| Infusion of CD133+ autologous stem cells followed by haploidentical NK cells | Recruiting | 02130869 | St. Jude Children's Research Hospital | 2014–present |
| Infusion of donor NK cells following haploidentical hematopoietic cell transplant | Recruiting | 02100891 | Monica Thakar Medical College of Wisconsin | 2014–2016 |

*EBV, Epstein–Barr virus; HER2, human epidermal growth factor receptor 2; CMV, cytomegalovirus.*

Some studies have shown that the cancer-testes antigen NY-ESO-1 is expressed on a subset of pediatric tumors, which lead to a trial using T cells engineered to express NY-ESO-1-specific TCR (NCT02775292) (84) (**Table 2**). HER2 CAR therapy has also been proposed to treat sarcomas (85). CAR T cells targeting glypican-3, a proteoglycan expressed on a small number of solid tumors (86), are being tested in clinical trials to treat pediatric solid tumors (87) (NCT02932956). Data are not yet available for these trials.

The lack of available immunotherapy for pediatric solid tumors may be due to the paucity of identified TAAs, and few basic studies designed to understand tumor immunity during development from infancy to young adulthood in either mouse or human. As a result, most of these trials are based on those that have been designed to treat adult solid tumors.

## ADOPTIVE NK- AND NATURAL KILLER T (NKT)-CELL THERAPY

Natural killer cells and NKT cells have been shown to play crucial roles in antitumor immunity by directly killing tumor cells or indirectly through antibody-dependent cellular cytotoxicity. Based on results from preclinical studies (88–90), several clinical trials have been initiated to test the ability of *in vitro*-activated/expanded or engineered NK cells and NKT cells to control pediatric solid tumors including NB (91, 92) (**Tables 1** and **2**). Results of these trials are not yet available to indicate whether NK- or NKT-cell therapy would be a viable option.

Nonetheless, clinical data have demonstrated that despite the large number of NK cells infused, the antitumor effects of these cells have been modest in adults. NK and NKT cells express a number of inhibitory receptors that bind to MHC class I and other molecules. Additionally, NK and NKT cells are sensitive to various inhibitory molecules within the TME (93). Moreover, we show that the frequency of NK and NKT cells in NB tumors is low, and no differences are detected in tumors from wt or *Cbx3*/HP1γ-deficient mice yet NB tumor growth is greatly abrogated in *Cbx3*/HP1γ-deficient mice (70). Our findings imply that NK or NKT cells may not play an important role in controlling NB tumor growth.

## DISCUSSION AND FUTURE DIRECTIONS

In the past 40–50 years, pediatric oncologists have made significant, basic advances toward our understanding of molecular pathways driving the development of tumors in children. This achievement hinges on the belief that cancer pediatric patients represent a distinct demographic group, and the biology of their tumors is fundamentally different from that of adults.

Adult cancer immunotherapy is experiencing a renaissance while that of children is still at its infancy. The momentum that drives adult cancer immunotherapy is built upon decades of basic research designed to understand how an adult immune system responses to tumors developing within an adult host. Thus, there is a need to recognize that pediatric cancer patients represent a distinct demographic group whose immune system is fundamentally different than that of mature adults.

Fetal and adult T cells are distinct populations that arise from different hematopoietic stem cell populations present at different developmental stages (94), and human NK cells follow similar developmental evolution (95, 96). Notably, fetal $CD4^+$ T cells are poised to differentiate into $CD4^+$ Treg cells upon allogeneic stimulation. Indeed, human fetus and cord blood (CB) contains an abundance of phenotypically naïve $CD25^+CD4^+$ Treg cells, but functionally mature, capable of suppressing T- and NK-cell proliferation and function (97–101). Similar suppressive mechanism is observed in the mouse fetus (102). Thus, T- and NK-cell lineages in the developing human or mouse are biased toward immune tolerance mediated by active suppression of early immunity; in some instances, this suppression persists at least until early adulthood (101). The tolerogenic tendency of fetal/neonatal immune system can be attributed to marked differences in response to alloantigens between human fetal and adult DCs. Fetal DCs strongly promote Treg-cell induction and inhibit T-cell tumor-necrosis factor-α production when cultured with alloantigens (103). This may explain why CAR T cells once in the NB TME often suffer from exhaustion. In addition to functional disparities, neonatal and adult immune systems differ quantitatively. Overall, there is a greater number of circulating $CD4^+$ T cells and a lower number of $CD8^+$ T cells in neonates compared to adults (104). Consequently, the ratio of CD4:CD8 is higher in neonates than adults. Together these results suggest that for some children, the persistence of fetal immune suppression, mediated by fetal $CD4^+$ Treg cells, and the lower number of $CD8^+$ T cells may render their immune system incapable of surveilling and eradicating tumor. Therefore in the future, it might be essential to study the effects of persistent fetal immune suppression on tumor development and growth. If the persistence of fetal immune suppression does influence antitumor immunity in pediatric patients, it would be crucial to determine the developmental age at which intervention can be mounted to prevent tumorigenesis and growth without breaking tolerance. Clinically, it might be important to collect data on the immune status of children bearing solid tumors in addition to dissecting their tumor immune environment. Results from these studies might help direct the design of T- and NK-cell therapies that can circumvent suppression and prevent exhaustion induction.

In adult tumors, mutation load appears to correlate with tumor immunity. However, the number of somatic mutations in pediatric solid tumors is low. In the future, it would be necessary to determine mechanisms controlling tumor immunity independent of somatic mutation loads in pediatric patients.

For this demographic group, perhaps the most effective strategy to control solid tumor growth is one that does not require identifying TAAs or neoantigens and their corresponding reactive $CD8^+$ T cells, can enhance effector activity of $CD8^+$ T cells, and can simultaneously eliminate immune suppression within the TME.

We believe these are crucial issues that need to be addressed in order to move the field of pediatric adoptive cancer immunotherapy to the fore. Until there is a will to allow for the funding of such studies and those that are outside traditional belief, children with cancers will continue to be treated as adults, and may not benefit from the cancer immunotherapy renaissance.

## AUTHOR CONTRIBUTIONS

TPL and THT conceived and cowrote this manuscript.

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# Chimeric Antigen Receptors T Cell Therapy in Solid Tumor: Challenges and Clinical Applications

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Adoptive cellular immunotherapy (ACT) employing engineered T lymphocytes expressing chimeric antigen receptors (CARs) has demonstrated promising antitumor effects in advanced hematologic cancers, such as relapsed or refractory acute lymphoblastic leukemia, chronic lymphocytic leukemia, and non-Hodgkin lymphoma, supporting the translation of ACT to non-hematological malignancies. Although CAR T cell therapy has made remarkable strides in the treatment of patients with certain hematological cancers, in solid tumors success has been limited likely due to heterogeneous antigen expression, immunosuppressive networks in the tumor microenvironment limiting CAR T cell function and persistence, and suboptimal trafficking to solid tumors. Here, we outline specific approaches to overcome barriers to CAR T cell effectiveness in the context of the tumor microenvironment and offer our perspective on how expanding the use of CAR T cells in solid tumors may require modifications in CAR T cell design. We anticipate these modifications will further expand CAR T cell therapy in clinical practice.

**Keywords: immunotherapy, T cell therapy, chimeric antigen receptor, CAR, solid tumors**

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## INTRODUCTION

For many years, the cornerstones of cancer treatment have been surgery, chemotherapy, and radiation, and more recently targeted therapies. Although these approaches have contributed to improved outcomes, most malignancies still carry a poor prognosis. Targeted anticancer approaches provide individualized therapy to combat the complexity of most malignancies and increase the probability of success. Currently, interest is increasing in immunotherapies, which harness the power of a patient's immune system to fight disease. One approach to cancer immunotherapy entails genetically engineering a patient's T cells to express chimeric antigen receptors (CARs) that recognize and attack tumor cells. The CAR consists of an antibody or ligand-derived targeting ectodomain fused with a hinge, a trans-membrane domain, and intracellular T cell signaling domains. When expressed by a T cell, CARs confer antigen specificity determined by the targeting domain (1, 2). In contrast to conventional T cell receptors (TCRs), which recognize antigens in a major histocompatibility complex (MHC)-dependent manner, CARs can potentially redirect the effector functions of a T cell toward any protein or non-protein target expressed on the cell surface. This strategy thereby avoids the requirement of antigen processing and presentation by the target cell and is applicable to non-classical T cell targets like carbohydrates (3). Circumventing human MHC-restriction renders the CAR T cell approach as a universal treatment, broadening the potential applicability of adoptive T cell therapy.

Four generations of CAR are being investigated in preclinical and ongoing clinical studies. The CAR "generation" typically refers to the intracellular signaling domains incorporated in the receptor molecule. First-generation CARs include only CD3ζ as an intracellular signaling domain; second-generation CARs include in addition to CD3ζ, a single co-stimulatory domain, such as CD28, 4-1BB (CD137), CD27, or OX40; third-generation CARs contain CD3ζ and two co-stimulatory domains, such as CD28, 4-1BB, or other co-stimulatory molecules. CARs may be further manipulated through the introduction of additional genes, including those encoding potent antitumor cytokines (e.g., IL-12 and Il-15) or co-stimulatory ligands (e.g., 4-1BBL), thus producing "armored" fourth-generation CAR T cells (4, 5).

Chimeric antigen receptors targeting the B cell receptor-associated protein CD19, developed for the treatment of B cell leukemia and lymphomas, have been the most clinically tested to date. Exciting progress with CD19-CAR T cell therapy across multiple institutions employing different therapeutic designs has led to the successful commercialization of this adoptive immunotherapy. In August 2017, the US Food and Drug Administration (FDA) approved the first CAR T cell therapy, tisagenlecleucel (Kymriah, Novartis Pharmaceuticals Corp.), for the treatment of certain pediatric and young adult patients with B cell acute lymphoblastic leukemia. This CAR T cell therapy has achieved remarkable outcomes in children and young adults with relapsed and often refractory disease, with complete response (CR) rates of 70–90% (6). Soon after the first approval, in October 2017, the FDA approved the CAR T cell therapy axicabtagene ciloleucel (Yescarta, Kite Pharma, Inc.) for the treatment of adult patients with certain types of B cell lymphoma. In lymphomas and other B cell malignancies, CAR T cell therapy, while effective, has shown lower CR rates, near 55% (6). This highlights the impact of tumor-specific parameters on the effectiveness of CAR T cells. Both CARs specifically bind CD19, an antigen that works well as a target for hematological malignancies because it is nearly uniformly expressed on malignant cells, save for conditions of therapeutic selective pressure, where antigen loss has been observed (7). Because CD19 appears on all B cells, both healthy and malignant, CD19-CAR T cell treatment may cause B cell aplasia, but the condition can be managed with intravenous immunoglobulins and close infection monitoring.

Despite progress in the treatment of hematological cancers with CAR T cells, treatment of solid tumors has proven more difficult. Here, we review CAR T cell therapy in solid tumors and discuss challenges and corresponding strategies to overcome them.

## CHALLENGES FOR CAR T CELL THERAPY IN SOLID TUMORS

The limited success of CAR T cell therapy against solid tumors may be due to many factors, including: (i) the lack of a unique tumor-associated antigen (TAA) in most cancers; (ii) the inability of *ex vivo* expanded CAR T cells to persist and proliferate following adoptive transfer; (iii) inefficient trafficking of CAR T cells to tumor sites; (iv) heterogeneous expression of the targeted antigen(s) leading to outgrowth of antigen-negative tumor variants; (v) the lack of survival and growth factors (e.g., IL-2); (vi) the presence of immunosuppressive molecules and cells; and (vii) the metabolically hostile tumor microenvironment. **Table 1** lists several fundamental characteristics of solid tumors that present obstacles to CAR T cell therapy.

### Tumor Antigen Expression and Heterogeneity

A primary challenge in developing CAR T cell therapy is identifying a tumor antigen that can be targeted safely and effectively [reviewed in Ref. (35)]. Ideally, CAR T cell therapy should target a tumor-restricted antigen to avoid the risk of "on-target/off-tumor" toxicity that may result in an immune reaction against healthy tissues, and at least two criteria should be considered (36). First, the proposed TAA should be differentially expressed on tumor cells compared with essential normal tissue. The CAR response is highly specific and can potentially bind to antigens even at low expression levels in normal tissues. Fine-tuning CAR design to recognize differential expression of antigens on tumor cells continues to evolve, and this represents a dynamic area of research aimed to expand the reach of CAR T cell therapy. Second, the TAA should be broadly expressed on the majority of tumor cells, as the success of CAR T cell therapy is largely dependent on expression of antigens on tumor cells (8, 36). Many of the TAAs identified [e.g., EGFR/EGFRvIII, IL13Rα2, HER2, CD171, mesothelin (MSLN), GD2, and carcinoembryonic antigen (CEA)] are expressed by a wide range of solid tumors, and this affords opportunity for combination therapies using CARs targeting multiple antigens. **Table 2** lists antigens that have served as targets for solid-tumor T cell therapeutic studies to date.

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase (170 kDa) that belongs to ErbB oncogene family (52–54). A wide range of normal and tumor cells express EGFR, and deregulation of EGFR is associated with epithelial tumors, such as pancreatic cancer, lung cancer, head and neck squamous cell carcinoma, colorectal cancer, and breast cancer (55, 56). Upregulation of EGFR is associated with poor prognosis in clinical settings (57, 58). Many studies have reported genomic alterations of *EGFR* in glioblastomas affecting both the extracellular and intracellular domains (59, 60). As the most common oncogenic EGFR mutant, with expression on ~30% of glioma cells (60, 61), EGFRvIII contains a deletion of extracellular amino acids 6–273 (62, 63), resulting in constitutive tyrosine kinase activity that promotes aggressive growth and tumor metastasis (64–66). This mutated extracellular EGFRvIII domain presents a tumor-specific, immunogenic epitope for CAR targeting (67, 68). Researchers have evaluated EGFRvIII-CARs for immunotherapy of glioma (38, 68), with the targeting domain derived from EGFRvIII-specific monoclonal antibodies. EGFRvIII-CAR T cells produced interferon-γ, effector cytokines, and were able to kill EGFRvIII+ tumor cells, demonstrating that EGFRvIII-CAR T cells can eliminate glioma cells (38, 67, 68).

Another promising target for brain malignancy is IL13 receptor α2 (IL13Rα2), a monomeric high affinity IL-13 receptor that is overexpressed in the majority of glioblastoma tumors and not

**TABLE 1** | Challenges for chimeric antigen receptor (CAR) T cell therapy in solid tumors.

| Challenge(s) | | Overcoming strategy(s) | Reference |
|---|---|---|---|
| Tumor microenvironment | Soluble molecules | Use of gene edited CAR T cells that disrupt sensitivity to inhibitory pathways such as adenosine and prostaglandin E2 signaling, PD-1, IDO, and TIM-3 inhibitory molecules | (8–13) |
| | Immunosuppressive immune cells | The concomitant application of CAR T cells with blockage and depletion of various immunosuppressive molecules and cells such as Tregs and myeloid-derived suppressor cells | (10, 14–16) |
| | | Use of armored-CAR T cells | |
| | Physical and metabolic barriers | Generation of CAR T cells which degrade the extracellular matrix and target tumor-associated stromal cells to facilitate infiltration of T cells into solid tumor masses | (17, 18) |
| Trafficking | | Use of CAR T cells overexpressing chemokine receptors or combined application of CAR T cells with an oncolytic virus armed with the chemokines that match the chemokines receptors expressed by T cells | (19–22) |
| | | Genetic addition of molecules which improve CAR T localization | |
| | | Local delivery of CAR T cells | |
| Target antigen heterogeneity | | Use of CARs targeting multiple antigens | (18, 23, 24) |
| | | Use of dual-specific T cells | |
| | | Monitoring of patients for expression of tumor antigen | |
| Intrinsic regulatory mechanisms of T cells | | Use of PD-1 switch receptors to blunt inhibitory effect of PD-1 signaling | (25–34) |
| | | Blocking inhibitory immune receptors to augment adoptive T cell transfer | |
| | | Gene-editing of CAR T cells to disrupt expression of inhibitory receptors | |
| | | Use of CAR T cells overexpressing antiapoptotic proteins | |
| | | Use of CAR T cells downregulating apoptotic proteins | |
| | | Use of dominant negative TGF-β receptor | |
| | | Use of drug/radio resistant CAR T cells | |
| | | Use of more persistent T cells | |
| | | Use of gene edited T cells | |

**TABLE 2** | A summary of solid tumor antigens being targeted using CAR T cell therapy.

| Antigen | Type of cancer | Endomains | Gene transfer method | Reference |
|---|---|---|---|---|
| CD171 | Recurrent/refractory neuroblastoma | CD3ζ | Electroporation | (37) |
| EGFRvIII | Glioma | CD28+CD3ζ, 4-1BB | Gamma-retrovirus | (38) |
| Epidermal growth factor receptor | Gastric cancer | – | Gamma-retrovirus | (39) |
| Carbonic anhydrase IX | Metastatic renal cell carcinoma | FcRγ | Gamma-retrovirus | (40) |
| α-folate receptor | Ovarian | FcRγ | Gamma-retrovirus | (41) |
| HER2 | Sarcoma | CD28-CD3ζ | Gamma-retrovirus | (42) |
| HER2 | Glioblastoma | CD28-CD3ζ | pigyBac | (43) |
| HER2 | Osteosarcoma | CD28-CD3ζ | SFG retroviral | (44) |
| αHER2/CD3 | Gastric cancer | CD28-CD3ζ | Gamma-retrovirus | (45) |
| Carcinoembryonic antigen | Liver metastases | CD28-CD3ζ | Gamma-retrovirus | (46) |
| IL13Rα2 | Glioblastoma | CD3ζ | Electroporation | (47) |
| IL13Rα2 | Glioblastoma | 4-1BB, CD3ζ | Lentivirus | NEJM |
| HER2 | Metastatic colon cancer | 4-1BB, CD28, CD3ζ | Gamma-retrovirus | (48) |
| GD2 | Neuroblastoma | CD3ζ | Gamma-retrovirus | (49) |
| GD2 | Neuroblastoma | CD28, CD3ζ, OX40 | SFG retroviral | (50) |
| ErbB2 + MUC1 | Breast cancer | CD28, CD3ζ | SFG retroviral | (51) |
| Vascular endothelial growth factor receptor 2 + gp100 + TRP-1 + or TRP-2 | Melanoma | – | Gamma-retrovirus | (24) |
| FAP | Colon and ovarian cancer | CD8α, CD3ζ, 4-1BB | Gamma-retrovirus | (17) |
| HER2 + CD19 | Medulloblastoma | CD28 + CD3ζ | SFG retroviral | (23) |
| Mesothelin (MSLN) | Malignant Pleural Mesothelioma | CD3ζ and 4-1BB | Lentiviral | (22) |
| NKG2D | Breast cancer | CD28 + CD3ζ | Gamma-retrovirus | (21) |
| MSLN | Pancreatic cancer | CD3ζ and 4-1BB | Gamma-retrovirus | (8) |
| MSLN | Malignant pleural mesothelioma | CD3ζ and 4-1BB | Gamma-retrovirus | (8) |

expressed at significant levels on normal brain tissue (69, 70). In addition, IL13Rα2 expression is a prognostic indicator of poor patient survival (70). This disease-associated expression profile supports the development of CAR T cells targeting IL13Rα2 for the treatment of glioblastoma and possibly other solid tumors (71). To target IL13Rα2 both antibody- and ligand-based CARs are being evaluated. Our group and others have developed ligand-based CARs utilizing membrane bound IL13 muteins for preferential recognition of IL13Rα2 over the more ubiquitously expressed IL13Rα1 (71). Ligand-based CARs represent a novel class of CAR

design. City of Hope is currently in clinical trial evaluating an IL13-ligand CAR T cell platform, and early findings suggest encouraging evidence for safety and therapeutic bioactivity (47, 72).

HER2, a trans-membrane glycoprotein belonging to the EGFR family, is another attractive target antigen for cancer immunotherapy (73, 74). HER2 is overexpressed in osteosarcoma, medulloblastoma, glioblastoma, and ovarian and breast cancer, among others (75–78). Several studies point to the critical role of HER2 in various cancer pathological processes (79), and HER2 overexpression is associated with poor clinical outcomes (80, 81). Ahmed et al. evaluated HER2-CAR T cell therapy for medulloblastoma (78), demonstrating that HER2-CAR T cells are able to target and kill HER2+ medulloblastoma cells *in vitro* and in an established medulloblastoma orthotopic xenogeneic SCID mouse model (78). The researchers reported in a study of osteosarcoma that HER2-CAR T cells, proliferated, produced immunostimulatory T helper 1 (Th1) cytokines, and killed HER2+ osteosarcoma cells *in vitro*, and HER2-CAR T cells caused regression of established osteosarcoma xenografts in locoregional as well as metastatic mouse models (44).

Mesothelin is a tumor differentiation antigen (40 kDa) that is normally present on the mesothelial cells of pleura, peritoneum, and pericardium (82, 83) and is highly expressed in many human cancers, including malignant mesothelioma, pancreatic, ovarian, and lung adenocarcinoma (84–87). MSLN overexpression is associated with proliferation of tumor cells, invasion, and poor survival rates of patients (88–90). The limited expression in normal tissues and high expression in many cancers renders MSLN a potential CAR T cell target (86). Riese et al. evaluated MSLN-CAR T cell treatment for thymoma in a mouse model (91) using a novel strategy designed to improve T cell function by eliminating negative regulators. Given that CAR signaling derives from TCR intracellular domains that function to initiate signal transduction, deletion of negative regulators may augment CAR signaling and effector T cell function. The researchers examined CAR activity in T cells that lacked one or both isoforms of diacylglycerol (DAG) kinase (dgk), normally highly expressed in T cells. The enzymes dgkα and dgkζ metabolize the second messenger DAG and limit Ras/ERK activation. The researchers found that, similar to pharmacologic inhibition of dgk enzymes, dgk-deficient CAR T cells were more effective in limiting the growth of implanted tumors, both concurrent with and after establishment of the tumor. These results indicate that modification of CAR T cells (herein, deletion of negative regulators of TCR signaling) could improve the activity and function of CAR T cells in a solid tumor model. This work highlights the importance of CAR T cell modifications that extend beyond the CAR molecule to T cell-specific functional machinery—modifications that may broaden clinical use and improve the efficacy of CAR T cells (91).

Many preclinical studies of CAR T cells that target stroma and/or TAAs in solid tumor models have evaluated such targets as carbonic anhydrase IX (CAIX), GD2, vascular endothelial growth factor receptor 2 (VEGFR2), folate receptor alpha (FR-α), FAP, and CEA. Several platforms have advanced to the clinic, and **Table 3** lists clinical trials corresponding to solid-tumor targets, many of which used first-generation CARs. For example, Lamers et al. assessed first-generation CAIX–CAR T cell therapy in renal carcinoma patients and observed "on-target/off-tumor" side effects and a low persistence of CAR T cells, possibly due to host immune response against CARs (92, 93). Other studies have reported low persistence of first-generation CD171–CAR T cells in neuroblastoma patients and FR-α-CAR T cells in ovarian cancer patients (37, 41). Although results from first-generation CAR T cell therapy trials were disappointing (37, 92, 93), the studies provided data and insights on CAR optimization, leading to the generation of second- and third-generation CARs that may overcome some of the challenges in solid tumor therapy.

A large number of surface antigens are variably expressed by tumor cells, and among barriers associated with solid-tumor CAR T cell therapy, cell surface antigen heterogeneity features prominently in failures to achieve durable responses (36). O'Rourke et al. found heterogeneity of EGFRvIII expression to be a major barrier in targeting it as a single antigen (67). In a clinical study of EGFRvIII–CAR T cell therapy for glioblastoma, they noted wide regional variation of EGFRvIII expression in tumor samples after EGFRvIII–CAR T cell infusion. Most subjects had loss or decreased expression of EGFRvIII in tumors despite no change in the degree of EGFR amplification or other tumor mutations. The study poses the question whether targeting EGFRvIII alone can provide durable clinical benefits or whether antigen escape will negate the clinical impact. CAR T cell targeting of the tumor antigen IL13Rα2 in patients with glioblastoma has encountered similar hurdles with antigen heterogeneity. Brown et al. reported that treatment with IL13Rα2–CAR T cells mediated a CR in one patient, but the disease eventually recurred 228 days after the first CAR T cell treatment at sites distinct and nonadjacent to the original tumors. Preliminary results suggest the cause of recurrence is decreased expression of IL13Rα2 (72). These and many other studies point to the importance of target antigen overexpression and distribution on most, if not all, tumor cells. As a criterion for patient enrollment on CAR T cell therapy trials, prescreening for the intensity and percentage of target antigen expression on tumor cells by immunohistochemistry and/or immunofluorescent techniques may be predictive of response (17, 23, 24, 51).

Targeting of multiple tumor antigens simultaneously or in a combinatorial strategy could lead to better "killing coverage" and potentially block the emergence of target antigen-null tumor cells (17, 23, 24, 51). Preclinical experiments with trivalent CAR T cells co-targeting HER2, IL13Rα2, and EphA2 showed promise in overcoming glioblastoma variability (94). Analysis of primary glioblastoma patient samples demonstrated the trivalent CARs captured nearly 100% of tumor cells in most tumors and exhibited improved cytotoxicity and cytokine release over monospecific and bispecific CAR T cells. Treatment with the multi-specific CAR T cells *in vivo* controlled established autologous glioblastoma patient-derived xenografts and improved survival of treated animals (94). Another study of dual-targeted CAR T cells specific for MUC1 and ErbB2 demonstrated their effectiveness against solid tumors, particularly breast cancer (51). Proliferation of the dual MUC1/ErbB2

**TABLE 3** | Various clinical trials using CAR T cell therapy in solid tumors.

| Type of cancer | Antigen | Identifier | Phase | Status |
|---|---|---|---|---|
| Glioblastoma | Epidermal growth factor receptor (EGFR) | NCT02331693 | I | Recruiting |
| | EGFRvIII | NCT02844062 | I | Recruiting |
| | EGFRvIII | NCT01454596 | I/II | Recruiting |
| | EGFRvIII | NCT02209376 | I | Recruiting |
| | EGFRvIII | NCT02664363 | I | Not yet recruiting |
| | IL13Rα2 | NCT00730613 | I | Completed |
| | IL13Rα2 | NCT01082926 | I | Completed |
| | IL13Rα2 | NCT02208362 | I | Recruiting |
| | HER2 | NCT02442297 | I | Recruiting |
| | HER2 | NCT01109095 | I | Active, not recruiting |
| Pancreatic | Mesothelin (MSLN) | NCT02959151 | I/II | Recruiting |
| | MSLN | NCT02465983 | I | Active not recruiting |
| | MSLN | NCT02706782 | I | Recruiting |
| Breast | HER2 | NCT02547961 | I/II | Recruiting |
| | MSLN | NCT02792114 | I | Recruiting |
| HER2-positive cancer | HER2 | NCT00889954 | I | Active, not recruiting |
| HER2-positive sarcoma | HER2 | NCT00924287 | I/II | Completed |
| | HER2 | NCT00902044 | I/II | Completed |
| MSLN-positive tumors | MSLN | NCT02930993 | I | Recruiting |
| | MSLN | NCT02159716 | I | Active, not recruiting |
| | MSLN | NCT02590747 | I | Recruiting |
| | MSLN | NCT01583686 | I/II | Recruiting |
| Neuroblastoma | GD2 | NCT00085930 | I | Completed |
| | GD2 | NCT02107963 | I | Completed |
| CD133-positive malignancies | CD131 | NCT02541370 | I | Recruiting |
| Malignant pleural mesothelioma | FAP | NCT01722149 | I | Recruiting |
| Liver metastases | Carcinoembryonic antigen (CEA) | NCT01373047 | I | Completed |
| Pancreatic ductal adenocarcinoma | MSLN | NCT01897415 | I | Active, not recruiting |
| Pleural mesothelioma | MSLN | NCT01355965 | I | Completed |
| Gastric cancer | HER2 | NCT02713984 | I/II | Recruiting |
| | HER2 | NCT01935843 | I/II | Recruiting |
| | CEA | NCT02349724 | I | Recruiting |
| | CEA | NCT01723306 | II | Recruiting |

CAR T cells required coexpression of MUC1 and ErbB2 on target tumor cells, and the CAR T cells were effective in killing ErbB2(+) tumor cells. These findings suggest that multivalent CARs may be an effective strategy to box-in heterogeneous tumors and thereby block resistance through tumor escape (51). However, tumor antigen expression loss in glioblastoma patients following CAR T cell therapy specific to one antigen implies that selection of clonal variants resistant to treatment occurs. With the integration of multivalent targets, there may be potential for further selection and the development of treatment resistance over time.

## The Suppressive Solid Tumor Microenvironment

Clinical and preclinical studies have shown that reversing immune inhibitory pathways triggered in many cancers may require CAR T cell modifications beyond the inclusion of co-stimulatory signaling. In contrast to certain blood cancers that have responded well to CAR T cell therapy, solid tumors not only lack conventional co-stimulatory molecules, which are expressed on malignant and normal B lymphocyte targets in hematological malignancies, but also have evolved mechanisms to actively suppress the immune system (95, 96). A number of immunosuppressive pathways can limit the full potential of adoptive CAR T cell therapy. Inhibitory immune receptors are often expressed on T cells following persistent tumor antigen encounter, and these include T-cell membrane protein-3 (TIM-3), lymphocyte-activation protein-3 (LAG-3), T cell Ig and ITIM domain (TIGIT), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and programmed death-1 (PD-1). The upregulation of these receptors limit the persistence and activity of the antitumor response of CAR T cells (36).

Tumors employ multiple tactics to evade or misdirect tumor-specific immune response. Many soluble factors that suppress anti-tumor immune responses have been identified in tissue extracts, serum, and ascites fluid of cancer patients. Tumor cells and macrophages express prostaglandin E2 (PGE2), a soluble factor derived from arachidonic acid and produced by inducible cyclo-oxygenase 2 enzyme (8, 36) that exerts its immunosuppressive

effect through subversion of CD8 differentiation, suppression of T cell proliferation, and inhibition of CD4 T cell helper functions (97). The PGE2/EP2/protein kinase A (PKA) signaling pathway mediates immunosuppression through PGE2 (98), which in combination with adenosine activates PKA and blocks TCR activation. A small peptide called the "regulatory subunit I anchoring disruptor" (RIAD) dampens the negative effects of PKA on TCR activation—a function that researchers leveraged to improve T cell function. Through generation of CAR T cells expressing RIAD, Albelda and colleagues showed that inhibition of upstream immunosuppressive mediators of PKA activation such as PGE2 and adenosine could lead to increased TCR signaling, more cytokine release, and increased CAR T cell infiltration, leading to enhanced tumor cells killing (19). Increased inflammatory activity is a hallmark of the tumor microenvironment and creates an abundance of reactive oxygen species (ROS) that substantially impair antitumor activity. Ligtenberg and colleagues hypothesized that CAR T cells coexpressing catalase (CAT) would perform better than regular CAR T cells. They showed that CAT–CAR T cells produced more intracellular catalase, leading to a reduced oxidative state with less ROS accumulation in both the basal and activated states. The CAT–CAR T cells maintained antitumor activity despite an inhospitable environment of high $H_2O_2$ (99).

Many tumors produce transforming growth factor beta (TGFβ), which inhibits T cell activation, proliferation, and cytotoxicity (**Figure 1**). Chou et al. demonstrated that cell-intrinsic abrogation of TGFβ signaling can enhance T cell persistence and function in a murine model of autochthonous prostate cancer. Moreover, it has been shown that T cells rendered insensitive to TGFβ by transduction with TGFβ dominant negative receptor II were highly effective in eliminating established melanoma-bearing mice (100). This idea has been translated to clinical studies of tumor-infiltrating lymphocytes engineered to express a TGFβ1-dominant negative transgene (NCT01955460). This approach offers an alternative to therapeutic anti-TGFβ monoclonal antibodies (fresolimumab/GC1008).

The recent development of checkpoint inhibitors such as ipilimumab targeting CTLA-4 and nivolumab targeting PD-1 provide further opportunities to enhance antitumor immune response with the potential to produce durable clinical responses though opportunistic autoimmunity (36). John et al. demonstrated that combining CAR T cells and PD-1 blocking antibodies could potently enhance CAR T cell therapy. They found that such combination can significantly decrease the percentage of Gr1+ CD11b+ myeloid-derived suppressor cells, and this was strongly correlated with therapeutic responses in established disease (9). Alternatively, CAR T cells can be further modified intrinsically to enhance their function particularly in the context of the targeted tumor microenvironment. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system provides a robust and multiplexable genome editing tool, enabling researchers to precisely engineer specific genomic sequences. The CRISPR/Cas9 system and the simpler Cas9/sgRNA system enable the efficient construction of knockout alleles through the induction of frameshift mutations. With

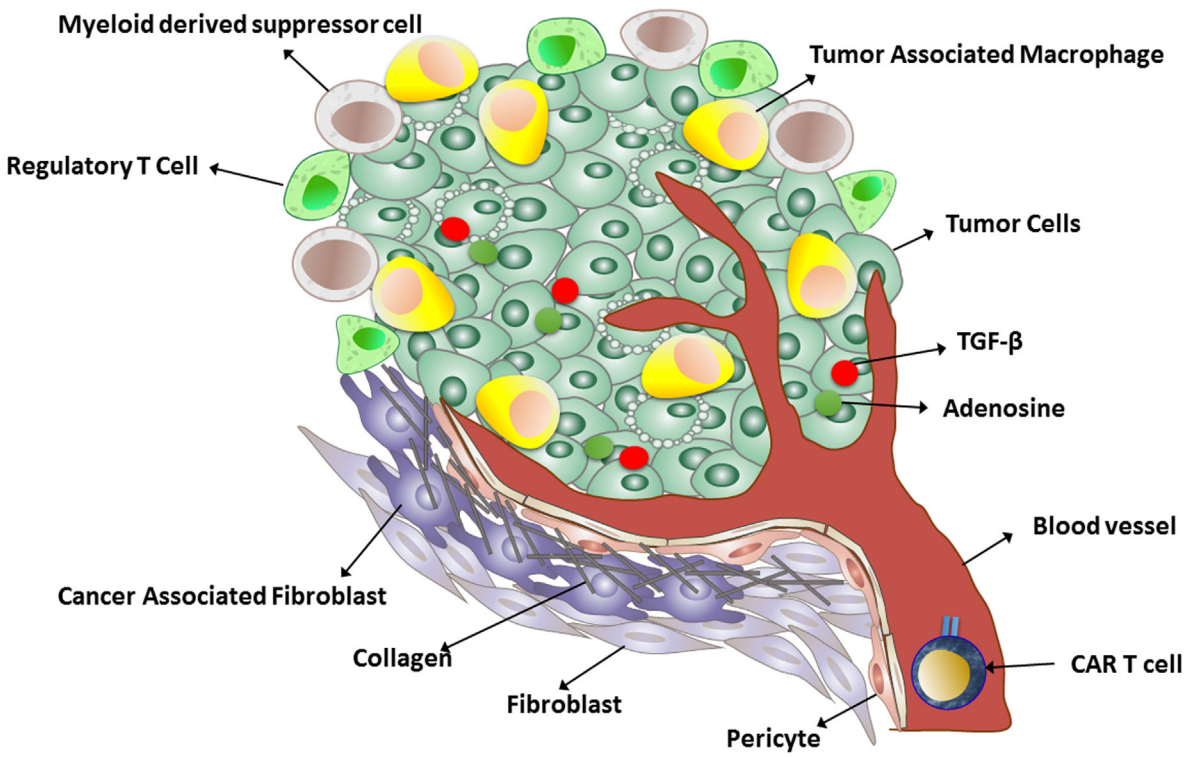


**FIGURE 1** | A schematic representation of the immunosuppressive tumor microenvironment.

this gene-editing tool, it is possible to generate knock out PD-1 and/or CTLA-4 CAR T cells. This strategy not only prevents potential toxicity of anti-PD-1 or CTLA-4 administration (e.g., opportunistic autoimmunity) but it also would not interfere with normal homeostatic functions of these molecules within the body. **Table 1** illustrates various challenges faced by CAR T cell therapy in solid tumors.

## Immune Stimulatory CAR T Cell Modifications

T cell costimulation mediated by CAR second-generation intracellular domains, such as CD28, CD137, CD27, or OX40, fail to overcome many inhibitory effects, especially once T cell anergy/exhaustion have taken effect. Several proposed countermeasures to immunosuppression may enhance antitumor CAR T cell activity in the solid tumor microenvironment, and these include increasing intrinsic CAR T cell activity *via* intracellular modifications, selecting immature or memory T cell subsets for T cell product manufacturing, and targeting cellular and molecular components of the tumor microenvironment. One promising approach entails the use of immunostimulatory cytokines that may revert or block tumor-associated inhibition and activate adoptively transferred T cells. Strategies that provide high levels of immunostimulatory cytokines locally at the antigen site have demonstrated preclinical and clinical efficacy. Animal models using poorly immunogenic tumors revealed that T cells genetically engineered to produce cytokines like IL-7, Il-15, and Il-12 were effective in eradicating tumors (101, 102). Another approach combines blocking immune suppression simultaneous with enhancing stimulatory cytokine production. To protect CAR T cells from the immunosuppressive cytokine IL-4, Mohammed et al. generated a hybrid cytokine receptor in which an IL-4-receptor exodomain was fused to an IL-7-receptor endodomain (103). Transgenic expression of this molecule in CAR T cells reverted the inhibitory effects of tumor-derived IL-4 and promoted T cell proliferation, resulting in enhanced antitumor activity.

Research has shown that locally produced IL-15 improved CAR T cell expansion and prolonged persistence *in vivo* by increasing the expression of antiapoptotic molecules, such as Bcl-2, through activation of the phosphoinositide 3-kinase signaling pathway (104). Local production of other cytokines, such as IL-7 and IL-12, have shown promising results in preclinical studies and clinical studies. Preclinical models showed that IL-7 (and IL-15) induced expansion of CAR T memory stem cells (CD8+ CD45RA+ CCR7+) with greater antitumor activity, which is mediated by increased resistance to cell death following repetitive exposures to the antigen, and maintenance of their migration ability to secondary lymphoid organs. Some studies also coupled CAR T cells with the constitutive or inducible release of IL-12. CAR T cells expressing IL-12 promote a Th1 immune response, reverse anergy in tumor-infiltrating cells, and inhibit Treg-mediated suppression of antitumor effector functions of T cells. CAR T cell expression of IL-12 also dampened production of immunosuppressive cytokines such as IL-10 and TGF-β by tumor-associated myeloid cells. Koneru et al. showed that IL-12 secreting tumor-targeted chimeric antigen receptor T cells (also known as armored-CAR T cells) could eradicate human ovarian xenografts. The authors showed that IL-12 secreting CAR T cells exhibit enhanced antitumor efficacy as determined by increased survival, prolonged persistence of T cells, and higher systemic IFN-γ (14). However, it should be noted that the authors measured the secretion of human IL-12 (p70) in the serum of CAR T cell-treated SCID-Beige mice [with impaired lymphoid development and reduced NK cell activity but normal macrophage and dendritic cells (DCs)] with established ovarian tumors. Because SCID-Beige mice have normal macrophages and DC populations and these cells are endogenous sources of mouse IL-12 production upon tumor challenge, these mice could in fact produce IL-12 after tumor challenge. Therefore, the total concentration of serum IL-12 may have consisted of both endogenous and exogenous IL-12, and may not have reflected IL-12 solely produced by armored-CAR T cells (105). Pegram and colleagues demonstrated that IL-12 producing CD19-CAR T cells eradicate systemic tumors without the need for prior conditioning. Moreover, they showed that such engineered T cells acquire intrinsic resistance to Treg cell-mediated inhibition (106). Chinnasamy et al. demonstrated that adoptive transfer of syngeneic CAR T cells co-transduced with VEGFR2 and constitutively expressing single-chain IL-12 resulted in the regression of established tumors of different histologies without the need for IL-2 administration. Indeed, the VEGFR2–CAR T cells changed the immunosuppressive tumor environment by altering/reducing both the systemic and the intratumoral CD11b+ Gr1+ myeloid suppressor cell subsets that expressed VEGFR2 (107). Alternatively, CAR T cells can be engineered to express cytokine receptors such as IL-7Ra that drive proliferation in response to endogenous IL-7. Perna et al. showed that IL-7 supports the proliferation and antitumor activity of IL-7Rα expressing CAR-GD2+ EBV-CTLs both *in vitro* and *in vivo* even in the presence of fully functional Tregs (108).

## Tumor Trafficking and Infiltration

Insufficient trafficking of CAR T cells to the tumor site represents another barrier for CAR T cell therapy. Studies have shown that improved migration ability of infused CAR T cells to tumor sites may increase their antitumor immune response (109), and efficiency of adoptively transferred T cells infiltrating the tumor site correlates with clinical responses in patients (110–112). Trafficking to the tumor site requires expression and binding of adhesion receptors on both T cells and the tumor endothelium lining. In addition, CAR T cell chemokine receptors must match the chemokines secreted by tumors (8, 36). Chemokine/receptor mismatch has been shown to account for insufficient tumor localization of T cells. Many human tumors either secrete low levels of chemokines or chemokines for which effector T cells lack receptors. Consequently, adoptively transferred T cells may fail find malignant cells. Peng et al. showed that T cell migration to tumor sites could be improved by overexpression of CXCR2, which recognizes tumor-produced CXCL1 (113). Moon and colleagues demonstrated that overexpression of CCR2b in MSLN-targeted CAR T cells led to a more than 12.5-fold increase in CAR T cell migration to mesothelin+ malignant pleural mesothelioma in mice, resulting in enhanced antitumor effects (22). Di Stasi

and colleagues showed that expression of CCR4 on CD30-CAR T cells enhanced the migration of these cells toward Hodgkin's lymphoma-secreting CCL17 in a xenograft animal model (114). Another study reported that expression of CCR2b on GD2-CAR T cells led to a more than 10-fold increase in CAR T cells homing toward CCL2 secreting neuroblastoma cells (115). A separate study also demonstrated that adoptive transfer of NKG2D-based CAR T cells could recruit and activate endogenous antigen-specific CD4+ and CD8+ T cells at the tumor site in a CXCR3-dependent manner to achieve optimal eradication of ID8 ovarian cancer (116).

To address both insufficient T cell migration and the immunosuppressive milieu of solid tumors, researchers combined CAR T cells with an oncolytic virus harboring the chemokine RANTES and the cytokine IL15. The local administration of biological agents, such as cytokines and oncolytic virus, has been previously translated to the clinic with success (117, 118). The experiments of Nishio et al. showed that the modified oncolytic virus provided a direct lytic effect on infected malignant cells, and it facilitated migration and survival of CAR T cells. They reported that the combination induced a potent, dose-dependent, cytotoxic effect on neuroblastoma tumor cells, while leaving the GD2-CAR T cells unharmed. The intratumoral release of both RANTES and IL15 attracted CAR T cells and supported their local survival, leading to increased overall survival of tumor-bearing mice (119). Together these studies suggest that CAR T cell modifications may enhance the efficacy and homing capabilities of adoptively transferred T cells.

Another strategy to increase CAR T cells at the solid tumor site is to break down the tumor stroma (**Figure 1**). In an interesting study, Garuana and colleagues modified CAR T cells to overexpress heparanase enzyme to degrade the main components of the subendothelial basement membrane and the extracellular matrix (ECM), including the heparan sulfate proteoglycans (HSPGs), in order to facilitate CAR T infiltration into tumor stroma. The ECM is an integral component of the stroma, and therefore, T cells attacking stroma-rich solid tumors must be able to degrade HSPGs in order to access tumor cells and exert antitumor effects. The authors found that engineered CAR T cells expressing heparanase showed improved capacity to degrade the ECM and promoted T cell infiltration and antitumor activity (50). The studies support the concept that generation of CAR T cells with a chemokine receptor or enzyme could facilitate infiltration into the tumor stroma and enhance antitumor efficacy. Although these approaches have been shown to be effective in animal models, introducing chemokine receptor transgenes into CAR T cells for adoptive cell therapy has yet to be tested in humans.

An entirely different approach to promote homing of CAR T cells to solid tumor sites involves delivery of CAR T cells directly to the tumor site, a departure from the more common intravenous (i.v.) route of administration. Adusumilli and colleagues showed that compared to i.v. administration, local CAR T cell administration resulted in greater T cell antitumor potency with reduced T cell doses, partially due to early CD4+ T cell activation and the systemic benefits that ensued (20). Brown et al. described promising results of locoregional CAR T cell delivery for the treatment of glioblastoma (72). A comparison in one patient of two intracranial CAR T cell delivery routes—infusion into the resected tumor cavity and infusion into the ventricular system—pointed to the potential impact of the route of administration. In this patient, both routes (intracavitary and intraventricular) had low toxicity profiles but differed in subsequent tumor growth at distant sites. While intracavitary therapy appeared to control local tumor recurrence, glioblastoma progressed at distant sites, including the onset of new lesions. By contrast, after intraventricular administration of CAR T cells, regression of all central nervous system tumors, including spinal tumors, was achieved (72).

## CLINICAL STUDIES—LOOKING AHEAD

In early clinical studies with first-generation CAR T cells, therapeutic T cells showed little persistence, so the efficacy and safety were difficult to assess. Although targeting solid tumors is still in the early stages, trials have already shown antitumor activity in solid tumors such as neuroblastoma. Louis et al. developed CAR T cells targeted to the validated tumor antigen GD2, for which the safety of monoclonal antibody therapy was previously demonstrated (49, 120). As one of the first CAR T cell therapy trials, the first-generation GD2-CAR T cells were administered to children with advanced neuroblastoma, with 3 of 11 patients experiencing CRs, no substantial toxicity observed, and sustained clinical benefit for several patients reported (49, 121). The results are especially encouraging in light of CAR T cell advances that incorporate co-stimulatory signaling motifs in addition to CD3ζ, as was used in this trial. Unlike the favorable safety profile observed with GD2-CAR T cells, another CAR T cell trial in a patient with colon cancer metastatic to the lungs and liver resulted in death of the patient. The CAR targeting domain was based on the humanized monoclonal antibody trastuzumab (herceptin), specific to the tumor antigen Her2. The outcome was attributed to Her2 expression on normal lung and/or cardiac tissue (49). Importantly, this trial administered substantially higher numbers of CAR T cells than most other trials, raising the question of whether lower doses of HER2-CAR T cells might be safe. One takeaway from this experience is that antigens safely targeted by monoclonal antibody therapy cannot be assumed safe for CAR T cell therapy.

Glioblastoma is the most common and most malignant of brain tumors. It grows aggressively in the CNS and no current treatment is curative. CAR T cell preclinical work has shown promise, and current CAR T cell clinical trials in glioblastoma target three different antigens, EGFRvIII, HER2, and IL13Rα2 (67, 72, 78). O'Rourke et al., reported on 10 patients with glioblastoma who were treated with EGFRvIII-CAR T cell therapy. Manufacturing CAR T cells from patients with recurrent GBM was feasible, and no cross reactivity of EGFRvIII-CAR T cells with wild-type EGFR was observed. However, clinical benefit was indeterminate because treatment-related changes common to immunotherapy such as inflammation were difficult to distinguish by imaging from tumor progression. The research team did observe that the i.v.-infused CAR T cells trafficked to the brain and demonstrated antigen-specific activity. Two barriers to therapy were clear from the study: heterogeneity of EGFRvIII expression, as described earlier, and the immunosuppressive tumor microenvironment,

which intensified upon CAR T cell administration. An increase in non-CAR polyclonal T cells was observed in the tumor environment, which phenotypic analysis indicated to be comprised mostly of immunosuppressive regulatory T cells based on their expression of CD4, CD25, and FoxP3. In addition, immunosuppressive molecules such as IDO1, PD-L1, and IL-10 were upregulated after CAR T cell infusion. These findings suggest that EGFRvIII-CAR T cells induced an immunosuppressive response, and that measures to counter such a response, such as immune checkpoint blockade might work synergistically with CAR T cell therapy.

Safety concerns over targeting HER2 with CAR T cells were raised by the death of a patient who had received third-generation HER2-CAR T cells ($10^{10}$ cells) after lymphodepleting chemotherapy, as described above. Ahmed et al., developed a second-generation HER2-CAR, and in patients with sarcoma, CAR T cell treatment (up to $10^8/m^2$ cells) demonstrated no evident toxic effects, some indicators of antitumor activity, but limited T-cell persistence. To optimize the persistence of adoptively transferred T cells, the team engineered CARs into virus-specific T cells (121) in which costimulation results from native TCR (αβTCR) engagement with latent virus antigens on professional antigen-presenting cells. The group has established the safety of adoptively transferred polyclonal virus-specific T cell lines, enriched for cytomegalovirus, Epstein–Barr virus, and adenovirus, in hematopoietic stem cell transplant recipients. A phase 1 dose-escalation study established the safety of autologous HER2-CAR virus-specific T cells in 17 patients with progressive glioblastoma. Although the CAR T cells did not expand, they were detectable in the peripheral blood for up to 12 months. Of eight patients, one had a partial response and seven had stable disease. The median OS was 11.1 months after T cell infusion and 24.5 months after diagnosis. The results highlight the need for improvement in expansion, function, and persistence of the HER2–CAR T cells. Manipulations of the immune system to thwart immunosuppression and/or targeting multiple antigens to overcome glioblastoma heterogeneity may improve response rates and outcomes. Preconditioning regimens, such as lymphodepletion, may aid in increasing T cell responses. However, more work is needed to delineate how these treatments can augment CAR T cell therapy.

A clinical trial of autologous CAR T cells targeting IL13Rα2 provided the first evidence for a CAR T cell–mediated CR to therapy in glioblastoma. After receiving IL13Rα2-CAR T cell therapy, a patient with recurrent multifocal glioblastoma experienced dramatic improvements in his quality of life, including the discontinuation of systemic glucocorticoids and a return to normal life activities. Notable in this case was the potential role of the endogenous immune system in the antitumor responses. Immediate increases in endogenous immune cells and inflammatory cytokines after each intraventricular infusion of CAR T cells may have reflected recruitment and stimulation of the host immune system and may explain how a CR was achieved despite non uniform expression of IL13Rα2 on the tumors. After each intraventricular infusion of CAR T cells, rapid and pleiotropic changes in levels of inflammatory cytokines in the cerebrospinal fluid were observed, with significant increases in the interferon-γ-inducible chemokines CXCL9 and CXCL10, which have antitumor potential but did not affect neurologic function or the general well-being of the patient. This clinical experience, along with the studies of EGFRvIII-CAR T cells and HER2-CAR T cells against glioblastoma provide initial evidence of the safety and antitumor activity of CAR T cell immunotherapy in patients with malignant brain tumors.

## CONCLUDING REMARKS

New generations of optimized CARs could contribute to improved clinical responses, as could the combination of CAR T cells with other immunotherapeutic modalities such as checkpoint inhibitors, oncolytic viruses, vaccines, or cytokines, which may synergistically enhance therapeutic efficacy in solid tumors.

The generation and optimization of engineered cells derived from different cell populations offers other immunotherapeutic avenues. To date most studies employ T cells bearing αβ receptors, but γδ T cells possess a combination of innate and adaptive immune properties that may be conducive to cancer immunotherapy (122–124). Studies show that γδ T cells play a key role in tumor immunosurveillance and antitumor immune responses (124–126). In contrast to T cells bearing αβ receptors, γδ T cells are not susceptible to antigen processing and presentation defects, which is one strategy for cancer immune evasion. Moreover, the absence of co-stimulatory molecules resulted in the appearance of tumor clone(s) resistant to αβ (but not γδ) T cell-mediated cytotoxicity. Further, γδ T cells are able to directly lyse stressed cells (e.g., malignant transformations), produce a range of inflammatory cytokines and chemokines, present antigen to αβ T cells (i.e., T cell priming), and induce DC maturation (127, 128). Another favorable characteristic of γδ T cells is the migration of specific subsets to mucosal epithelial surfaces. This could be a crucial factor for successful immune or tumor-surveillance functions. Tissue-specific trafficking of γδ T cells to epithelial tissues as well as to tumors originating from such tissues has important implications for the design of unique immunotherapeutic strategies (129–132). As noted above, one of the potential challenges of adoptive T cell therapy is insufficient trafficking of effector T cells to tumor sites. Inherent γδ T cell tropism to epithelia tissues may overcome the barrier of insufficient trafficking of effector T cells to epithelial solid tumor sites. Moreover, expression of NKG2D ligands on tumor cells derived from these tissues can enhance the antitumor activity of the adoptively transferred T cells, potentially acting synergistically with CAR stimulation and reducing the likelihood of immune escape through antigen loss. Taken together, many properties of γδ T cells make them an attractive candidate platform for CAR T cell therapy for solid tumors.

Encouraging results in which CAR T cells mediate robust antitumor responses have been observed in certain blood cancers as well as isolated cases of patients with solid tumors, but engineered T cells have yet to yield high response rates responses in patients with solid tumors. Better understanding of the various solid tumor features that are problematic for adoptive T cell therapy will guide the development of new generations of T cells that may prove more effective in overcoming the challenges of solid tumor malignancies.

## AUTHOR CONTRIBUTIONS

Conception and design of paper was done by CB and BB. Initial draft was completed by HM. Editing and further drafts were done by AR and JS. All authors reviewed final version of paper.

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# Corrigendum: Chimeric Antigen Receptors T Cell Therapy in Solid Tumor: Challenges and Clinical Applications

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## A Corrigendum on

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In the original article, there was an error. We neglected to disclose a conflict of interest.

A correction has been made to the **Conflict of Interest statement**:

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The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
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# Natural Killer Cell-Based Immunotherapy in Gynecologic Malignancy: A Review

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Harnessing the immune system has proven an effective therapy in treating malignancies. Since the discovery of natural killer (NK) cells, strategies aimed to manipulate and augment their effector function against cancer have been the subject of intense research. Recent progress in the immunobiology of NK cells has led to the development of promising therapeutic approaches. In this review, we will focus on the recent advances in NK cell immunobiology and the clinical application of NK cell immunotherapy in ovarian, cervical, and uterine cancer.

**Keywords: natural killer cells, immunotherapy, gynecologic malignancy, ovarian cancer, cervical cancer**

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## INTRODUCTION

Exploiting the immune system has proven an effective therapeutic approach in treating a variety of malignancies. Identified in 1975, natural killer (NK) cells exist in the blood as preactivated cytolytic lymphocytes and are recognized as the most efficient antitumor effector (1–3). Distinct from T and B cells, NK cell effector function is not mediated by high-resolution antigen specificity but through signaling of multiple germ line-encoded activating and inhibiting receptors. Over the past 40 years, research has defined the regulation of NK cells and established essential roles they play in anticancer immunity. Strategies to harness and augment NK cells for cancer therapy are a relatively new and rapidly developing field. At this point, the success of NK cell-based immunotherapy has largely been confined to hematologic malignancies and has yet to translate to solid organ tumors (4). As a subset of solid organ tumors, gynecologic malignancies are a heterogeneous group of tumors derived from vulvar/vaginal, cervical, uterine, fallopian, and ovarian tissues. The treatment regimens for gynecologic cancers continue to develop with great room for improvement. With increased understanding of NK cell biology, there is renewed interest in NK cell-based immunotherapy directed against gynecologic malignancies. In this review, the advances in our understanding and clinical application of NK cell immunotherapy against ovarian, cervical, and uterine cancer is summarized.

## CHARACTERISTICS OF NK CELLS

The innate and adaptive immune systems function together to recognize and effectively eliminate aberrant cells, including cancer. Historically seen as part of the innate immune response, NK cells are large, granular lymphocytes. They were found to have the ability to kill tumor cells without any prior sensitization (thus "natural") or restriction of major histocompatibility complex (MHC) molecule expression (1, 2, 5).

Phenotypically, NK cells are defined *via* flow cytometry by the absence of CD3 and the presence of CD56 surface expression and comprise approximately 5–10% of circulating lymphocytes (6, 7). NK cells can be divided into $CD56^{bright}CD16^{-}$ or $CD56^{dim}CD16^{+}$ populations with different functional properties. Developmentally immature $CD56^{bright}CD16^{-}$ NK cells are capable of producing abundant cytokines, particularly interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), immediately after activation but possess little direct cytolytic function. In contrast, mature $CD56^{dim}CD16^{+}$ NK cells are characterized by the direct killing of transformed cells *via* perforin/granzyme release or death receptor pathways (Fas, TNF-related apoptosis-inducing ligand, TRAIL) (8–10).

As discussed below, NK cells are involved in tumor immunosurveillance and mediate antitumor responses (11). Their activity is highly regulated by a variety of germ line-encoded inhibitory and activating receptor expression (12, 13). Collectively, the complex balance of inhibitory and activating signals promotes self-tolerance or drives potent effector function of NK cells.

## NK CELL EFFECTOR FUNCTIONS

Natural killer cells identify and eliminate foreign, infected, damaged, or malignant cells through a variety of mechanisms. The most well-known is through receptor-mediated cytotoxicity. NK cells express a series of activating receptors capable of binding stress-induced ligands expressed on tumor cells. They also express a number of inhibitory receptors that interact with ligands to induce activation-limiting signals. When activating signals over-ride inhibitory mechanisms, the NK cell mediates exocytosis of stored lytic molecules. The membrane-disrupting protein perforin and serine protease granzymes then function in coordination to gain access to the target cell and induce apoptosis through the activation of caspases (14, 15).

Natural killer cells also appear to be the principal effectors for a process called antibody-dependent cell-mediated cytotoxicity (ADCC) (16). ADCC occurs when targets that become coated by antibody are recognized by NK cells *via* ligation to the low-affinity receptor for the Fc portion of human immunoglobulins, CD16 (FcγRIIIa). Upon binding, downstream signal transduction mechanisms lead to NK cell degranulation, cytokine secretion, and tumor cell lysis (15). The recent advances in our understanding of ADCC and NK function can be applied to augment NK cell immunotherapy. For example, monoclonal antibodies (mAbs) targeting CD20 (rituximab), Her2/neu (herceptin), epidermal growth factor receptor (cetuximab and panitumumab), and disialoganglioside (GD2) demonstrate significant antitumor contributions from NK cell-dependent ADCC in addition to the direct antitumor effect of the antibody (17). This strategy maintains the specificity against key molecular tumor targets important for cell proliferation or tumor growth with the added contribution of ADCC *via* NK cell effector function (16). Highlighting this role of ADCC, previous studies have demonstrated depletion in NK cell populations decreases the efficacy of mAb therapy (18). There is further evidence showing that specific FcγR polymorphisms impact responsiveness to mAb therapy and may even predict clinical outcomes for certain tumors (19–22). Today, mAb are being developed with enhanced affinity for CD16 to better activate NK cells and improve antitumor response (23, 24). Unfortunately, strategies to include the use of mAb to enhance ADCC in gynecologic malignancies have not been thoroughly investigated.

Natural killer cells can also initiate the transduction of death signals within target cells through death ligand/receptor ligation (25). NK cells are capable of expressing Fas ligand or TRAIL (26, 27). Interaction of these ligands with their respective antigens on tumor cells activates caspases and induces apoptosis (14). Recent studies have demonstrated the proteasome and histone deacetylase inhibitors upregulate the expression of death receptors and enhance NK cell-mediated cytotoxicity of tumor cells through the death receptor pathways (28–30). This is particularly interesting because this strategy was effective in both hematologic and solid tumors.

Finally, specific subsets of NK cells are capable of producing important immunoregulatory cytokines (31). NK cells expressing $CD56^{bright}$ are the primary source of NK cell-derived IFN-γ, TNF-α, and other cytokines that play a major role during the innate immune response to infection or tumorigenesis (8). The NK cells provide an early source of IFN-γ to induce $CD8^{+}$ T cells to become cytotoxic T lymphocytes (CTLs) and drive a Th1 response of $CD4^{+}$ T cells to further promote CTL differentiation (32, 33). These interactions are illustrated in **Figure 1**.

## IMPORTANT NK CELL RECEPTORS

Due to the capability of immediate response, NK cells are tightly regulated through a combinatorial array of surface receptors. Functionally, these receptors are classified as activating or inhibitory with their ligands either members or homologs of MHC class I molecules. Structurally, they belong either to the immunoglobulin (Ig)-like receptor superfamily or the C-type lectin-like receptor (CTLR) superfamily (34). We will review a few of the selected inhibitory and activating receptors on human NK cells.

The NK cell response is dominated by a variety of germ line-encoded inhibitory receptors from three families: killer immunoglobulin-like receptors (KIRs), C-type lectins (including NKG2A-CD94), and leukocyte immunoglobulin-like receptors (35–37). Stochastic surface expression within these families leads to subsets of NK cells with a diverse repertoire of receptors (35). Ligands for these receptors are both "classical" and "non-classical" class I molecules encoded within the MHC, termed human leukocyte antigens (HLAs) (38–40). For example, inhibitory KIRs recognize the classical HLA-A, HLA-B, and HLA-C proteins but do not distinguish self from non-self peptides. The binding of inhibitory KIR on NK cells to their HLA cognate suppresses cytotoxicity and cytokine secretion. The diverse groups of inhibitory receptors are all glycoproteins that signal through the canonical immunoreceptor tyrosine-based inhibitory motif (ITIM) to suppress NK cell response. Only when sufficient activating signals are present does the NK cell initiate effector function.

The *CD94* and *NKG2* family of genes encode CTLRs that recognize non-classical MHC class I molecules (HLA-E, -F, -G, and -H) and play a dominant role in NK cell function. CD94 can heterodimerize with NKG2A and signal through ITIM to function as

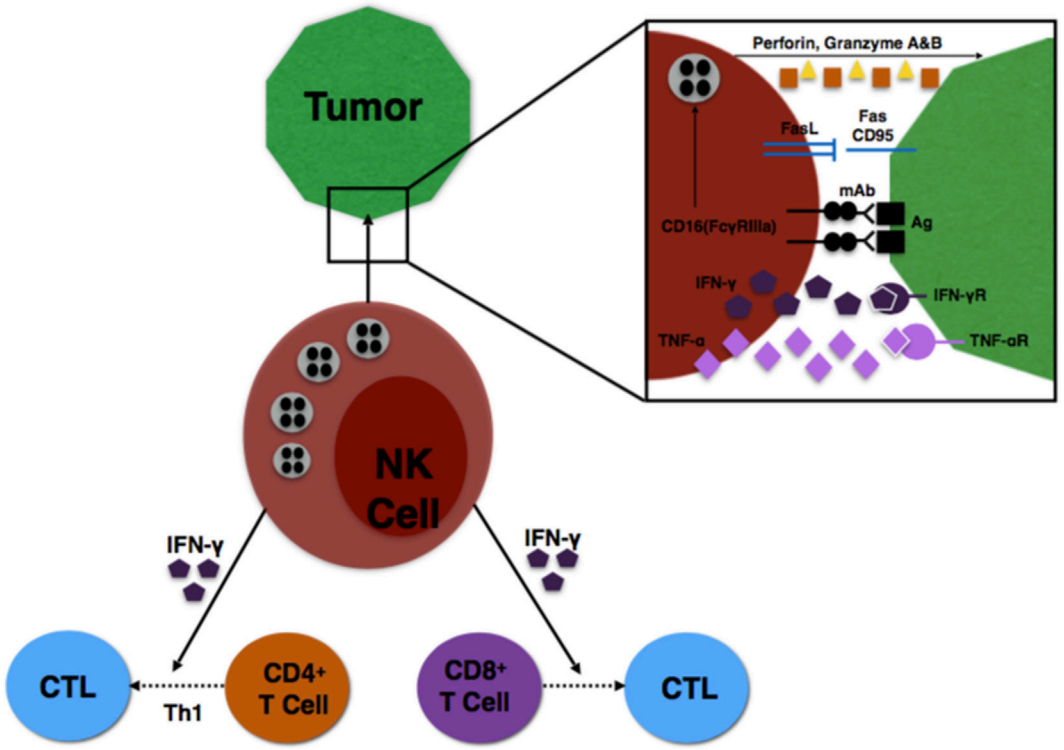


**FIGURE 1** | Illustration of NK cell interaction with tumor cells, as well as NK influence on T cell differentiation.

an inhibitory receptor when bound to HLA-E. However, CD94/NKG2C heterodimers serve as activating receptors. The inhibitory CD94/NKG2A and the activating CD94/NKG2C receptors are found in overlapping subsets of NK cells in the peripheral blood (38). Not surprisingly, CD94/NKG2A inhibitory receptor binds HLA-E with higher affinity compared to CD94/NKG2C activating receptor (41).

The role and function of NK inhibitory receptors are well defined. Recent research has elucidated the NK effector functions of activating and coactivating receptors. Unlike B and T cells, NK cells do not possess an activating receptor that dominates their development and effector function. Instead, they express a complement of invariant activating receptors that include the NK specific natural cytotoxicity receptors (NCRs) (NKp46, NKp30, and NKp44), C-type lectin-like (NKG2D, CD94/NKG2C), 2B4, DNAM-1, NTB-A, NKp80, CD59, and CD16 among others (36, 38, 42, 43).

The *NKG2D* gene encodes one activating receptor that, unlike its name, shares very little homology with *NKG2A* or *NKG2C*. NKG2D is a type II transmembrane-anchored glycoprotein constitutively expressed on all human NK cells and recognizes cell surface glycoproteins structurally related to MHC class I molecules. NK cells stimulated through NKG2D initiate cell-mediated cytotoxicity and cytokine release. Known human ligands include MICA, MICB, ULBP1, ULBP2, ULBP3, and ULBP4 and are upregulated by stress and stalled DNA replication *via* DNA-damage checkpoint pathways (34, 44). MICA and MICB are stress-induced antigens frequently expressed by tumors (45). However, progressive stages of cancer are associated with tumor shedding of MICA/B, which appears to systemically impair the immunological competence of individuals with cancer by causing downregulation of NKG2D. This impairment of effector function promotes tumor immune evasion (46, 47). Targeting the tumor pathways that lead to the upregulation of NKG2D ligands or alternatively maintain and/or upregulate NKG2D receptors may be a productive method to enhance NK cell-based immunotherapy.

The activating DNAM-1 receptor (CD226) belongs to the Ig superfamily and is constitutively expressed on all human NK cells (48). The specific ligands CD112 (nectin-2) and CD155 (polio virus receptor) bind and augment NK cell-mediated cytotoxicity and cytokine release (49, 50). Similarly to MICA, ovarian cancer cells ubiquitously expressing the ligand CD155 show reduced DNAM-1 expression and impaired NK cell function (51). As our understanding of these signaling and inhibitory pathways expands, our potential targets for NK immunotherapy grow.

Other triggering receptors are the NCRs, which include NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337) (52–56). These receptors have a variety of ligands with various structures. NKp46, the main activating receptor for human NK cells, binds the hemagglutinins on influenza virus-infected cells (57). The human cytomegalovirus pp65 tegument protein was identified as the original ligand for NKp30 and was shown to be responsible for suppression of

NK cell cytotoxicity (58). Later on, HLA-B associated transcript 3 protein (BAG6) and B7-H6 (NCR3LG1) were identified as novel surface ligands of NKp30 (59–61). Importantly, B7-H6 is present on a broad spectrum of tumors and may play a role in antitumor immunity (62). In addition, tumor shedding of B7-H6 was demonstrated to be a novel mechanism of immune escape (63). A recent study in patients with ovarian cancer demonstrated B7-H6 tumor ligands were associated with decreased NKp30 expression of tumor-associated NK cells (64). These NK cells demonstrated impaired IFN-γ production and cytolytic function. Together, these findings indicate how NK cells may recognize and kill target cells without the decreased expression of MHC class I protein and serve as a template for designing molecules to stimulate NK-mediated cytotoxicity for tumor immunotherapy. It also exemplifies the important role the tumor microenvironment plays on NK cell function and immune surveillance, which will be elucidated below.

## NK ROLE IN IMMUNOSURVEILLANCE

In 1909, the German physician Paul Ehrlich predicted the immune system routinely identified and eliminated aberrant cells that would otherwise lead to cancer (65). The theory was revisited with great interest 50 years later following a deeper understanding of tumor immunobiology (66–70). Eventually, the concept of tumor immunosurveillance was experimentally validated with the advancement in mouse genetics and the generation of mAb production (71–73). The complex relationship between the tumor and immune system further expanded to incorporate tumor immunoediting, a process where tumor cells reduce their immunogenicity thereby rendering the immune system incapable of recognizing and destroying the aberrant cells (74). Today, it is accepted both innate and adaptive immunity play vital roles in continuously monitoring tissues to eliminate aberrant tumor cells (73).

Some of the earliest experimental evidence detailing the role NK cells play in tumor control and immunosurveillance was obtained in mice. An early study demonstrated beige (bg) mice with 75 NK cell activity resulted in increased tumor growth rate and metastasis compared to control mice with normal NK cell function (75). The presence of NK cell activity also correlated with better control of *in vivo* tumor growth and metastasis, particularly against histocompatibility complex (MHC) class I-deficient variants (76). In addition, mice depleted of NK cells by the anti-asialo-$GM_1$ mAb resulted in a twofold to threefold increase in 3′-methylcholanthrene (MCA)-induced tumorigenesis compared to wild-type controls (77). Similarly, mice with defective NK function due to a deficiency of $NK1.1^+CD3^-$ cells but with functionally normal B, T, and NK/T cells showed impaired *in vivo* rejection of tumor cells (78).

More recently, a prospective cohort of 3,625 individuals were assessed for natural cytotoxic activity of peripheral blood lymphocytes and then followed for 11 years to observe the incidence of cancer (79). These results indicate individuals with impaired NK cell function display an increased risk of developing cancer. In an alternative study, NK cells were identified to also play a role in surveillance against DNA damage through checkpoint pathways (44). The DNA damage response is activated early in tumorigenesis and induces surface expression of ligands for an activating receptor of NK cells, providing a link between the innate immune system and tumor surveillance (80). Together, these studies highlight the contribution NK cell effector function plays in immune protection from tumor development.

It is now established that NK cells participate in first-line defense against tumor development. NK cells are able to discriminate self and non-self due to a wide array of cell surface receptors that control their response, particularly the MHC class I molecules (38). In a 1986 observation, NK cells eliminated MHC class I-deficient cells but not cells with normal MHC class I expression (81). This seminal observation was termed the "missing-self" hypothesis; in the absence of critical surface proteins cells are recognized and eliminated by NK cells (82, 83). This feature is important because solid tumors undergoing malignant transformation frequently reduce MHC class I expression and this process represents one of the main mechanisms for tumor cells to avoid detection by the adaptive immune system (84–88). Thus, one key function of NK cells is to monitor the integrity of MHC class I expression on tumor cells. The significance uncovered in the "missing-self" hypothesis was NK cell effector function is actively inhibited with engagement of MHC class I molecules on cells. More importantly for solid tumor malignancies, newer evidence suggests inhibitory MHC class I receptors only dampen, rather than eliminate, the effector function of NK cells (89–91). This suggests a sufficient activating signal either through a single potent stimulation or the simultaneous engagement of multiple activating receptors is capable of mediating NK cell effector function to eliminate target cells despite MHC class I expression. Thus, a modified "missing-self" hypothesis states "NK cells patrol for abnormal cells that lack MHC class I or overexpress ligands for activating NK cell receptors" (38).

## THE TUMOR MICROENVIRONMENT AND ITS IMPACT ON NK FUNCTION

The malignant transformation of normal cells results from a multifactorial process resulting in genomic instability and a modification of immunosurveillance mechanisms that induce tolerance (92). As tumors evolve, they develop different strategies to escape the immune response: (i) the secretion of immunosuppressive cytokines or soluble tumor-derived inhibitory factors, (ii) the expression of co-inhibitory or loss of co-stimulatory receptors, and (iii) the loss or downregulation of MHC class I molecules (84). To be effective, NK cells must first extravasate through the vessel endothelial lining and migrate to the tumor tissue (93). Any localizing defect can lead to insufficient numbers of NK cells to the primary or metastatic sites. Once in the extravascular space, NK cells encounter hypoxia, acidic pH, and low glucose conditions that are hostile to immune effector cell function (94). Peripheral blood NK cells and tissue NK cells are notably different; tumor NK cells are functionally defective, incompletely activated, or anergic compared to peripheral blood effectors. In addition, the effectors found within solid

tumors are often in limited supply. Further understanding the mechanisms of tissue immunity and its impact on NK cells will be important in our ability to treat solid tumors therapeutically with immunotherapy.

Here, we will discuss how the tumor microenvironment limits the effectiveness of the NK cell antitumor response with attention to ovarian cancer, cervical cancer and endometrial cancer.

## Epithelial Ovarian Carcinoma

Ovarian cancer is the most lethal gynecologic malignancy, with an estimated 14,080 deaths expected in the United States for 2017 (95). Despite optimal treatment with surgery and adjuvant chemotherapy, the recurrence rate approaches 70–80% (96). Although the disease tends to remain confined to the abdominal cavity, women with recurrent ovarian cancer progress and ultimately die. Therefore, there is an urgency to develop new and effective therapies.

Important to effective therapeutic development is the understanding of the immunologic interactions within the tumor and its physiologic impacts, which often includes the development of profuse ascites. It is well documented that the ascites fluid from patients with advanced stage ovarian cancer suppress the function of otherwise normal immune effectors, including NK cells (97–101). The ascites contains large numbers of growth factors and cytokines that promote the proliferation of tumor cells (102–104). While fresh NK cells ($CD56^+CD3^-CD16^+$) isolated from the ascites fluid are found in relatively high concentrations compared to peripheral blood, they are functionally deficient (105, 106). This population of NK cells demonstrates decreased CD16 expression and have reduced proliferative, cytolytic, and cytokine production compared to peripheral blood NK cells (107). Signaling proteins vital to interpreting the activating and inhibitory signals become defective and alter the expression of cytokine transcripts and proteins (100). As detailed above, tumor-associated ligands MICA/B and B7-H6 are often found within the peritoneal fluid of serous ovarian cancer patients and impair NK cell effector function (46, 64). These deficiencies act in combination and likely influence the ability to control the spread and proliferation of tumor cells within the peritoneal cavity of patients with advanced ovarian cancer.

For ovarian cancer, the role of the immune response has been well documented with immunohistochemistry. Multiple studies document a positive correlation between the number of tumor-infiltrating lymphocytes within the tumor and overall survival (OS) (108–110). The absence of CTL infiltration (CTL) also predicts platinum resistance (111). More recently, genomic profiling studies also support using immunophenotype as a method to predict response to therapy and clinical outcomes (112–114). However, most of the published studies document limited infiltration of NK cells within the primary ovarian tumor and cells that suppress immune response and support tumor growth dominate (108, 115–120). The presence of infiltrating NK cells impact on OS is also controversial. Infiltrating NK cells have largely not been associated with better outcomes, and in one case, predicted worse OS (121). However, it was recently shown that $CD103^+$ tumor-infiltrating NK cells were almost always found with $CD8^+$ T cells and were the second best predictor of positive outcomes in primary ovarian cancer (122). In light of this evidence, further study regarding the role of infiltrating NK cells is warranted. Regardless of the uncertainty of the role of NK cells and OS, freshly isolated NK cells from the tumor are drastically impaired through a variety of mechanisms (123, 124).

Within the tumor tissue, NK cells have complex interactions with other immune cells with suppressive functions, including myeloid-derived suppressor cells (MDSCs) and regulatory T cells ($T_{reg}$) (125). The MDSCs typically suppress effector function of T cells, thereby promoting tumor growth. In mice, a subset of MDSCs expresses the NKG2D ligand Rae-1 and is capable of elimination through NK cell-mediated cytolysis (126). If present, NK cells are also potent producers of IFN-γ and prevent the macrophage polarization toward the M2 phenotype that support tumor progression (127).

Another mechanism interfering with NK effector function within the microenvironment is the secretion of immunosuppressive cytokines. The hypoxic environment induces transcription of interleukin (IL)-8, a chemokine important for tumor growth, angiogenesis, and metastasis (128–130). Women with ovarian cancer have significantly elevated concentrations of IL-8 compared to benign controls (131). In addition to promoting angiogenesis and tumor growth, *in vitro* studies demonstrate an immunosuppressive effect of IL-8 by inhibiting TNF-induced apoptosis (132). In addition, the cytokine transforming growth factor-β (TGF-β) can contribute to the immunosuppressive microenvironment (133). Overproduction of TGF-β by tumor cells suppresses CD16-mediated NK cell IFN-γ production and ADCC (134). These immunosuppressive cytokines attenuate NK cell effector function and limit the antitumor response.

## Cervical Carcinoma

Cervical cancer is a human papillomavirus (HPV)-induced cancer. Mediated by an adaptive immune response against viral proteins, the greatest success involving immunotherapy in gynecologic malignancies is the development of vaccines against HPV and prevention of cancer (135). The two early viral proteins E6 and E7 are defined tumor-associated antigens and are processed and expressed on MHC class I molecules. However, HPV-induced cervical cancers often show altered expression of MHC class I molecules resulting in the inability of CTLs to recognize the peptide epitopes (88, 136–138). The HPV16 E6 and E7 oncoproteins also inhibit NK cell IL-18-induced IFN-γ production likely contributing to viral pathogenesis (139). In addition, HPV infection is non-lytic and produces only a modest inflammatory infiltrate of macrophages and lymphocytes (140). These changes likely affect the efficacy of the innate immune response and provide opportunities to escape immune surveillance. However, the "loss of self" may render these HPV-related cancers susceptible to NK cell attack.

Very little is known in regard to NK cell function and phenotype in women with cervical cancer. Freshly isolated peripheral blood NK cells in women with cervical cancer and benign healthy controls demonstrated no significant functional differences until the patient had distant metastatic disease

(stage IVb) (141). Another study demonstrated infiltrating NK cells of patients with cervical cancer were present and of the $CD56^{bright}CD16^{-}$ phenotype. They also observed the upregulation of the DNAM-1 ligand CD155 and the NKG2D ligand MICA in cervical cancer but not in cervical intraepithelial neoplasia or normal controls (142). A follow-up study demonstrated keratinocyte expression of HPV16 E6 and E7 produced rapid induction of intracellular adhesion molecule-1 protein levels (143). NK cells recognize expression of these ligands and adhesion molecules and may be a promising strategy to target for the treatment of cervical cancer.

Interestingly, certain combinations of KIRs and HLA loci associated with NK cell activation increase the risk of developing cervical cancer (144). Specifically, the presence of the activating KIR3DS1 on NK cells in the absence of ligands for inhibitory KIRs results in an increased risk of cervical neoplasia. In constrast, NK cell effector inhibition mediated by KIR2DL1 and KIR3DL1 in the absence of KIR3DS1 results in protection from cervical neoplasia. KIR receptors and HLA ligands interact through an epistatic relationship in which HLA ligands activate a genetic molecular cascade through the KIR receptor that influences NK functionality. This KIR/HLA interaction suggests that an inappropriate inflammatory response, mediated by NK cell KIR-ligand interactions, may lead to tumor progression. The precise role of NK cells in the context of cervical cancer is far from being defined.

### Endometrial Carcinoma

Endometrial carcinoma arises from the lining of the uterine cavity and is the fourth most common malignancy in women (95). Most women are diagnosed with low-grade, early stage disease and are cured following surgery. There is very little information on the microenvironment of uterine NK (uNK) cells and cancer. There is much more known about NK cell biology and pregnancy.

Uterine NK cells are a tissue-specific, specialized population of cells that make up large percentage of both endometrial and decidual lymphocytes (145). uNK cells are almost exclusively $CD56^{bright}CD16^{-}$, although they contain cytotoxic granules (146). uNK cells are thought to contribute to immunosuppressive mechanisms during pregnancy when immune tolerance is vital. As a result, these uNK cells also display less cytotoxicity against tumor targets compared to peripheral NK cells (147). uNK cells are not only immunosuppressive during pregnancy in order to protect the fetus but also may play a key role in modulating fetal growth, with activated uNK cells at the maternal–fetal interface producing factors that play a role in the regulation of trophoblast invasion and uterine vascular remodeling. These roles are critical to placental formation and healthy gestation (148, 149). A number of interesting questions remained unanswered with respect to NK cells and uterine cancer: (i) does the immunosuppressive nature of the maternal–fetal interface contribute to developing uterine carcinoma? (ii) Do peripheral NK cells migrate into the uterine cavity with tumorigenesis? (iii) Are uterine tumor cell lines susceptible to NK cell killing? More complete knowledge of the biology and function of uNK cells in endometrial cancer is required prior to developing strategies of NK cell immunotherapy for this malignancy.

## NK IMMUNOTHERAPY IN GYNECOLOGIC MALIGNANCIES

Advances in understanding the NK cell biology and function over the last few decades have resulted in promising new immunotherapeutic approaches for gynecologic malignancies, in particular ovarian cancer. There is scant research published on NK cell-based immunotherapy in cervical and uterine carcinoma at this time. In this section, we will review recent advances in NK cell-based immunotherapy for all gynecologic malignancies highlighting the opportunities and challenges for each cancer.

### Early Trials Using Biologic Response Modifiers and Cytokine Therapy

Early clinical trials in ovarian cancer patients aimed to improve the antitumor activity of immune cells through intraperitoneal injection of biological response modifiers, including *Corynebacterium parvum*, bacillus Calmette-Guerin, leukocyte interferons (IFN), and irradiated autologous and allogeneic tumor cells (150). Overall, these agents had limited success in treatment response with relative toxic side effects. Another novel strategy using an attenuated strain of influenza virus to infect ovarian cancer tumor cell lines was later developed. The nonviable extracts from the tumor cells, termed viral oncolysate, were isolated and then injected intraperitoneally (IP) into patients with ovarian cancer with both clinical and pathological responses noted (151, 152). Follow-up studies noted the viral oncolysate enhanced the NK cell response (153). Although better tolerated, the treatment had limited clinical responses.

Advancement in recombinant DNA technologies led to the purified production of cytokines and their use to treat a variety of malignancies. Today, cytokines are easy to manufacture and administer. While the central goal of cytokine therapy is to potentiate the autologous antitumor response *in vivo*, they lack specific immunomodulatory effects (154). **Table 1** lists selected clinical trials in ovarian cancer evaluating cytokine therapy and NK cell response if reported. The first generation of cytokines were recombinant (r) IFN-α, rIFN-γ, and rIL-2. The published results of IP therapy with recombinant IFN with or without chemotherapy have been comprehensively reviewed by Freedman and colleagues (155). They also reported on eight clinical trials evaluating IP therapy with IL-2 alone or in combination with cellular therapy (discussed below). The results demonstrated IP immunotherapy with cytokines was tolerated but had varying levels of success. Two important features were identified: (i) response was dependent on remaining tumor burden prior to initiation of therapy and (ii) efficacy of first-line therapy is critical in these patients. Most of these early trials did not assess NK cell response to therapy.

Recently, a randomized phase III trial of 847 women with stage III or IV ovarian cancer evaluating front-line combination carboplatin/paclitaxel plus subcutaneous *Escherichia coli*-derived recombinant IFNγ-1b was stopped early following a second

**TABLE 1** | Natural killer (NK) cell findings from clinical trials of cytokine immunotherapy for ovarian cancer.

| Year | No. of patients | Population | Treatment | Phase | Clinical response | NK cell response | Reference |
|---|---|---|---|---|---|---|---|
| 1982 | 5 | Recurrent disease | IM interferons (IFN) | I | 1 patient had PR; 2 patients with SD at 12 months | Increase in NK cell activity in peripheral blood in all three patients examined | Einhorn et al. (192) |
| 1984 | 14 | Persistent disease EOC on second look laparotomy | Intraperitoneally (IP) IFN-α | I | 11 pts underwent surgical reevaluation after therapy: 4 had CR (36%), 1 PR (9%), and 6 with disease progression (55%) | NK cytotoxicity was elevated in the IP cavity | Berek et al. (98) |
| 1987 | 40 (1 OvCa) | Advanced cancer | Continuous IL-2 | I | PR in 1 OvCa pt | NR | West et al. (193) |
| 1996 | 108 | Persistent disease EOC on 2nd look laparotomy | IP IFN-γ | II | 98 evaluable pts: 31 (32%) with surgical RR, including 23 (23%) with CR | NR | Pujade-Lauraine et al. (194) |
| 2000 | 148 | First-line therapy | Cisplatin/cyclophosphamide ± SubQ IFN-γ | III | Progression-free survival (PFS) at 3 years: 38% in control to 51% in treatment; CR 56% in control vs 68% in treatment; similar toxicity | NR | Windbichler et al. (195) |
| 2005 | 44 | Maintenance following second-line | Low-dose SubQ IL-2 + RA | II | Treatment decreased VEGF and improved immune function; absolute difference of 42% between treatment and matched controls in both PFS and overall survival (OS) at 2 years; well tolerated | Treatment improved lymphocyte and NK cell counts | Recchia et al. (159) |
| 2008 | 847 | First-line therapy | Carboplatin/paclitaxel ± SubQ IFN-γ | III | Stopped early due to interim analysis: shorter OS (60 vs 70%) in pts receiving IFN-γ at time of analysis; more adverse events | NR | Alberts et al. (156) |
| 2009 | 31 | Platinum-resistant/refractory disease | IP IL-2 | II | 24 evaluable pts: 6 (25%) with surgical RR, including 4 CR (17%); well tolerated | NR | Vlad et al. (196) |
| 2010 | 65 | Maintenance following second-line | Low dose SubQ IL-2 + RA | II | Overall RR 57%, including 4 (6%) CR; median PFS 23.2 months and median OS 52.8 months | Treatment improved NK cell counts and decreased VEGF | Recchia et al. (158) |

interim analysis (156). At the time of analysis, patients receiving IFNγ-1b plus chemotherapy compared to chemotherapy alone demonstrated significantly shorter OS (60 vs 70%). It should be noted IFNγ-1b has biological activity identical to natural human IFN-γ (157). The authors speculate IFNγ-1b may have resulted in activation of $T_{regs}$ and immunosuppression. They also suggest treatment with IFNγ-1b is more toxic and leads to decreased treatment adherence or dose reductions of chemotherapy. The ability to complete all six cycles of chemotherapy was compromised in patients receiving IFNγ-1b (77 vs 83%). Regardless, it was concluded that IFNγ-1b does not have a role in first-line treatment of advanced ovarian cancer.

Finally, a phase II trial of advanced ovarian cancer patients treated with second-line therapy followed by maintenance low-dose subcutaneous IL-2 with oral 13-cis-retinoic acid reported an overall response rate of 57% (158, 159). Treatment was associated with an improvement of peripheral NK cell counts and a decrease in VEGF compared to baseline. In this cohort of 65 patients, the progression-free survival (PFS) and OS was 29 and 38%, respectively.

Similar to IL-2, the cytokine IL-15 can potently increase NK cell numbers. While IL-2 and IL-15 share common signaling mechanisms, they differentially control the development, activation, and proliferation of NK cells (160). IL-2 activates a broad range of T cells, including $T_{regs}$. In contrast, IL-15 preferentially stimulates $CD8^+$ T cells and non-terminally differentiated NK cells and has been shown to enhance NK cell function in the ovarian cancer setting (101, 161). Several clinical trials evaluating IL-15 are underway (162).

In summary, biologic response modifiers and cytokine therapy have demonstrated conflicting results in the limited clinical trials. In addition, the small number and heterogeneity of study participants limit interpretations. Additional investigations examining the role of cytokines in ovarian cancer and reporting standard immune and clinical responses is warranted.

## Adoptive Transfer of NK Cells

Initial efforts in adoptive transfer of immune cells aimed to improve the autologous antitumor responses through cytokine stimulation (155, 163–165). Immune cells removed from the peripheral blood of patients were activated with various cytokines and then infused back into the same patient. **Table 2** lists selected clinical trials in ovarian cancer evaluating adoptive transfer of NK cell-related therapy and response if reported.

The early phase I clinical trials evaluating the adoptive transfer of autologous lymphokine-activated killer (LAK) cells with high-dose IL-2 therapy demonstrated limited clinical responses with high rates of peritoneal fibrosis (165–167). Similar to LAK immunotherapy, cytokine-induced killer (CIK) cells arise from peripheral blood mononuclear cell cultures with stimulation of anti-CD3 mAb, IFN-γ, and IL-2 (168). CIK cells are characterized by a mixed T-NK phenotype ($CD3^+CD56^+$) and demonstrate enhanced cytotoxic activity compared to LAK cells against ovarian and cervical cancer (169, 170).

**TABLE 2** | Natural killer (NK) cell findings from clinical trials of adoptive cellular transfer for ovarian cancer.

| Year | No. of patients | Population | Treatment | Phase | Clinical response | NK cell response | Reference |
|---|---|---|---|---|---|---|---|
| 1989 | 20 (7 OvCa) | NR | Autologous IP lymphokine-activated killer (LAK) + IL-2 | I | 2/7 OvCa pts had PR; extended therapy was hampered by IP fibrosis | NR | Urba et al. (165) |
| 1990 | 24 (10 OvCa) | Recurrent disease | Autologous IP LAK + IL-2 | I | 2/10 laparoscopic documented PR; 8/10 no response; progressive IP fibrosis | NR | Steis et al. (166) |
| 1990 | 10 | Recurrent disease | Autologous IP LAK + IL-2 | I | 1/10 (10%) RR; dose-limiting toxicity was ascites accumulation | LAK activity correlated with $CD3^{-}CD56^{+}$ lymphocytes | Stewart et al. (167) |
| 1991 | 7 | Advanced or recurrent disease | Cyclophosphamide, ACT of tumor-infiltrating lymphocytes (TIL) | II | 5/7 (71%) had RR, including 1 (14%) CR | NR | Aoki et al. (197) |
| | 10 | | Cisplatin, ACT of TIL | | 9/10 (90%) had RR, including 7 (70%) CR | | |
| 2011 | 20 (14 OvCa) | Refractory disease (4+ prior therapies) | Allogeneic IV NK + IL-2 | II | Well tolerated overall, but 2 severe adverse events including 1 death; 4/14 (29%) OvCa pts had RR, 8/14 (57%) with SD, and 1/14 (7%) with PD | No sustained *in vivo* expansion of NK cells was noted | Geller et al. (173) |
| 2014 | 92 | First-line therapy | Primary debulking surgery, carboplatin/paclitaxel ± autologous IV cytokine-induced killer (CIK) cells | III | Progression-free survival: 37.7 vs 22.2 months favor CIK ($p = 0.004$); overall survival 61.5 vs 55.9 months (NS); well tolerated | NKT ($CD3^{+}CD56^{+}$) cells increased; NK cells decreased in CIK culture; no changes in peripheral NK cells | Liu et al. (171) |
| 2016 | 20 (2 OvCa) | Advanced or recurrent disease | Allogeneic IV NK | I | Well tolerated; 1 had SD and 1 had PD | *Ex vivo* expanded and activated NK cells were generated and safely administered | Yang et al. (174) |
| 2017 | 1 | First-line therapy | Allogeneic IV NK | Case report | PR, with CA-125 decreasing 11,270 to 580 after 6 treatments | Expanded NK cells in culture | Xie et al. (175) |

A recent phase III clinical trial investigated adoptive transfer of autologous CIK cells following primary debulking surgery and adjuvant carboplatin/paclitaxel chemotherapy (171). Advanced epithelial ovarian cancer patients ($n = 92$) were paired to receive maintenance monthly CIK transfusions ($n = 46$) vs standard of care observation ($n = 46$). Median PFS was 37.7 months in the treatment group and 22.2 months in the control group ($p = 0.004$). Median OS in the treatment group was 61.5 months, compared to 55.9 months in the control group ($p = 0.289$). The therapy was well tolerated with no grade III or IV adverse reactions. Interestingly, the proportion of $T_{regs}$ in peripheral blood decreased following two courses of immunotherapy ($p = 0.006$). While only a small, non-randomized phase III study, these results are promising and follow-up studies are warranted.

Recently, insights into the molecular mechanisms regulating NK cell function shifted the focus toward allogeneic NK cell immunotherapy. The mismatch between donor KIR repertoire and recipient MHC class I molecules can improve the antitumor activity of NK cells (172). In a phase II clinical trial, we studied haplo-identical related IV infused NK cells in patients with recurrent ovarian ($n = 14$) and breast cancer ($n = 6$) (173). Following a lymphodepleting chemotherapy regimen ± radiation, women received adoptive transfer of a CD3/CD19-depleted NK cell product and were treated with subcutaneous IL-2 injections. No successful NK cell persistence or expansion was noted, likely as a result from recipient $T_{reg}$ expansion and reconstitution following therapy. Only two other small reports using allogeneic NK cell therapy in ovarian cancer have been published (174, 175). Together, these studies suggest allogeneic NK cell therapy is feasible. However, further investigation into strategies to augment *in vivo* NK cell persistence and expansion are needed.

Future efforts generating novel NK cell products for adoptive transfer are likely to be investigated in the ovarian cancer setting. *Ex vivo* inhibition of GSK3 kinase in peripheral blood enhances CD57 expression and late-stage maturation of NK cells (176). These NK cells demonstrated significantly higher production of cytokines (TNF-α and IFN-γ) and ADCC when exposed to cancer cells. Recruitment for an ovarian cancer clinical trial using the product generated from this method has opened at the University of Minnesota. Another recent study evaluated the potency of NK cells derived from human $CD34^{+}$ hematopoietic stem and progenitor cells (HSPC) against a mouse xenograft model for ovarian cancer (177). Mice that received IP HSPC-NK cell infusions had significantly reduced tumor progression compared to controls. Finally, efforts to generate ovarian cancer specific NK chimeric antigen receptors are underway (178). These engineered proteins consist of a fused single-chain variable fragment (scFv) to an intracellular signaling domain to enhance NK effector function. A combination of these different techniques of generating NK cell products hold great promise and may make IP adoptive transfer effective against ovarian cancer following primary cytoreductive surgery and adjuvant chemotherapy.

## Other Immunotherapeutic Options to Enhance NK Cell Function

Other immunotherapeutic strategies are currently being characterized for antitumor activity (162). The development of drugs known to influence NK cell presence and function include mAb therapy, immunomodulatory drugs, vaccines (peptide, viral-based, tumor antigens, dendritic cells), and the adoptive transfer of T cells, dendritic cells, and macrophages. Even commonly used cytotoxic agents increase expression of NK cell-activating ligands and enhance NK cell recognition and killing more than others (179). A thorough discussion of each method is outside the scope of this review, but we will comment on several key issues.

Antibody-based immunotherapy has transformed the treatment of many malignancies, but is not yet standard of care for ovarian cancer. The mAbs function through two separate mechanisms. First, treatment is aimed at antigens present on tumor cells to facilitate an antitumor response through opsonization and activation of ADCC. Several tumor-associated antigens targeted with mAb for ovarian cancer have been identified, including NY-ESO-1, CA 125 (MUC16), MUC1, and epithelial cell adhesion molecule (EpCAM) (154). mAbs can also function in a non-immune-mediated manner to block vital growth and survival pathways, such as Her2/Neu, membrane folate receptor, and VEGF. Clinical trials evaluating the efficacy of mAbs should include investigations into both mechanisms.

A newer approach involves engineered bispecific antibodies and bispecific/trispecific killer engagers (BiKEs or TriKEs), which are molecules that cross-link antigens on tumor cells with CD16 on NK cells, activating and enhancing ADCC (180). One example utilized anti-CD16 scFv spliced to anti-EpCAM scFv (181). This BiKE promoted immune synapse and ADCC between NK cells and EpCAM-expressing tumor cells. More recently, a fully humanized TriKE utilized a modified IL-15 to cross-link the anti-CD16 scFv and EpCAM scFv (182). The 1615EpCAM TriKE was specific and active against EpCAM bearing ovarian cancer cells and mediated NK proliferation, sustained ADCC activity, improved lytic degranulation, and cytokine production. A TetraKE construct incorporating the cancer stem cell marker anti-CD133 scFv was recently engineered to simultaneously target EpCAM and CD133 bearing cells (183). These engineered small molecules combine the specificity of mAbs with the NK cell expansion and survival benefits of cytokine therapies *via* IL-15 into a single product. This novel strategy to target NK cells for antigen-specific immunotherapy has recently been reviewed and will hopefully prove effective in supplementing traditional therapies against gynecologic malignancies (184, 185).

Immune checkpoints are inhibitory pathways that serve to prevent self-tissue damage. During tumorigenesis, cancer cells often express ligands to bind and induce immune suppression. A number of antibodies have been developed to block checkpoint pathways expressed on certain T cells, B cells, monocytes, and NK cells, including CTL-associated protein 4, programmed death protein 1 (PD-1), TIM-3, NKG2A/CD94 complex, and CD96/CD226/TIGIT receptors (186, 187). A recent publication identified a population of NK cells within the ascites of women with ovarian cancer where PD-1 is highly expressed suggesting therapies targeting PD-1/PD-L may be effective (188). In fact, *in vitro* studies have shown that PD-1 and CD96/TIGIT blockade augments NK cell-mediated tumor lysis (189, 190). Future research is needed to clarify the effects checkpoint inhibitors have on the NK cell response and the potential to enhance adoptive NK cell immunotherapy.

## CONCLUDING REMARKS

Here, we provide an extensive overview of NK cell-based immunobiology and therapy in gynecologic malignancies. Over the past several decades, insight into biology controlling activation or inhibition has advanced the prospect of NK cell-based immunotherapy, which is just now being realized. Today, there are strategies to harness NK cell function for immunotherapy, including: (i) adoptive transfer of alloreactive NK cells, (ii) blocking NK inhibitory signals with mAb, (iii) promoting death ligand expression, and (iv) enhancing specificity *via* activation of ADCC. In addition, using drugs or cytokines to promote NK cell proliferation and function or inhibit NK cell suppressors are potential strategies. Complementary approaches also exist to manipulate the genetics geared to maximize NK cell function against specific tumor targets. One example includes viral transduction and gene transfection through electroporation technologies with the goal of increasing production of cytokines or cell receptors (191).

There are several crucial issues that require consideration for adoptive NK cell-based cancer immunotherapy that need to be highlighted. These include (i) standardizing protocols and techniques in NK cell preparation, (ii) establishing firm criteria for donor selection to improve clinical response, (iii) identifying the best method of conditioning recipients to avoid rejection and promote survival of transferred NK cells, (iv) combining NK cell-based immunotherapy with other therapies to eliminate cancer cells, and (v) enhancing understanding of tissue immunity and the tumor microenvironment (3).

Finally, early clinical studies have demonstrated promise of NK cell-based immunotherapy for gynecologic malignancies. Future research will be important to identify patients that will most likely benefit from immunotherapy and define the specific role and timing of therapy. In addition, combination approaches need to be explored and optimized before therapeutic breakthroughs can realistically be envisioned.

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LU and CD wrote the manuscript with contributions from MF and MG. JM, MF, and MG edited the manuscript.

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# Differences in Expansion Potential of Naive Chimeric Antigen Receptor T Cells from Healthy Donors and Untreated Chronic Lymphocytic Leukemia Patients

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**Introduction:** Therapy with chimeric antigen receptor T (CART) cells for hematological malignancies has shown promising results. Effectiveness of CART cells may depend on the ratio of naive ($T_N$) vs. effector ($T_E$) T cells, $T_N$ cells being responsible for an enduring antitumor activity through maturation. Therefore, we investigated factors influencing the $T_N/T_E$ ratio of CART cells.

**Materials and methods:** CART cells were generated upon transduction of peripheral blood mononuclear cells with a CD19.CAR-CD28-CD137zeta third generation retroviral vector under two different stimulating culture conditions: anti-CD3/anti-CD28 antibodies adding either interleukin (IL)-7/IL-15 or IL-2. CART cells were maintained in culture for 20 days. We evaluated 24 healthy donors (HDs) and 11 patients with chronic lymphocytic leukemia (CLL) for the composition of cell subsets and produced CART cells. Phenotype and functionality were tested using flow cytometry and chromium release assays.

**Results:** IL-7/IL-15 preferentially induced differentiation into $T_N$, stem cell memory ($T_{SCM}$: naive CD27+ CD95+), CD4+ and CXCR3+ CART cells, while IL-2 increased effector memory ($T_{EM}$), CD56+ and CD4+ T regulatory ($T_{Reg}$) CART cells. The net amplification of different CART subpopulations derived from HDs and untreated CLL patients was compared. Particularly the expansion of CD4+ $CART_N$ cells differed significantly between the two groups. For HDs, this subtype expanded >60-fold, whereas CD4+ $CART_N$ cells of untreated CLL patients expanded less than 10-fold. Expression of exhaustion marker programmed cell death 1 on $CART_N$ cells on day 10 of culture was significantly higher in patient samples compared to HD samples. As the percentage of malignant B cells was expectedly higher within patient samples, an excessive amount of B cells during culture could account for the reduced expansion potential of $CART_N$ cells in untreated CLL

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patients. Final $T_N/T_E$ ratio stayed <0.3 despite stimulation condition for patients, whereas this ratio was >2 in samples from HDs stimulated with IL-7/IL-15, thus demonstrating efficient $CART_N$ expansion.

**Conclusion:** Untreated CLL patients might constitute a challenge for long-lasting CART effects *in vivo* since only a low number of $T_N$ among the CART product could be generated. Depletion of malignant B cells before starting CART production might be considered to increase the $T_N/T_E$ ratio within the CART product.

**Keywords: chimeric antigen receptor, immunotherapy, CD19, T cell subpopulations, naive T cells, cytokines, chronic lymphocytic leukemia, T cell expansion**

## INTRODUCTION

The advent of T cells expressing chimeric antigen receptor T (CART) cells for the treatment of cancer patients represents a milestone in the field of immunotherapy (1). Various clinical trials have been undertaken in recent years, especially for relapsed or refractory CD19+ hematologic malignancies such as acute lymphoblastic leukemia, chronic lymphocytic leukemia (CLL), and non-Hodgkin lymphoma (2–7). For clinical CART application, reproducibility and safe generation of CART cells is of crucial relevance. T cells from patients (autologous) or donors (allogeneic) are genetically modified *via* viral or non-viral vectors to express a recombinant transmembrane receptor on the cell surface. The so-called chimeric antigen receptor (CAR) recognizes CD19+ malignant B cells with the extracellular antibody-derived and antigen-specific recognition domain (single chain variable fragment) (8, 9). The cytoplasmic signaling domain is constituted of a CD3zeta stimulatory domain combined to costimulatory signaling components such as CD28 (10, 11), CD137/4−1BB (12), or OX40, either alone for so-called second generation or in combination for third generation CARs (13). However, while some patients have displayed long-lasting CART responses (14), expansion and particularly persistence of CART cells in other patients have lasted only for few weeks (5, 15). Since clinical response correlates with long-term detection of the engineered T cells (16), short-term CART cells are limited in their capacity to fully eradicate cancer cells (17). The phenotype of T cells administered to patients often correlates with antitumor reactivity *in vivo* (18): Particularly, less-differentiated naive ($T_N$) and central memory ($T_{CM}$) T cells in contrast to the more differentiated effector memory ($T_{EM}$) and effector ($T_E$) T cells seems to play an essential role in CART persistence (19–21). Effectiveness of CART cells might therefore depend on the proportion of naive vs. effector cells ($T_N/T_E$ ratio) in the final CART product. It still remains to be elucidated why for some patients a high proportion of naive cells within their CART product can be expanded, whereas for others efficient expansion of this subtype could not be achieved despite optimal culture conditions.

Hence, we monitored the evolution and amplification of CART subpopulations ($T_N$, $T_{CM}$, $T_{EM}$, and $T_E$) and particularly the $T_N/T_E$ ratio derived from both healthy donors (HDs) and untreated CLL patients during CART culture. For CART generation, the most commonly used cytokine stimulation cocktails are either interleukin (IL)-7/IL-15 (22–25) or IL-2 (5). In order to assess a specific influence of those stimulating cytokines on the net amplification of CART subtypes, CART cells were generated simultaneously under both conditions. The starting cell material, i.e., peripheral blood mononuclear cells (PBMCs), from HDs as well as CLL patients was screened to analyze whether a correlation exists between the phenotypic composition of PBMCs and the subsequent expansion of highly effective, long-lasting naive CART cells during culture. We demonstrate that the expansion of naive T cells is clearly associated with the specific cellular composition of PBMCs used for CART generation and identify factors determining optimal generation of clinical CART products.

## MATERIALS AND METHODS

### CD19.CART Generation and Culture

Cryopreserved human PBMCs from HDs (from the blood bank Mannheim, DRK-Blutspendedienst Baden-Württemberg-Hessen) and untreated CLL patients (from Heidelberg University Hospital, protocol number: S-254/2016) were thawed and activated with anti-CD3/anti-CD28 antibodies (Biozol, Eching, Germany) at a concentration of $5 \times 10^5$ cells/ml culture medium. For activation of PBMCs, non-tissue culture-treated 24-well plates (Corning, Wiesbaden, Germany) had been previously coated over night with 0.5 ml of 1 µg/ml anti-CD3 and 1 µg/ml anti-CD28, diluted in Aqua ad iniect (Fresenius, Bad Homburg, Germany). Culture medium consisted of 50% RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) and 50% Click's Medium (EHAA) (Irvine Scientific, Santa Ana, CA, USA), with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) and 2 mM L-glutamine (Thermo Fisher Scientific). Two different cytokine cocktails were added on day 2 of culture: IL-7/IL-15 (R&D Systems, Minneapolis, MN, USA) vs. IL-2 (Novartis, Nuremberg, Germany), at concentrations according to our GMP protocol (IL-7: $4.4 \times 10^3$ U/ml, IL-15: 100 U/ml, IL-2: 100 U/ml). On day 3, activated T cells ($5 \times 10^5$ cells in 0.5 ml + 1.5 ml retroviral supernatant per well) were transduced with a CD19.CAR-CD28-CD137zeta third generation retroviral vector (kindly provided by Dr. Malcolm Brenner at the Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, USA) in a 24-well non-tissue culture-treated plate, previously coated with 7 µg/ml RetroNectin (Takara Bio, Shiga,

Japan) in Dulbecco's phosphate-buffered saline (PBS) (Sigma-Aldrich, Taufkirchen, Germany). Viability of CART cells was assessed through Trypan blue staining. Medium change with fresh addition of cytokines was performed on days 3, 7, 10, 14, and 17 ($1 \times 10^6$ cells/ml). CART cells were cultivated in 6-well tissue culture plates (Sarstedt, Nümbrecht, Germany) and transferred to T75 tissue culture flasks (Sarstedt) when the total cell number reached $15 \times 10^6$ cells. On days 14–17, an aliquot of CART cells was cryopreserved in FBS + 10% DMSO (Sigma-Aldrich). Total culture period lasted 20 days. The net amplification of CART cells and specific subpopulations from day 7 up to day 20 was calculated using the following formula: net amplification = absolute cell number × % CD3+ CART × % specific subpopulation.

## Cell Lines

CD19+ Burkitt lymphoma (Daudi) cells (Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were expanded in cell culture medium (RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine) in T75 tissue culture flasks (Sarstedt) at 37°C and 5% $CO_2$ in a humidified incubator (Memmert, Schwabach, Germany) and split 1:3 every three days. Cells were used as target cells for the cytotoxicity and cytokine expression assays.

## Flow Cytometry

Flow cytometric analyses were performed on days 0, 7, 10, 14, 15, 17, and 20 of CART culture. Dead cells were excluded using the LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific). Surface marker staining was performed in order to assess the corresponding subpopulation markers on $T_N$ (CD45RA+ CCR7+), $T_{CM}$ (CD45RA− CCR7+), $T_{EM}$ (CD45RA− CCR7−), and $T_E$ (CD45RA+ CCR7−) cells. The following fluorochrome-conjugated antibodies were used: CD45RA-APC, CD8-PerCP, CD8-Pacific Blue, CD95-PE-Cy5, programmed cell death 1 (PD-1)-Alexa Fluor 488, Tim-3-Brilliant Violet 421, CXCR3-Alexa Fluor 488, CD56-Alexa Fluor 488, γ/δ TCR-Brilliant Violet 421, CD25-PE-Cy7, CD20-APC-Cy7 (all from Biolegend, San Diego, CA, USA), CCR7-PE-Cy7, CD3-PE eFluor 610, CD4-Alexa Fluor 700, HLA-DR-PerCP, CD62L-eFluor 450 (all from eBioscience, San Diego, CA, USA), CD3-V500, CD27-FITC, CD45-FITC, CD19-APC, and CD34-PE (all from BD Biosciences, Franklin Lakes, NJ, USA). CART cells were stained with an anti-human goat $F(ab)_2$ IgG (H+L)-PE antibody (Dianova, Hamburg, Germany). As the $F(ab)_2$ IgG (H+L) antibody recognizes IgG molecules, present on both the CAR receptor and B cells, the following gating strategy was applied: first gate assessed viable cells, second gate CD3+ cells and third gate CD3+ CART cells (Figure S1 in Supplementary Material). Surface marker staining was performed for 30 min at room temperature. Intracellular staining was used for detection of expression of TNF-α (BV421, BD Biosciences), IFN-γ (Alexa Fluor 488, Biolegend), and IL-2 (BV510, BD Biosciences) by CART cells after coculturing with CD19+ Daudi cells for 6 h. Brefeldin A was used for blocking cytokine secretion. The percentage of FoxP3+ $T_{Reg}$ cells after stimulation with IL-7/IL-15 vs. IL-2 was measured using the FoxP3 Staining Buffer Set (Miltenyi Biotec, Bergisch-Gladbach, Germany). After staining, cells were fixed with PBS + 1% paraformaldehyde + 3 mM ethylene-diamine-tetra-acetic acid. All acquisitions of data were performed on an LSRII device (BD Biosciences) and data were analyzed using FlowJo software (FlowJo, Ashland, OR, USA).

## Chromium-51 Release Assay/Cytotoxicity Assay

Cytotoxic efficacy of the manufactured CART cells was assessed by a standard chromium (Cr-51) release assay as previously described (26). CD19+ Burkitt lymphoma (Daudi) cells (DSMZ, Braunschweig, Germany) (target cells) were labeled for 2 h with Cr-51 (Hartmann Analytic, Braunschweig, Germany) and coincubated for 4 h with CART cells (effector cells) in 96-well U-bottom microplates (Greiner Bio-One, Frickenhausen, Germany). Maximum Cr-51 release of target cells was measured following complete lysis by adding PBS + 1% Triton X-100 (Sigma-Aldrich), whereas background was assessed *via* spontaneous release of Cr-51 of target cells kept in culture medium without the addition of CART cells. Effector to target cell ratios ranged from 10:1 to 1:1. Cr-51 release was measured in a liquid scintillation counter (PerkinElmer, Waltham, MA, USA) after adding Ultima Gold liquid scintillation cocktail (PerkinElmer) to a culture supernatant aliquot. Experiments were performed in triplicates. Cytotoxicity was calculated as percentage of specific lysis according to the following formula: % specific lysis = (Cr-51 release in the test well − spontaneous Cr-51 release)/(maximum Cr-51 release − spontaneous Cr-51 release) × 100.

## Statistical Analysis

Statistical analyses were performed using SPSS software (IBM, Armonk, NY, USA). Equal variances between values from HDs vs. untreated CLL patients were determined by an F-test and subsequent *p*-values were calculated using the two-tailed independent samples t-test. In order to compare the influence of the cytokine cocktails IL-7/IL-15 vs. IL-2, a paired samples t-test was used. Graphs were designed by Excel (Microsoft, Redmond, WA, USA) and Keynote (Apple, Cupertino, CA, USA). If not otherwise mentioned, results are displayed as mean ± SD. Correction for multiple comparisons was performed using the Holm-Bonferroni method.

# RESULTS

## Screening of HDs and Patients

The cellular composition of cryopreserved human PBMCs for CART production from 24 HDs and 11 untreated CLL patients was analyzed: the percentage of B cells as well as CD3+ T cells within lymphocytes, the proportions of CD4+ and CD8+ cells and the CD4:CD8 ratio were measured. Moreover, the CD4+ naive, CD8+ naive and the total naive cells within the CD3+ fraction as well as the absolute naive cell number were determined (**Table 1**). Within the cells of HDs, CD4:CD8 ratios were heterogeneously distributed ranging from 0.4 to 5.3. In 8 out of

**TABLE 1** | Screening of HDs and CLL patients (P) for lymphocyte subsets within PBMCs.

| **A healthy donors** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***n* = 24** | **Subsets within lymphocytes (%)** | | | | | | | | **Absolute number naive CD3+ T cells (per 100 lymphocytes)** |
| | **B cells** | **CD3+** | **% total CD3+ T cells** | | | | | | |
| | | | **CD4+** | **CD8+** | **CD4:CD8 ratio** | **CD4+ naive** | **CD8+ naive** | **Total naive** | |
| HD 1 | 11.4 | 82.0 | 66.9 | 29.9 | 2.2 | 30.0 | 9.2 | 39.4 | 32.3 |
| HD 2 | 17.4 | 77.3 | 44.6 | 44.5 | 1.0 | 21.7 | 14.2 | 36.4 | 28.1 |
| HD 3 | 19.7 | 76.2 | 31.2 | 55.2 | 0.6 | 6.9 | 26.7 | 34.1 | 26.0 |
| HD 4 | 10.1 | 76.6 | 61.2 | 35.5 | 1.7 | 20.0 | 10.3 | 30.5 | 23.4 |
| HD 5 | 11.0 | 75.3 | 49.6 | 45.6 | 1.1 | 15.0 | 14.7 | 30.0 | 22.6 |
| HD 6 | 8.1 | 82.1 | 57.5 | 37.7 | 1.5 | 13.7 | 12.3 | 26.2 | 21.5 |
| HD 7 | 26.6 | 64.1 | 43.1 | 50.2 | 0.8 | 10.0 | 23.0 | 33.4 | 21.4 |
| HD 8 | 15.5 | 80.5 | 35.6 | 59.8 | 0.6 | 7.8 | 17.3 | 25.3 | 20.4 |
| HD 9 | 8.8 | 86.4 | 81.1 | 15.2 | 5.3 | 20.4 | 2.9 | 23.4 | 20.2 |
| HD 10 | 8.1 | 82.4 | 54.3 | 36.7 | 1.5 | 17.4 | 5.8 | 23.4 | 19.3 |
| HD 11 | 20.0 | 74.6 | 48.0 | 45.5 | 1.1 | 10.5 | 14.7 | 25.5 | 19.0 |
| HD 12 | 16.5 | 78.3 | 55.7 | 42.3 | 1.3 | 18.9 | 5.1 | 24.2 | 18.9 |
| HD 13 | 19.1 | 66.1 | 46.7 | 49.3 | 0.9 | 12.5 | 15.2 | 27.9 | 18.4 |
| HD 14 | 22.8 | 69.3 | 54.8 | 41.5 | 1.3 | 17.0 | 8.7 | 26.0 | 18.0 |
| HD 15 | 9.3 | 90.0 | 36.6 | 55.0 | 0.7 | 8.8 | 10.3 | 19.5 | 17.6 |
| HD 16 | 20.5 | 64.1 | 69.4 | 28.0 | 2.5 | 18.6 | 4.5 | 23.2 | 14.9 |
| HD 17 | 36.3 | 58.0 | 43.8 | 45.8 | 1.0 | 10.4 | 11.7 | 22.6 | 13.1 |
| HD 18 | 17.8 | 72.4 | 62.8 | 33.7 | 1.9 | 12.3 | 5.5 | 17.8 | 12.9 |
| HD 19 | 20.2 | 58.4 | 49.4 | 38.0 | 1.3 | 15.5 | 5.8 | 21.5 | 12.6 |
| HD 20 | 24.1 | 76.0 | 28.0 | 65.0 | 0.4 | 5.8 | 10.5 | 16.5 | 12.5 |
| HD 21 | 15.8 | 64.2 | 48.4 | 42.4 | 1.1 | 14.2 | 4.9 | 19.2 | 12.3 |
| HD 22 | 17.4 | 74.2 | 44.9 | 51.2 | 0.9 | 10.6 | 2.5 | 13.3 | 9.9 |
| HD 23 | 16.2 | 74.4 | 48.3 | 48.3 | 1.0 | 5.6 | 4.4 | 10.2 | 7.6 |
| HD 24 | 54.5 | 41.5 | 43.6 | 51.3 | 0.9 | 8.3 | 9.3 | 18.1 | 7.5 |
| **B CLL patients** | | | | | | | | | |
| ***n* = 11** | | | | | | | | | |
| P 1 | 92.8 | 6.9 | 51.7 | 40.1 | 1.3 | 13.6 | 6.3 | 22.0 | 1.5 |
| P 2 | 96.1 | 3.7 | 55.2 | 38.4 | 1.4 | 24.8 | 11.5 | 37.5 | 1.4 |
| P 3 | 92.0 | 6.3 | 64.5 | 26.0 | 2.5 | 17.0 | 1.5 | 19.2 | 1.2 |
| P 4 | 71.5 | 23.8 | 32.3 | 65.6 | 0.5 | 0.3 | 3.5 | 3.9 | 0.9 |
| P 5 | 94.7 | 2.3 | 57.1 | 31.5 | 1.8 | 22.3 | 5.2 | 29.8 | 0.7 |
| P 6 | 92.5 | 5.5 | 42.4 | 48.2 | 0.9 | 6.7 | 5.0 | 13.1 | 0.7 |
| P 7 | 93.8 | 5.6 | 41.0 | 56.0 | 0.7 | 9.0 | 2.8 | 12.8 | 0.7 |
| P 8 | 95.3 | 3.6 | 55.6 | 32.8 | 1.7 | 13.7 | 1.7 | 17.3 | 0.6 |
| P 9 | 92.5 | 2.0 | 60.2 | 31.8 | 1.9 | 18.4 | 3.7 | 24.8 | 0.5 |
| P 10 | 80.6 | 11.9 | 44.0 | 51.1 | 0.9 | 1.4 | 2.5 | 4.6 | 0.5 |
| P 11 | 95.9 | 2.6 | 29.3 | 64.4 | 0.5 | 2.3 | 2.3 | 5.7 | 0.1 |

*Cryopreserved PBMCs from HDs (n = 24) (**A**) and untreated CLL patients (n = 11) (**B**) were analyzed. The number of B cells as well as CD3+, CD4+, and CD8+ T cells, the CD4:CD8 ratio, CD4+ and CD8+ naive T cells and the absolute number of naive CD3+ T cells per 100 lymphocytes are listed. Data from HDs and patients with chronic lymphocytic leukemia (CLL) are shown from highest to lowest frequency of naive T cells (last column).*

24 (33%) HDs the CD4:CD8 ratio was <1, i.e., more CD8+ than CD4+ cells were found within the peripheral blood. The CD3+ cell proportion within total PBMCs ranged from 41.5 to 90.0% (mean ± SD: 73 ± 11%). The percentage of naive cells within the CD3+ fraction ranged from 10.2 to 39.4% (mean ± SD: 24 ± 7%) and did not correlate with the percentage of CD3+ cells or with the CD4:CD8 ratio.

Within cells of patients, CD4:CD8 ratios were also heterogeneously distributed ranging from 0.5 to 2.5. In 5 out of 11 (45%) patients the CD4:CD8 ratio was <1. Given that analyzed patients were untreated CLL patients containing high percentages of B cells within their PBMCs, the CD3+ cell proportion was significantly lower compared to HDs ($p = 2.35E\text{-}19$) and ranged from 2.0 to 23.8% (mean ± SD: 7 ± 6%). The percentage of naive cells within the CD3+ fraction ranged from 3.9 to 37.5% (mean ± SD: 17 ± 11%) and was significantly lower in comparison to HDs ($p = 0.026$).

Following this screening for subpopulations within the PBMCs, we selected individuals from both HDs (HD 7, HD 10, HD 21; $n = 3$) and untreated CLL patients (P 2, P 4, P 5; $n = 3$) for the production of CART cells.

## Generation of CART Cells from HDs and Patients

Following screening, CART cells were generated from six different donors with comparable starting frequencies of $T_N$, i.e., three

HDs and three untreated CLL patients. Thawed cryopreserved PBMCs were stimulated with anti-CD3/anti-CD28 antibodies plus either IL-7/IL-15 or IL-2 and cultured *ex vivo* for up to 20 days. The evolution of specific CART subpopulations over time was assessed by phenotypic analyses on days 0, 7, 10, 14, 17, and 20. Transduction efficiency on day 7 of culture was comparable and averaged 54 ± 32% and 52 ± 32% for IL-7/IL-15 and IL-2 cultures, respectively.

The net amplification factor of CART cells takes into account the absolute cell number, the percentage of CD3+ cells as well as the transduction efficiency from day 7 until day 20 of culture and was used to assess the expansion rate of CART cells throughout culture. **Figure 1** displays the expansion levels of CART cells for both HDs and untreated CLL patients after stimulation with IL-7/IL-15 or IL-2. When stimulated with IL-7/IL-15, the expansion rate of CART cells from HDs and patients showed no significant difference [net amplification on final culture day 20 (IL-7/IL-15): 12-fold (HDs) and 14-fold (CLL patients)]. When stimulated with IL-2, however, CART cells of patients showed a higher expansion rate compared to CART cells of HDs [on final culture day 20 (IL-2): 26-fold expansion (CLL patients) vs. 9-fold expansion (HDs)].

Subsequently, expansion of different subpopulations of CART cells during culture, i.e., $T_N$, $T_{CM}$, $T_{EM}$, and $T_E$, was investigated (**Figure 2**). The naive CART cells stimulated with IL-7/IL-15 were the subpopulation that showed the most pronounced differences when HDs and patients were compared, showing statistical significance on days 10, 14, and 17. While $CART_N$ cells from HDs expanded notably between day 7 and day 20 (expansion level day 10: 5-fold, day 14: 25-fold, day 17: 67-fold, day 20: 81-fold), $CART_N$ cells from untreated CLL patients showed a reduced capacity to expand [expansion level day 10: 2-fold ($p = 0.019$), day 14: 5-fold ($p = 0.002$), day 17: 8-fold ($p = 0.012$), day 20: 14-fold]. When stimulated with IL-2, expansion levels of $CART_N$ cells were lower compared to IL-7/IL-15 stimulation, e.g., on day 14: 25-fold expansion (IL-7/IL-15) vs. 5-fold expansion (IL-2) ($p = 0.004$).

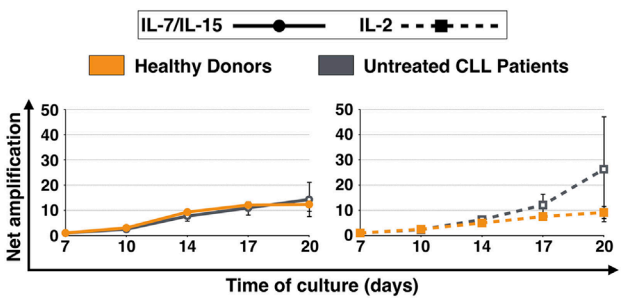



**FIGURE 1** | Net amplification of all chimeric antigen receptor T (CART) cells during culture. CART cells were generated from cryopreserved peripheral blood mononuclear cells of three healthy donors ($n = 3$, orange lines) and three untreated chronic lymphocytic leukemia patients ($n = 3$, gray lines) after activation of T cells with anti-CD3/anti-CD28 antibodies (days 0–3), transduction with a CD19.CAR-CD28-CD137zeta retroviral vector (day 3), and culture with either interleukin (IL)-7/IL-15 ($n = 3$, solid lines) or IL-2 ($n = 3$, hatched lines) (days 2–20). The net amplification of all CD3+ CART cells were determined by flow cytometry for each treatment condition and time point as indicated. Mean values of all twelve cell samples ± SDs are plotted.

The most stable subpopulation during culture was the central memory subset. $T_{CM}$ showed no difference of expansion when stimulated with the two different cytokine stimulation cocktails (day 20: twofold expansion for both IL-7/IL-15 and IL-2 stimulation).

Until day 14, also $T_{EM}$ CART cells displayed similar expansion patterns independent of the cytokine cocktail used. After day 14, however, $T_{EM}$ derived from patients showed higher proliferation levels compared to HDs [highest effect with IL-2: 44-fold expansion (patients) vs. 15-fold expansion (HDs)].

Finally, $T_E$ cells were the subpopulation of CART cells that expanded the most during culture: the amplification factor for $T_E$ was above 75 for HDs and the strongest proliferation was detected for untreated CLL patients [day 20: 296-fold expansion (IL-7/IL-15), 405-fold expansion (IL-2)].

The expansion of the naive CART subpopulation was further evaluated by analyzing the evolution of the CD4+ and CD8+ fractions of HDs and untreated CLL patients (**Figure 3**). For both CD4+ and CD8+ $CART_N$ cells, a more pronounced expansion during culture was detected when they were derived from HDs. CD8+ expansion differences were significant on day 14 under stimulation with IL-7/IL-15 [20-fold (HDs) vs. 7-fold expansion (patients) ($p = 0.002$)]. As for CD4+ $CART_N$ cells, they showed significantly higher proliferation values from day 10 until the end of the culture under stimulation with IL-7/IL-15 [day 10: 7-fold (HDs) vs. 2-fold (patients) expansion ($p = 0.0005$), day 14: 42-fold (HDs) vs. 3-fold (patients) expansion ($p = 0.0007$), day 17: 77-fold (HDs) vs. 6-fold (patients) expansion ($p = 0.004$), and day 20: 62-fold (HDs) vs. 8-fold (patients) expansion ($p = 0.005$)].

Although the effect under stimulation with IL-7/IL-15 was marked, stimulation with IL-2 also led to a higher proliferation rate of CD4+ and CD8+ $CART_N$ cells from HDs in comparison to untreated CLL patients (day 20: CD4 + $CART_N$: 10-fold expansion; CD8+ $CART_N$: 36-fold expansion). CD4+ $CART_N$ cells of untreated patients, in turn, were not stimulated at all and even decreased with a net amplification of <1, whereas CD8+ $CART_N$ cells showed a higher amplification rate (day 20: 23-fold expansion). Overall, the CD4+ naive CART expansion rate for HDs stimulated with IL-7/IL-15 on day 14 was significantly higher in comparison to IL-2 ($p = 0.008$).

## Detailed Phenotypic Analysis: IL-7/IL-15 vs. IL-2

We analyzed which CART phenotype was preferentially stimulated by IL-7/IL-15 vs. IL-2 on day 17. Out of the six donors tested, it was evaluated how many presented a higher absolute cell number for a specific marker under stimulation with the two cytokine cocktails (distribution for a total of 20 CART subtypes depicted in **Figure 4A**; the mean percentages of CART cells of each subgroup for HDs and untreated CLL patients is illustrated in **Figure 4B**).

Impact of IL-7/IL-15: six out of six donors showed higher absolute $T_N$, stem cell memory ($T_{SCM}$), CD4+ and CXCR3+ CART cell numbers compared to generation in IL-2. $T_{SCM}$ represent a subset

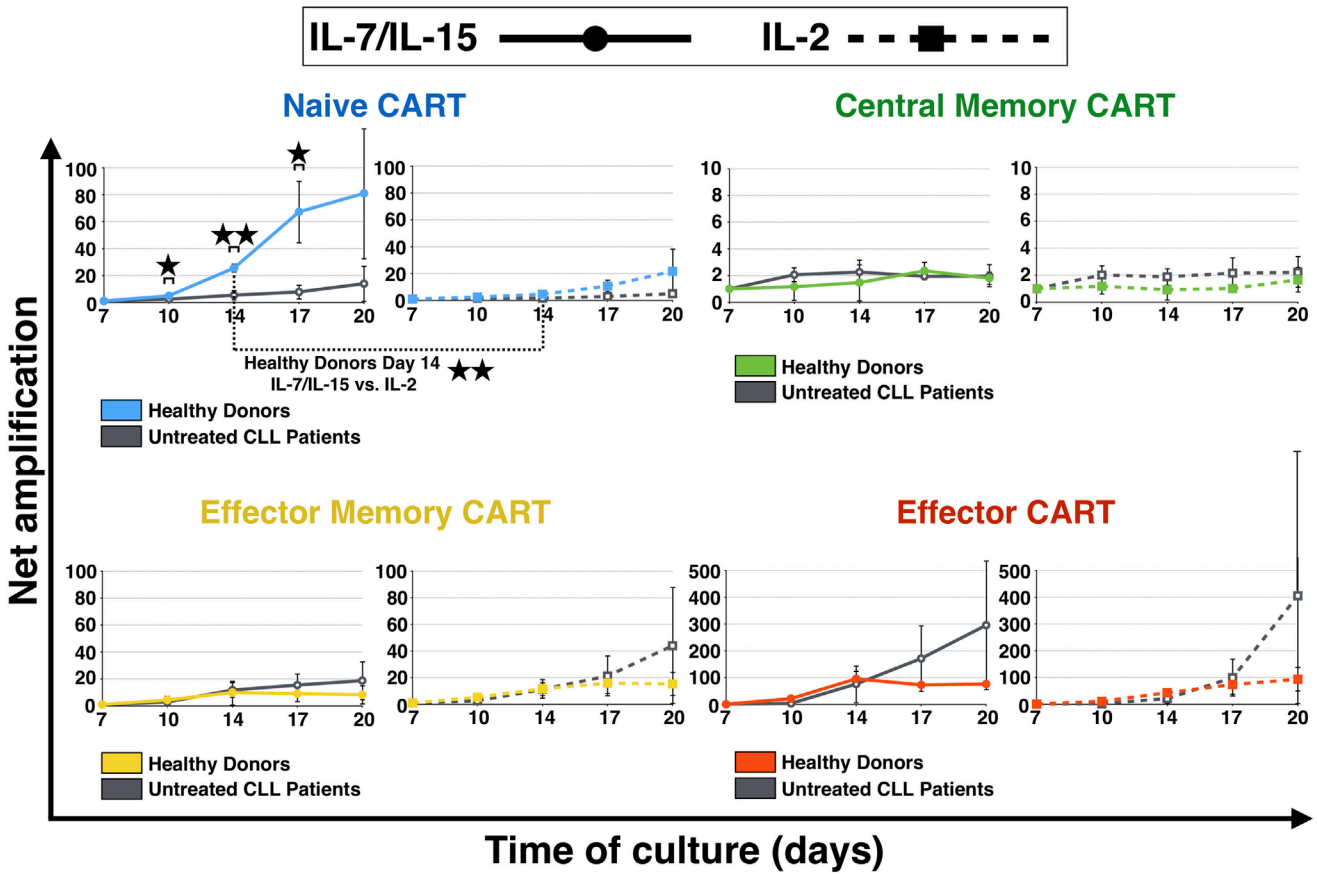



**FIGURE 2** | Net amplification of chimeric antigen receptor T (CART) subpopulations during culture. The net amplification of $T_N$ (CD45RA+ CCR7+), $T_{CM}$ (CD45RA − CCR7+), $T_{EM}$ (CD45RA − CCR7−), and $T_E$ (CD45RA+ CCR7−) CART cells from healthy donors ($n = 3$, colored lines) and untreated chronic lymphocytic leukemia patients ($n = 3$, gray lines) was determined. The cellular subsets were also compared according to the mode of stimulation, either with interleukin (IL)-7/IL-15 ($n = 3$, solid lines) or with IL-2 ($n = 3$, hashed lines). Mean values of the net amplifications of the various cellular subsets were calculated for each group and are represented with SD. Significance is represented as * for $p < 0.05$ and ** for $p < 0.01$. Exact data are specified in the text.

of naive T cells and are characterized by the expression of CD27 and CD95 (27, 28). 87% of $T_N$ cells in HDs and 69% in untreated CLL patients were $T_{SCM}$ cells.

Five out of six donors (three HDs and two untreated CLL patients) had increased numbers of CART cells expressing CD95, CD62L, and γ/δ TCR and five out of six (two HDs and three untreated CLL patients) displayed a high absolute cell number of $T_E$ and PD-1+ CART cells. Four out of six donors (three HDs and one untreated CLL patient) had higher levels of $T_{CM}$, HLA-DR+, Tim-3+, CD19+, and CD34+ CART cells and four out of six (two HDs and two patients) presented a higher number of CD4+ CD8+ CART cells. However, for both the CD19 and the CD34 markers, the percentage of positive CART cells was overall very low for both cytokine cocktails (expressed on levels <0.2%).

The three HDs presented a higher absolute cell number of CD8+ CART cells after stimulation with IL-7/IL-15, while the three untreated CLL patients had higher levels of CD8+ CART cells after stimulation with IL-2.

Impact of IL-2: six out of six donors (three HDs and three untreated CLL patients) displayed an increased proportion of CD4+ $T_{Reg}$ and CD56+ CART cells, five out of six (three HDs and two untreated CLL patients) presented a higher absolute cell number of $T_{EM}$ CART cells and four out of six donors (two HDs and two untreated CLL patients) had an increased proportion of CD4−CD8− cells within their CART product compared to generation in IL-7/IL-15.

## Functional Evaluation of CART Cells

The cytotoxic efficacy of CART cells as well as non-transduced T cells was measured *via* chromium (Cr-51) release assay. Daudi cells were chosen as CD19+ target cell line and fresh CART cells were compared to freshly thawed CART cells. Additionally, the difference in lytic activity of CART cells stimulated with IL-7/IL-15 vs. IL-2 was assessed and is represented in **Figure 5A**. The effector:target ratios were 1:1, 2.5:1 as well as 10:1 and the number of viable CART cells was adjusted to the respective ratio. Fresh

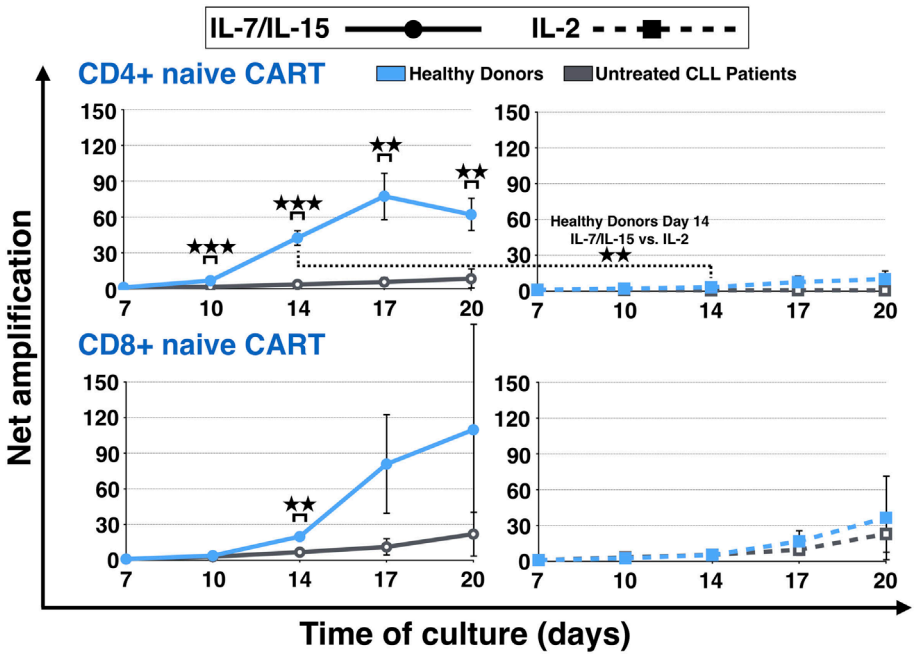



**FIGURE 3** | Net amplification of CD4+ and CD8+ naive chimeric antigen receptor T (CART) cells during culture. The net amplification of the CD4+ and CD8+ naive CART subtypes from healthy donors ($n = 3$, blue lines) and untreated chronic lymphocytic leukemia patients ($n = 3$, gray lines) was determined. The cellular subsets were also compared according to the mode of stimulation, either with interleukin (IL)-7/IL-15 ($n = 3$, solid lines) or with IL-2 ($n = 3$, hashed lines). Mean values of the net amplifications of the various cellular subsets were calculated for each group and are represented with SD. Significance is represented as ** for $p < 0.01$ and *** for $p < 0.001$. Exact data are specified in the text.

CART cells showed the highest cytotoxic efficacy and reached significance for both IL-7/IL-15 and IL-2 at the 10:1 ratio in comparison to non-transduced cells. At the 10:1 ratio, average lysis by fresh CART cells under stimulation with IL-7/IL-15 was 31 ± 13% (non-transduced T cells: 2 ± 4%, $p = 0.017$), whereas stimulation with IL-2 mediated lysis of 54 ± 19% (non-transduced T cells: 9 ± 12%, $p = 0.026$). Freshly thawed CART cells were less effective than fresh cells and showed a lytic activity of 21 ± 19 and 36 ± 27% at the 10:1 ratio, for stimulation with IL-7/IL-15 vs. IL-2, respectively. Moreover, the significantly higher lysis for fresh CART cells stimulated with IL-2 in comparison to those stimulated with IL-7/IL-15 was observable at every effector:target ratio (1:1 ratio: $p = 0.028$, 2.5:1 ratio: $p = 0.024$, 10:1 ratio: $p = 0.026$).

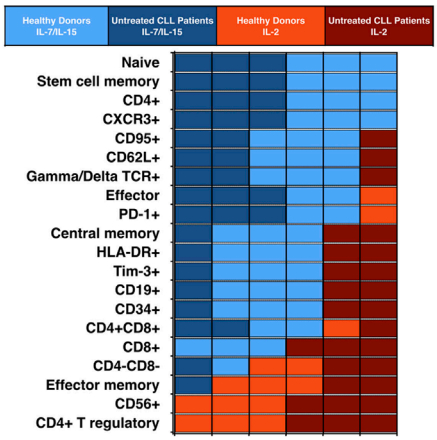
**Figure 5B** displays the ability of IL-7/IL-15 and IL-2 to induce cytokine-expressing CART cells on day 15 of culture. The absolute cell numbers were compared. IL-7/IL-15 led to a higher population of IFN-γ, IL-2, and TNF-α producing CD4+ as well as IFN-γ releasing CD8+ CART cells in three out of three donors (cells derived from one HD, two CLL patients) compared to IL-2. For IL-2 and TNF-α producing CD8+ CART cells, one donor displayed higher levels under stimulation with IL-2. **Figure 5C** displays the mean percentage of CART cells expressing IFN-γ, IL-2, and TNF-α on day 15. Significantly more CD8+ CART cells were expressing IFN-γ when compared to CD4+ cells. This higher cytokine production was observable for both IL-7/IL-15 ($p = 0.009$) and IL-2 stimulation ($p = 0.002$).

## Suboptimal Expansion of Naive CART Cells from Untreated CLL Patients

In order to better understand why it was difficult to expand $CART_N$ cells derived from PBMC samples from untreated CLL patients, we evaluated the expression of exhaustion markers on CART cells during culture. **Figure 6** illustrates the expression of the exhaustion marker PD-1 by the CART subpopulations at day 10. Samples from HDs stimulated with IL-7/IL-15 and IL-2 as well as samples from untreated CLL patients also stimulated with both cytokine cocktails were compared. Overall, CART cells derived from untreated patients displayed a higher percentage of PD-1 on their cell surface when compared to HDs [IL-7/IL-15: 23 ± 6% PD-1+ CART cells (HDs) vs. 47 ± 15% (untreated CLL patients); IL-2: 23 ± 7% PD-1+ CART cells (HDs) vs. 47 ± 22% (untreated CLL patients)]. The highest expression of PD-1 was detected on $T_N$ [71 ± 24% of exhausted cells (patients) vs. 16 ± 6% (HDs) ($p = 0.018$)] and $T_{CM}$ [62 ± 13% of exhausted cells (patients) vs. 25 ± 3% (HDs) ($p = 0.009$)] CART cells stimulated with IL-7/IL-15. With progressing differentiation, the level of exhaustion decreases, e.g., in $CART_{EM}$ and $T_E$ cells.

As a high percentage of $T_N$ cells within the final CART product correlates with long-lasting remissions in patients, we calculated the $T_N/T_E$ ratio from HDs and untreated CLL patients. The results are depicted in **Figure 7**. The $T_N/T_E$ ratio of HDs vs. untreated CLL patients became significant at the end of culture: on day 20, the $T_N/T_E$ ratio for HDs stimulated with IL-7/IL-15 reached the

**A** **Highest proliferation of specific subpopulations depending on stimulation with IL-7/IL-15 vs. IL-2**




**B** **Overall percentage of specific subpopulations for healthy donors vs. CLL patients**

| Percentage on Day 17 | Healthy Donors IL-7/IL-15 | Untreated CLL Patients IL-7/IL-15 | Healthy Donors IL-2 | Untreated CLL Patients IL-2 |
|---|---|---|---|---|
| $T_N$ | 49.07 +/- 7.46 | 2.31 +/- 1.27 | 13.48 +/- 4.22 | 1.26 +/- 0.62 |
| $T_{SCM}$ | 42.63 +/- 6.09 | 1.60 +/- 0.87 | 11.33 +/- 3.78 | 0.53 +/- 0.41 |
| CD4+ | 48.47 +/- 5.23 | 55.40 +/- 29.62 | 36.77 +/- 6.07 | 42.17 +/- 28.65 |
| CXCR3+ | 90.63 +/- 6.31 | 82.87 +/- 2.56 | 92.33 +/- 4.77 | 84.27 +/- 4.66 |
| CD95+ | 99.67 +/- 0.12 | 99.63 +/- 0.31 | 99.63 +/- 0.15 | 99.53 +/- 0.21 |
| CD62L+ | 86.67 +/- 3.65 | 45.93 +/- 27.68 | 50.90 +/- 13.81 | 39.23 +/- 22.07 |
| γ/δ TCR+ | 2.04 +/- 0.93 | 0.62 +/- 0.12 | 3.26 +/- 2.12 | 0.65 +/- 0.19 |
| $T_E$ | 21.53 +/- 6.39 | 22.43 +/- 10.26 | 26.80 +/- 6.56 | 14.87 +/- 5.90 |
| PD-1+ | 8.05 +/- 1.14 | 19.90 +/- 6.50 | 11.00 +/- 5.27 | 16.41 +/- 11.09 |
| $T_{CM}$ | 10.49 +/- 2.44 | 5.96 +/- 2.57 | 8.29 +/- 2.71 | 6.47 +/- 3.36 |
| HLA-DR+ | 37.97 +/- 18.30 | 50.70 +/- 14.47 | 56.13 +/- 16.03 | 66.73 +/- 18.58 |
| Tim-3+ | 39.20 +/- 6.11 | 53.63 +/- 16.86 | 45.03 +/- 3.92 | 60.40 +/- 9.77 |
| CD19+ | 0.04 +/- 0.00 | 0.07 +/- 0.03 | 0.03 +/- 0.01 | 0.07 +/- 0.05 |
| CD34+ | 0.07 +/- 0.02 | 0.12 +/- 0.06 | 0.07 +/- 0.03 | 0.11 +/- 0.09 |
| CD4+CD8+ | 2.56 +/- 2.47 | 1.36 +/- 0.79 | 3.40 +/- 2.22 | 1.78 +/- 0.70 |
| CD8+ | 47.93 +/- 3.00 | 42.63 +/- 29.67 | 57.47 +/- 3.72 | 55.10 +/- 29.97 |
| CD4-CD8- | 1.05 +/- 0.46 | 0.58 +/- 0.16 | 2.39 +/- 1.30 | 0.86 +/- 0.68 |
| $T_{EM}$ | 18.97 +/- 4.51 | 69.43 +/- 13.77 | 51.60 +/- 9.21 | 77.57 +/- 8.52 |
| CD56+ | 3.93 +/- 2.63 | 2.73 +/- 1.76 | 10.60 +/- 6.26 | 5.57 +/- 3.88 |
| CD4+ $T_{Reg}$ | 0.48 +/- 0.42 | 1.94 +/- 1.63 | 3.68 +/- 1.73 | 5.76 +/- 2.76 |

**Day 17**

**FIGURE 4** | Analysis for different subpopulations of chimeric antigen receptor T (CART) cells from healthy donors (HDs) vs. patients on day 17 of culture with interleukin (IL)-7/IL-15 vs. IL-2. **(A)** The absolute cell number of specific cell subpopulations after stimulation with IL-7/IL-15 vs. IL-2 was compared for each donor independently ($n = 6$). Each unit on the x-axis represents one donor. Fields are highlighted with blue or red color according to the highest cell number of CART cells for each subtype, i.e., blue when proliferation resulted in higher absolute cell numbers under culture conditions with IL-7/IL-15 and red when proliferation resulted in higher absolute cell numbers under culture conditions with IL-2. Light colors were used for HDs, dark colors for patients. **(B)** The mean values of the percentage of CART cells with a specific phenotype on day 17 are represented and grouped into four categories: blue for stimulation with IL-7/IL-15, red for stimulation with IL-2 (HDs: light blue/red; patients: dark blue/red).

value 2.51. In contrast, untreated CLL patients showed a ratio of 0.13 ($p = 0.0007$). This effect was also visible when CART cells were stimulated with IL-2 ($p = 0.004$), although IL-2 was not able to promote expansion of $CART_N$ cells as much as IL-7/IL-15.

## DISCUSSION

Chimeric antigen receptor T cells have been designated as major breakthrough in cellular therapy for patients with cancer, particularly for patients with relapsed or refractory hematologic malignancies such as CD19+ leukemia and lymphoma. Hitherto, research has focused on genetic engineering in terms of vector design and costimulatory molecules. Only recently the "point of departure", i.e., the characterization of PBMCs to be transduced, gained attention. Moreover, the influence of different cytokine cocktails on CART cultures has not yet been elucidated in depth. By analyzing the characteristics of HD cells that responded particularly well to cytokine stimuli and comparing CART products generated from HDs and untreated CLL patients under similar conditions, this study aimed to define the contribution of cytokine stimulation and culture conditions to resultant phenotype of CART products.

Since response failure in clinical CART therapy has been associated with an insufficient number of $T_N$ cells within the CART cells (25), we screened PBMCs from HDs and untreated CLL patients for percentage of $T_N$ cells. In comparison with HDs, CLL patients contained significantly lower $T_N$ cells within their PBMCs.

Following screening, HDs and CLL patients displaying comparable levels of $T_N$ cells were chosen for CART production. CART cells were successfully generated for all included individuals with similar transduction efficiencies under two stimulating cytokine conditions, i.e., IL-7/IL-15 and IL-2. When comparing the expansion of the subpopulations $T_N$, $T_{CM}$, $T_{EM}$, and $T_E$ during CART culture, significant differences were observed under distinct culture conditions: IL-7/IL-15 cytokine stimulation led to an increase of $T_N$, $T_{SCM}$, CD4+, CXCR3+, CD62L+, γ/δ TCR+, $T_E$, PD-1+, $T_{CM}$, HLA-DR+, Tim-3+, and CD4+ CD8+ CART cells, whereas an IL-2 culture condition stimulated $T_{EM}$, CD56+, immunosuppressive CD4−CD8− and CD4+ $T_{Reg}$ CART cells.

The functional evaluation *via* chromium release assay showed a higher cytotoxic activity of CART cells against CD19+ target cells after stimulation with IL-2 compared to stimulation with IL-7/IL-15. The higher cytotoxic efficacy of IL-2 stimulated CART cells can be explained given the higher proportion of differentiated effector CART cells ($T_{EM}$ and CD56+) specialized in killing. In contrast, stimulation with IL-7/IL-15 mediated a higher proportion of immature subtypes ($T_N$, $T_{SCM}$) within the CART product, thus leading to lower overall lytic capacity. *In vivo* killing of malignant cells by CART is more complex and depends on the different CART subpopulations acting over time (**Figure 8**): the first attack after infusion of CART cells into the patient is mediated by highly differentiated cells endowed with high lytic activity. However, these cells soon reach the end of differentiation and become anergic. Without a second line of attacking immune cells, patients would face relapse after loss of effector cells. Long-lasting $T_N$ or $T_{CM}$ CART subtypes with self-renewal capacity can act as a reservoir for a second line of effector CART cells (18, 19). Therefore, infusing a high percentage of $CART_N$ cells into

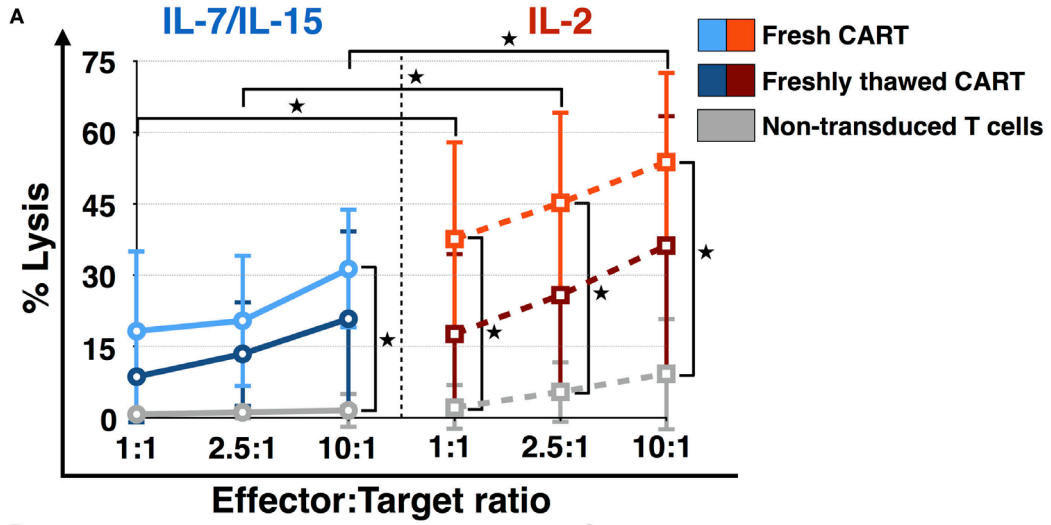



**FIGURE 5** | Functional characterization of chimeric antigen receptor T (CART) cells: cytotoxicity and cytokine expression. **(A)** The cytotoxic efficacy of CART cells against CD19+ Daudi cells (fresh $n = 3$, freshly thawed $n = 3$, non-transduced $n = 3$) was assessed after stimulation with interleukin (IL)-7/IL-15 vs. IL-2. The average lysis of CD19+ Daudi cells for different effector:target ratios (10:1, 5:1, 1:1) was determined by release of radioactive chromium-51 from labeled target cells. **(B)** Differences in the absolute cell number of CART cells expressing TNF-α, IFN-γ, and IL-2 after stimulation with IL-7/IL-15 vs. IL-2 are represented for each donor ($n = 3$). Fields are highlighted with blue or red color according to the highest percentage of CART cells for each subtype, i.e., blue for IL-7/IL-15 and red for IL-2. **(C)** The mean values of the percentage of CART cells with a specific phenotype on day 15 are represented (blue for stimulation with IL-7/IL-15, $n = 3$; red for stimulation with IL-2, $n = 3$). Significance is represented as * for $p < 0.05$ and ** for $p < 0.01$.

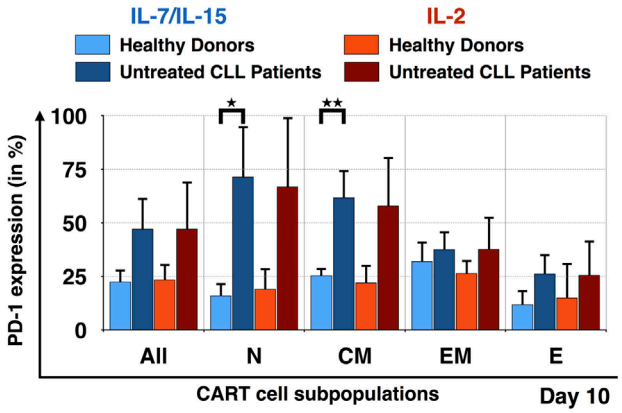



**FIGURE 6** | Exhaustion of naive T cell subpopulation for untreated chronic lymphocytic leukemia (CLL) patients in comparison to healthy donors (HDs). PD-1 expression on the surface of all chimeric antigen receptor T cells and the subpopulations $T_N$, $T_{CM}$, $T_{EM}$, and $T_E$ on day 10, for HDs ($n = 3$, light colors) and untreated CLL patients ($n = 3$, dark colors). The exhaustion marker expression was also compared according to the mode of stimulation, either with interleukin (IL)-7/IL-15 (blue) or with IL-2 (red). Significance is represented as * for $p < 0.05$ and ** for $p < 0.01$.

| $T_N/T_E$ Ratio | Day 7 | Day 10 | Day 14 | Day 17 | Day 20 |
|---|---|---|---|---|---|
| Healthy Donors IL-7/IL-15 | 3.05 | 0.87 | 1.12 | 2.51 | 2.51 |
| Untreated CLL Patients IL-7/IL-15 | 4.05 | 2.65 | 0.19 | 0.10 | 0.13 |
| Healthy Donors IL-2 | 3.75 | 0.73 | 0.40 | 0.50 | 0.71 |
| Untreated CLL Patients IL-2 | 4.20 | 3.61 | 0.34 | 0.08 | 0.07 |

**FIGURE 7** | Naive to effector chimeric antigen receptor T (CART) ratio ($T_N/T_E$) during culture. Representation of the $T_N/T_E$ ratio for CART cells from healthy donors ($n = 3$, light colors) vs. untreated chronic lymphocytic leukemia patients ($n = 3$, dark colors) during culture. Stimulation with interleukin (IL)-7/IL-15 is marked in blue, stimulation with IL-2 in red. Significance is represented as ** for $p < 0.01$ and *** for $p < 0.001$.

patients is highly desirable and represents a source for new effector cells over time, eventually contributing to higher survival rate of treated patients. Consequently, a high percentage of $T_N$ cells at the beginning of the CART culture correlates with *in vivo* CART expansion (29).

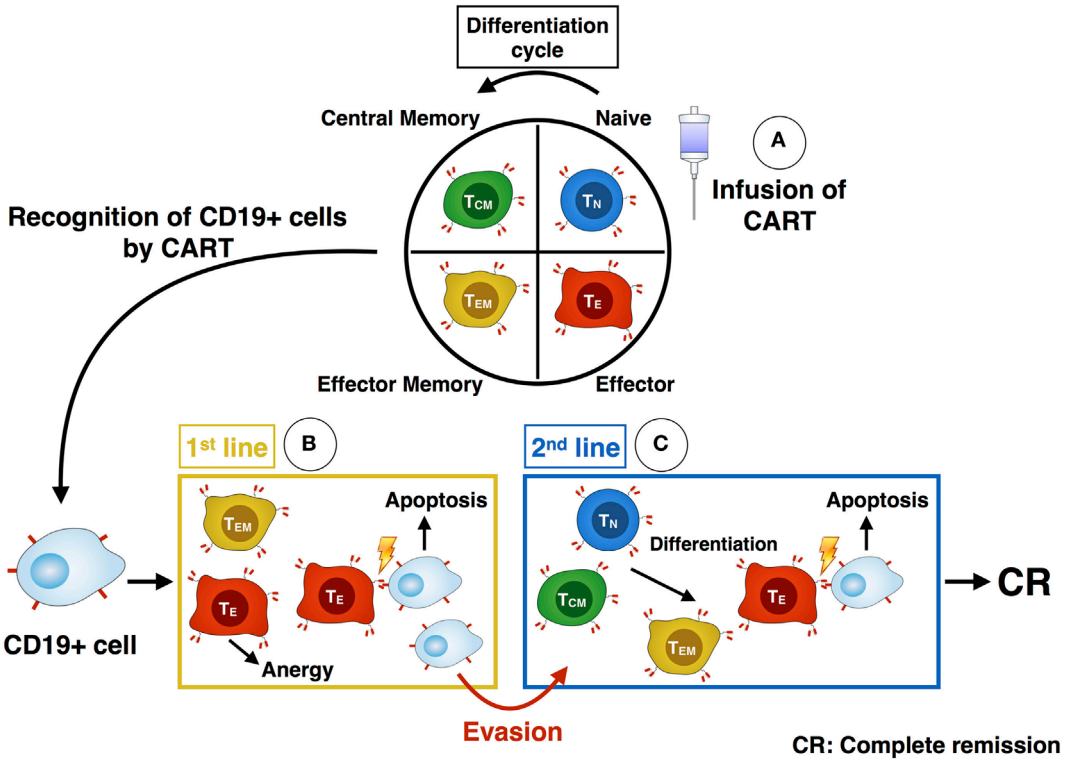



**FIGURE 8** | Illustration of the differentiation and killing process of chimeric antigen receptor T (CART) subpopulations. After administration of CART cells into the patient **(A)**, the more differentiated $T_{EM}$ and $T_E$ CART cells are responsible for the first killing of CD19+ cells **(B)**. As they become anergic, the less-differentiated $T_N$ and $T_{CM}$ subsets build up a second line of effector CART cells **(C)**. They differentiate into $T_{EM}$ and $T_E$ and are able to kill remaining malignant cells, which evaded the immune defense beforehand. Ideally, these cells eventually mediate a complete response within the patient.

We expanded $CART_N$ cells the most successfully in samples derived from HDs under stimulation with IL-7/IL-15. However, similar expansion rates of $CART_N$ cells could not be achieved for samples derived from untreated CLL patients, independent of the addition of IL-7/IL-15 or IL-2. As a consequence, $T_N/T_E$ ratio on day 20 of culture was high for HDs, but very low for CART cells derived from untreated patients.

The main reason for this might be the cellular composition of the starting material: whereas other parameters were similar between the tested individuals from both groups, untreated CLL patients showed a high percentage of malignant B cells contained within their PBMCs. These were cultured together with the CD3+ T cells, but received no activation signal through anti-CD3/anti-CD28 antibodies and underwent apoptosis. In fact, on day 17 of culturing B cells had vanished (percentage of CD19+ cells $< 0.01$), resulting in a culture made up solely of CART and non-transduced T cells.

As reported by Gattinoni et al. for an *in vivo* model, endogenous cells compete with transferred T cells for supportive cytokines IL-7/IL-15 (30). A similar effect might have occurred during our CART culture: B cells in samples from untreated CLL patients might have taken up most of the added cytokines, thus restricting the availability for CART cells. As a consequence, $CART_N$ expansion normally promoted by IL-7/IL-15 was disturbed. Lacking cytokine stimulation induced the exhaustive phenotype expressing high levels of PD-1 observed on day 10 of culture of the less-differentiated CART cells, i.e., $T_N$ and $T_{CM}$. Moreover, given the (1) high frequency of CLL cells present during the early culture period and (2) the known contribution of the microenvironment on CART cell generation, it is possible that CLL cells within the culture are recognized by the CART cells leading to differentiation from naive to effector cells. As the frequency of B cells within samples from HDs is significantly lower, this effect is less prominent in HD culture conditions.

In clinical studies, lymphodepletion is performed through a conditioning cytostatic regimen preceding CART cell infusion in order to make room for CART expansion and enhance the homeostatic microenvironment. This has been identified as a crucial factor for CART cell engraftment (31–34). However, patient samples used to generate the CART product are taken before lymphodepletion and the peripheral blood of patients might still contain high numbers of malignant B cells, interfering with the expansion of naive T cells. As a consequence, our data support the selection of T cells either by magnetic-activated cell sorting or by flow cytometry to obtain pure T cells and enable efficient expansion of $CART_N$ cells *ex vivo*.

In conclusion, we aimed to establish a preclinical potency assay for CART generation. A high percentage of less-differentiated $T_N$ cells contributes to a long-lasting remission and significant expansion of CART cells *in vivo* (25, 35–40). According to our results,

these conditions are fulfilled when a cocktail of IL-7 and IL-15 is used instead of IL-2 as stimulating cytokines. High expansion of long-lasting $CART_N$ cells was achieved when PBMCs from HDs were used. In contrast, untreated CLL patients remain a particular challenge for production of CART cells targeting the CD19 antigen, as clinical responses and persistence *in vivo* is limited in CART products with low proportions of $T_N$. High amounts of B cells in untreated CLL patients might be responsible for this. Hence B cell depletion before starting CART production might constitute a tool to overcome this problem. As CART cells emerge as frontline therapy, manufacture may be hindered in untreated CLL patients due to high frequencies of B cells in the initial culture. Our data therefore support either B cell depletion or T cell sorting early on, which could allow more $T_N$ to expand. In line with others (41–44), B cell depletion in hematologic malignancies also before infusing CART cells is advisable. Subsequent analyses of the PBMC content factors that determine CART efficacy might further improve the results of CART cell therapy. However, the additional impact of differences in CAR construct, target antigen, manufacture protocol and patient disease status also likely play a role. *In vitro* studies with larger cohorts of CLL patients vs. HDs as well as evaluation of clinical samples from patients treated with CD19.CART cells are ongoing and might result in future selection or purging strategies.

## ETHICS STATEMENT

The manuscript contains data on patient samples. This study was carried out in accordance with the recommendations of the Institutional Review Board (IRB) of the Heidelberg University Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol (S-254/2016) was approved by the IRB of the Heidelberg University Hospital.

## AUTHOR CONTRIBUTIONS

MS designed the study; J-MH performed the experiments and analyzed the data; J-MH, M-LS, and MS wrote the manuscript; LW, SS, AH, CK, and AS discussed experimental design; LS, CK, PW, SH, AH, CM-T, and PD reviewed the manuscript; UG and AL set up the CART culture protocol and reviewed the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01956/full#supplementary-material.

**FIGURE S1** | FACS data. Illustration of the gating strategy used during acquisition with the LSRII flow cytometer. The first gate assessed viable cells, the second gate CD3+ cells and the third gate CD3+ CART cells. Out of the CD3+ CART cells, the percentages of CD4+/CD8+ cells were evaluated. $T_N$, $T_{CM}$, $T_{EM}$, and $T_E$ subpopulations were defined and expression of the exhaustion marker programmed cell death 1 was assessed. As representative examples data from HD 7 and untreated chronic lymphocytic leukemia patient 2 (P 2) at day 17 of culture after stimulation with interleukin (IL)-7/IL-15 are displayed here.

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# Cathepsin G Is Expressed by Acute Lymphoblastic Leukemia and Is a Potential Immunotherapeutic Target

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Cathepsin G (CG) is a myeloid azurophil granule protease that is highly expressed by acute myeloid leukemia (AML) blasts and leukemia stem cells. We previously identified CG1 (FLLPTGAEA), a human leukocyte antigen-A2-restricted nonameric peptide derived from CG, as an immunogenic target in AML. In this report, we aimed to assess the level of CG expression in acute lymphoid leukemia (ALL) and its potential as an immunotherapeutic target in ALL. Using RT-PCR and western blots, we identified CG mRNA and protein, respectively, in B-ALL patient samples and cell lines. We also examined CG expression in a large cohort of 130 patients with ALL *via* reverse-phase protein array (RPPA). Our data show that CG is widely expressed by ALL and is a poor prognosticator. In addition to endogenous expression, we also provide evidence that CG can be taken up by ALL cells. Finally, we demonstrate that patient ALL can be lysed by CG1-specific cytotoxic T lymphocytes *in vitro*. Together, these data show high expression of CG by ALL and implicate CG as a target for immunotherapy in ALL.

**Keywords: cathepsin G, immunotherapy, nonameric peptide, acute lymphoblastic leukemia, cross-presentation, antigens, targeted therapy, serine proteases**

## INTRODUCTION

Patients with relapsed and refractory acute lymphoblastic leukemia (ALL) have a very aggressive malignancy with poor outcomes. Although the outcome of pediatric patients with ALL is excellent, the overall survival (OS) for adult patients with ALL is poor, especially in patients who relapse after initial therapy (1–3). Although hematopoietic stem cell transplantation (HSCT) provides cures in some cases, the 1-year OS following allogeneic (allo) HSCT has reached approximately 30% (4–6), with the majority of mortality being attributed to disease progression. Despite the poor outcomes with allo-HSCT, ALL has been shown to be responsive to immunotherapy, including donor lymphocyte infusion and adoptive T cell therapy (3, 7–10). In fact, ALL is the prototype malignancy for the use of chimeric antigen receptor T cells, highlighting the potential of immunotherapy to eliminate ALL and improve patient outcomes (7–9, 11).

A critical component of immunotherapy development, however, is the identification of tumor antigens against which immunotherapies can be engineered. The paucity of known tumor antigens

has been an obstacle to the development of vaccines, antibodies, and cellular immunotherapies. In addition to enabling the specific targeting of the tumor cells, identifying tumor-specific or tumor-associated antigens will minimize the off-target immune toxicities of immunotherapy, including allo-HSCT and cellular therapies. Our group has identified azurophil granule serine proteases, specifically cathepsin G (CG), neutrophil elastase (NE), and proteinase 3 (P3), as immunotherapeutic targets in acute myeloid leukemia (AML) and has developed immunotherapies that target these proteases. Although CD19 has proven to be an effective target in lymphoid malignancies, including ALL (7, 8, 11, 12), there remains a paucity of known immunotherapeutic targets in ALL. However, the response of ALL to targeted immunotherapy highlights the need to further discover ALL-specific/associated antigens. In this report, we focus on CG as a novel antigen for immunotherapy development in ALL.

Cathepsin G is a serine protease largely restricted to the myeloid lineage and is expressed in high levels within azurophil granules in AML blasts and leukemia stem cells and during the promyelocyte stage of neutrophil development. CG is involved in host immunity, cleavage of inflammatory mediators, degradation of extracellular matrix components, antigen presentation, and leukemogenesis (13–16). We previously identified CG as a myeloid leukemia-associated antigen (17, 18). We showed that CG is processed by AML blasts and that a number of human leukocyte antigen (HLA) class I peptides derived from CG were presented by AML. We also demonstrated that cytotoxic T lymphocytes (CTLs) that are expanded to target CG1 (FLLPTGAEA), a HLA-A2-restricted nonameric peptide derived from CG, eliminated AML *in vitro* and *in vivo* (17, 18). Finally, we detected CTLs specific for CG1 in the peripheral blood of AML patients after allo-SCT (17).

Using mass spectrometry, we identified CG1 in the HLA class I-immunoprecipitated fraction from one patient with ALL (18). In addition to our studies, there have been three other reports that suggested CG expression in lymphoid leukemia. CG was reported in chronic lymphocytic leukemia (19) and Hodgkin's lymphoma (20), and cellular immunity targeting CG eliminated leukemic cells in three patients with ALL (21). These data provided the impetus to further study the immunotherapeutic potential of targeting CG in lymphoid malignancies.

In this study, we demonstrate CG gene and protein expression in ALL cell lines and ALL patient samples. In addition to endogenous expression, we demonstrate that CG can be taken up by ALL. We show that ALL is susceptible to killing by CG1-specific CTLs (CG1-CTLs). Finally, we show that CG expression correlates negatively with ALL patient outcomes.

# MATERIALS AND METHODS

## Patient Samples and Cell Lines

Patient and healthy donor samples were obtained after appropriate informed consent through an institutional review board approved protocol at the University of Texas MD Anderson Cancer Center (MDACC). Patient, including the samples used in the reverse-phase protein array (RPPA) and UPN1-8, and healthy-donor peripheral blood mononuclear cells (PBMC) and polymorphonuclear lymphocytes (PMN) were isolated from buffy coats after single or double Ficoll gradient centrifugation, respectively, using Histopaque-1077 and Histopaque-1119 (Sigma-Aldrich). SUP-B15 (B lymphoblastic leukemia), SB (B lymphoblast leukemia), RS4;11 (B lymphoblastic leukemia), NALM6 (B lymphoblastic leukemia), Raji (Burkitt's lymphoma), and T2 (B cell/T cell hybridoma) cell lines were obtained from American Type Culture Collection. Cells were cultured in RPMI 1640 media with 2.5 mM L-glutamine (Hyclone) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptavidin (Invitrogen). All cells were cultured at 37°C and 5% $CO_2$. Cells lines were validated at the MD Anderson Sequencing and Microarray Facility *via* short tandem repeat DNA fingerprinting and checked for mycoplasma *via* PCR (PromoKine). Raji cells were transduced with HLA-A*0201 as described previously using a lentiviral vector encoding HLA-A*02:01 (18, 22). HLA-A2 expression was verified by flow cytometry prior to using the cell line. HLA-A*0201$^+$ Raji cells (Raji-A2) were subsequently used in western blots and cytotoxicity assays, as described.

## RNA Purification and RT-PCR

Purified RNA was extracted via the RNeasy Plus Mini Kit (Qiagen) and used per manufacturer's instructions. Synthesis of cDNA was performed using the Gene Amp RNA kit (PerkinElmer). The following primer was ordered from Sigma-Aldrich: *Cathepsin G* (forward 5′-AAACACCCAGCAACACATCA-3; reverse 5′-TATCCAGGGCAGGAAACTTG-3′). Actin (forward 5′-CCAGAGCAAGAGAGCTATCC-3′; reverse 5′-CTGTGGTGGTGAAGCTGTAG-3′) served as a loading control. Following denaturation for 5 min at 95°C, samples were amplified for 35 cycles using an iCycler IQ Thermal Cycler (Bio-Rad Laboratories). Samples were run on a 1.5% agarose gel and bands were imaged using GelDoc2000 (Bio-Rad Laboratories) and analyzed by Quantity One software (Bio-Rad Laboratories).

## Cell Lysates and Western Blots

Western blotting for CG was performed as previously described (17). Briefly, cell pellets were suspended in lysis buffer (10 mM/L HEPES [pH 7.9], 10 mM/L KCl, 0.1 mM/L EGTA, 0.1 mM/L EDTA, and 1 mM/L DTT) containing protease inhibitors and underwent freeze–thaw cycles for 15 min to generate whole-cell lysates. Cell lysates were separated on 10% SDS gels by electrophoresis, transferred onto polyvinylidene difluoride membranes, blocked in 5% milk, and stained with anti-CG (Abcam), anti-tubulin (Sigma) antibodies. Blot was rocked in ECL reagent and then imaged using ChemiDoc Touch Imaging System (Bio-Rad).

## CG Uptake by ALL and Normal B Cells

Analysis of the uptake of CG in B-ALL cell lines was carried out by standard flow cytometry methods, as we previously described (23–25). Cells were cocultured in reduced serum medium (0.5% FBS) with irradiated (7,500 cGy) PMN at a ratio of 3:1 (PMN: B-ALL); PMN served as the source for cell-associated CG. After co-incubation, cells were stained with Live/Dead Fixable Ghost Dye (Tonbo), B-ALL markers (**Table 1**), and IVIG (Privigen) to

**TABLE 1** | Patient characteristics.

| Patient | Blast% | Surface markers | Ph+ | Cytogenetics |
|---|---|---|---|---|
| UPN1 | 72 | CD10+, CD19+, CD34+, CD20+ | – | 46 XX, −12 |
| UPN2 | 95 | CD10+, CD19+, CD34+, TdT | – | 39–47, XX, −6, +5, +8, +11, −18, −19 |
| UPN3 | 96 | CD10−, CD19+, CD34+, CD20− | – | 46 XX, *t*(4;11) |
| UPN4 | 91 | CD10+, CD19+, CD34$^{dim}$, CD20$^{dim}$, TdT | – | 47 XY, +21 |
| UPN5 | 97 | CD10+, CD19+, CD34+, CD20+ | + | Diploid |
| UPN6 | 92 | CD10$^{dim}$, CD19+, CD34+, CD20$^{partial}$ | – | 46 XY |
| UPN7 | 87 | CD10+, CD19+, CD34+, CD20− | + | 46 XX, *t*(9;22), +21 |
| UPN8 | 92 | CD10+, CD19+, CD34+, CD20$^{dim}$ | – | 45, 46 XY, −6, −9 |

block the Fc receptor. After surface staining, cells were washed in PBS, fixed in 1% formaldehyde (ThermoFisher Scientific) in PBS and permeabilized in 5% Perm/Wash Buffer (BD). Uptake of CG was determined by staining with anti-CG-FITC (Bio-Rad). Flow cytometry was performed using LSRFortessa Analyzer (BD Bioscience), and analyzed *via* Flowjo software (Treestar).

Same methodology was used to determine uptake of CG by normal B cells. After co-incubation with PMN, PBMC were surface stained with lineage antibodies including CD3 (BioLegend), CD14 (BioLegend), CD16 (BioLegend), and CD19 (BD), fixed, permeabilized, and intracellularly stained with anti-CG antibody. B cells were differentiated based on light scatter characteristics as well as being surface CD3−/CD14−/CD16−/CD19+. Similar to B-ALL, normal B cells also appear to take up CG (Figure S4 in Supplementary Material).

## CG1-Specific CTLs

To expand CG1-specific CTLs, dendritic cells (DCs) were matured from adherent monocytes and then used as professional APCs (26, 27). Normal PBMC isolated from buffy coats were adhered to a six-well plate at 37°C in Macrophage Serum Free Medium 1× (Gibco). Lymphocytes from the same donor were separated and cocultured with 40 μg/mL of CG1. Cells were then stimulated with interleukin (IL)-7 (10 ng/mL) (rhIl-7, carrier free; BioLegend) and IL-2 (10 ng/mL) (rhIL-2; R&D) over 5 days. Adhered monocytes were matured into monocyte-derived DC through the addition of granulocyte macrophage colony-stimulating factor (GM-CSF) (100 ng/mL; Sanoti), IL-4 (50 ng/mL) (rhIL-4; Tonbo Biosciences), and tumor necrosis factor-α (25 ng/mL; BioLegend). After 5 days, DCs were detached from the six-well plates, cocultured with CG1 peptide (Bio-Synthesis, Inc.) at 40 μg/mL, and combined with the expanded lymphocyte population. Lymphocytes were expanded by coculture with mature DCs and stimulated with IL-7 (10 ng/mL) and IL-2 (25 ng/mL) for an additional 7 days. On day 14 of stimulation, cells were harvested and analyzed *via* flow cytometry by CG1 tetramer staining to determine the percentage of antigen-specific cells that were generated (17). CG1-CTL were identified using the following fluorescently conjugated tetramer and antibodies: PE-CG1/HLA-A*0201 tetramer (Baylor College of Medicine); APC-anti-CD3 (BioLegend); APC/Cy7-anti-CD8 (BioLegend); lineage (Lin) markers including pacific blue-anti-CD4 (BioLegend), CD14 (BioLegend), CD16 (BioLegend), and CD19 (BD) (Figure S1 in Supplementary Material). Prior to coculture with target cells in the cytotoxicity assays, bulk CTLs were enriched using a CD8+ T cell isolation MACS kit (Miltenyi Biotec).

## CG1-CTLs Cytotoxicity Assay

A standard cytotoxicity assay was used to determine specific lysis, as previously described (28, 29). T2 cells were cultured overnight with soluble CG (10 μg/mL), washed in RPMI 1640 (HyClone), and resuspended at $1.0 \times 10^5$ cells/mL. T2 and target ALL cells were then stained with calcein-AM (Invitrogen) for 15 min at 37°C. Stained cells were then washed three times in RPMI 1640, resuspended at $2.0 \times 10^5$ cells/mL, plated onto a Terasaki plate, and cocultured with CG1-specific CTLs at increasing effector:target ratios. After a 4-h incubation period, trypan blue was added to each well to quench fluorescence of dead cells. Fluorescence was measured using a Cytation 3 Imaging Reader (BioTek). Percent specific lysis was calculated by using the following formula:

$$\left(\begin{array}{l}1-[\text{fluorescence}_{\text{target + effector}}-\text{fluorescence}_{\text{media}}]\\ /[\text{fluorescence}_{\text{target alone}}-\text{fluorescence}_{\text{media}}]\end{array}\right)\times 100.$$

T2 cells pulsed with CG1 and irrelevant E75 peptide were used as positive and negative controls, respectively.

The HLA typing of patient samples UPN5 and UPN7 was performed at the MD Anderson HLA-typing laboratory and was obtained from the patients' medical records. HLA typing of UPN2 was not included in the patient's medical record; therefore, typing was performed by staining cells with anti-HLA-A*0201 antibody (clone BB7.2, BD Bioscience), as previously described (17, 30). To show the effects of CG uptake on cytotoxicity, Raji-A2 cells were cocultured with purified CG protein (Athens) for 24 h prior to performing the cytotoxicity assay.

## Samples and Sample Preparation for the RPPA Analysis

Peripheral blood and bone marrow (BM) specimens were collected from 130 patients with newly diagnosed ALL, and healthy donors evaluated at MDACC from 1992 to 2007. A paired relapse sample was also collected when available. Of the 130 patients, all were treated at MDACC and were evaluable. Among the treated patients, all received regimens that contained hyper-CVAD, with 23 also receiving rituximab, 9 with Philadelphia chromosome (Ph)-positive disease also received a tyrosine kinase inhibitor, and 1 Ph-positive case was treated with hyper-CVAD in combination with both rituximab and imatinib.

For RPPA, fresh samples were collected and enriched for leukemic cells using Ficoll-Hypaque (Sigma-Aldrich) density-gradient separation to yield a mononuclear fraction. The blast purity in the samples reached approximately 98% as determined by flow cytometry. Whole-cell lysates were prepared from samples that were normalized to a concentration of $1 \times 10^4$ cells/μL. RPPA was carried out as previously described (31, 32). Briefly, five serial dilutions of patient samples were printed onto slides along

with normalization and expression controls. Slides were stained with 232 strictly validated primary antibodies, including one against CG (Abcam ab8816, Cambridge, MA, USA). The stained slides were analyzed using MicroVigene software (Vigene Tech, Carlisle, MA, USA) to produce quantified data.

## Statistical Analysis

The associations between CG expression and categorical clinical factors were evaluated in R using standard *t*-tests, linear regression, and mixed-effects linear models. OS was measured from the date of diagnosis to the date of death due to any cause, and survival curves were generated using the Kaplan–Meier method. In addition, a Cox proportional hazards regression model was constructed to investigate the association between CG protein levels and OS. All statistical analyses for clinical outcome were computed using the Statistica software V.12 (StatSoft, Tulsa, OK, USA).

# RESULTS

## ALL Patient Samples and Cell Lines Demonstrate Variable Expression of CG Transcript and Protein

We first employed the Cancer Cell Line Encyclopedia (CCLE) database to investigate CG transcript expression by malignant tissues. CCLE data demonstrate a higher expression of CG transcript in B-ALL compared with other malignancies, although as predicted, the expression is lower than that observed in AML (**Figure 1A**). As predicted, CCLE also confirms higher

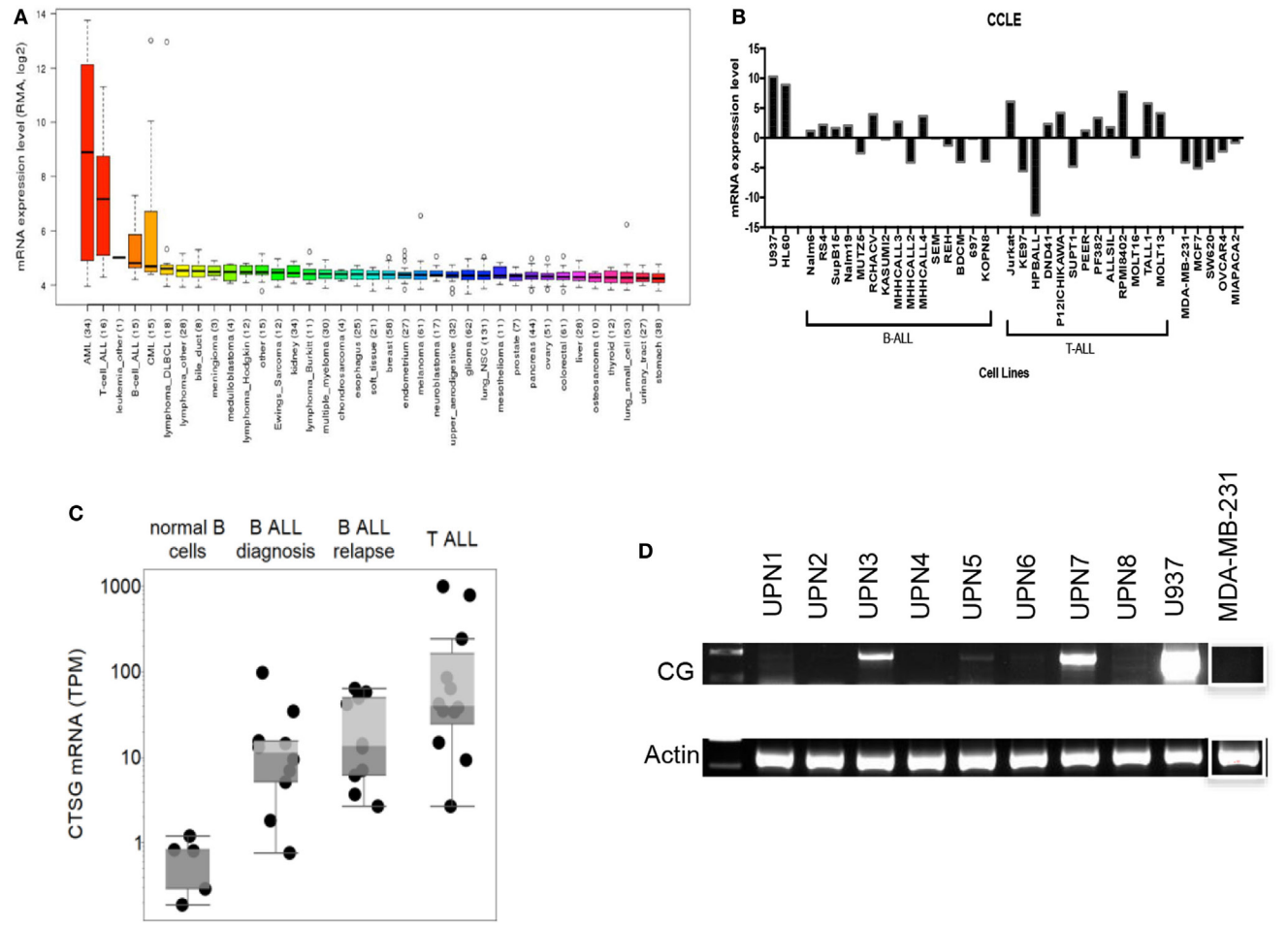


**FIGURE 1** | Cathepsin G (CG) transcript is detected in B-ALL patient samples and cell lines. **(A)** According to Cancer Cell Line Encyclopedia (CCLE), ALL cell lines express CG transcript at higher levels compared with other malignancies. High-expressing acute myeloid leukemia (AML) cell lines are shown for comparison. **(B)** CG mRNA expression is not ubiquitous to all ALL. CG levels are lower in B-ALL and T-ALL cell lines compared with U937 and HL60 AML cell lines, but are higher compared with solid tumors; MDA-MB-231 (breast cancer), MCF7 (breast cancer), SW620 (colorectal adenocarcinoma), OVCAR-3 (ovarian adenocarcinoma), and MIA PaCa-2 (pancreatic carcinoma). **(C)** The Cancer Genome Atlas shows higher CG expression at the transcript level in B-ALL compared to normal B cells. Additionally, mRNA expression is higher in relapsed B-ALL compared with B-ALL at time of diagnosis. T-ALL has the highest CG expression at the transcript level. **(D)** RT-PCR of patient B-ALL shows prominent CG expression in three B-ALL patient samples (UPN3, UPN5, and UPN7; **Table 1**). AML cell line U937 and breast cancer cell line MDA-MB-231 were used as positive and negative controls, respectively. Actin was used as a loading control. The white boxes outlining the MDA-MB-231 bands indicate that they were transposed from a different region of the same gel.

expression of CG by B and T cell ALL cell lines, compared with other tumor cell lines (**Figure 1B**; Table S1 in Supplementary Material). Furthermore, according to The Cancer Genome Atlas, the expression of CG mRNA is higher in relapsed B-ALL compared to B-ALL at the time of diagnosis. Also, T-ALL CG mRNA expression is greater than that seen in B-ALL and normal B cells (**Figure 1C**; Table S2 in Supplementary Material). This implicates CG as a possible marker of more aggressive ALL. We also confirm variable expression of CG transcript by RT-PCR using eight B-ALL patient samples and demonstrate the absence of CG transcript in some of the patient samples (**Figure 1D**; **Table 1**).

We then used western blot analysis and RPPA to determine the expression of CG by ALL at the protein level. Western blots show variable expression of CG in the ALL samples, although CG was detected in all eight patient samples (**Figure 2A**). Furthermore, along with demonstrating variable expression across patient samples, RPPA confirms that CG protein levels are increased in ALL blasts as compared to normal CD34$^+$ hematopoietic cells (**Figure 2B**). Although CG protein levels may seem lower in a sizeable proportion of ALL blasts compared with normal CD34$^+$ cells, the mean value of CG in ALL cells was 0.138 while the mean value of CG in CD34$^+$ cells was 0.122. Homoskedastic *t*-tests were performed assuming equal variance as well as unequal variance, which produced *P*-values of 0.96 and 0.92, respectively. This provides strong evidence that the means are not different. In addition, RPPA analysis demonstrates protein-level expression of CG across various ALL subtypes (**Figure 2C**), as well as differing levels of CG expression in normal and aberrant cytogenetic subgroups (**Figure 2D**).

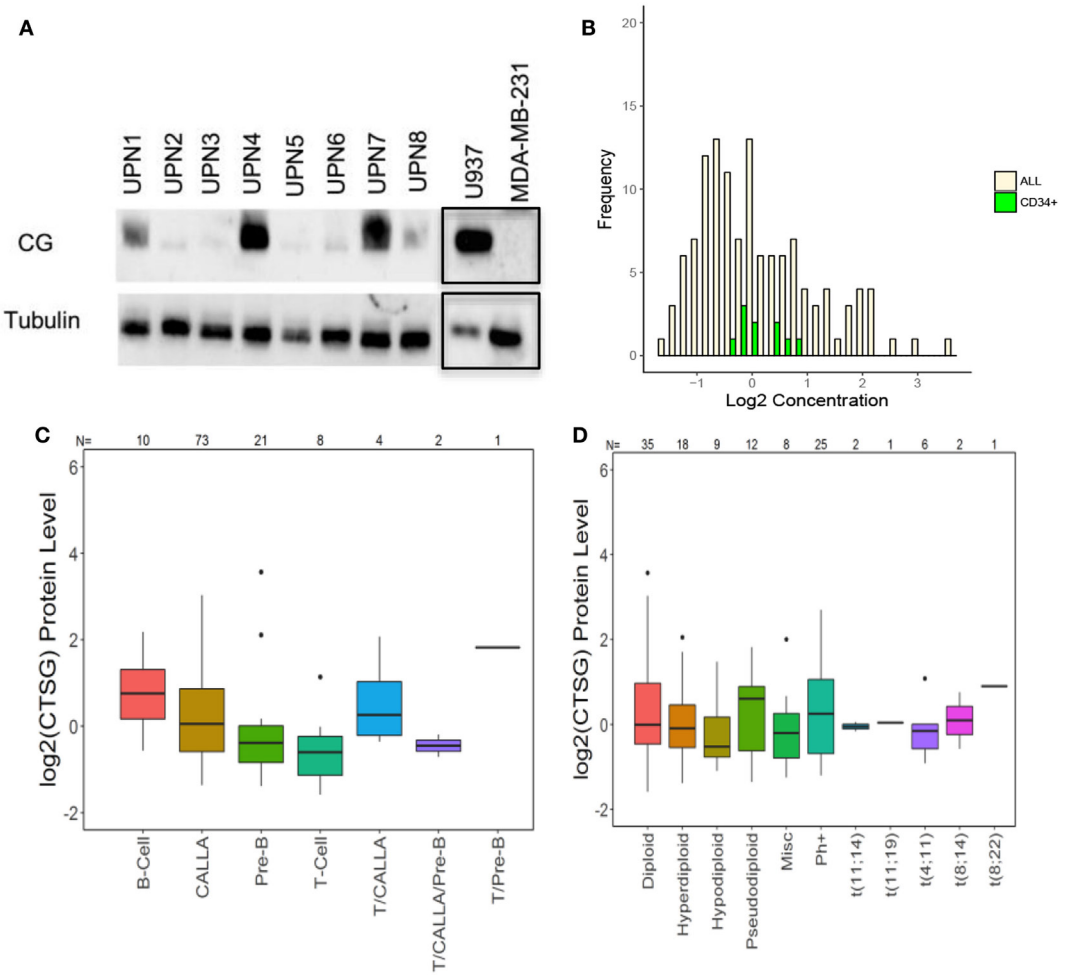


**FIGURE 2** | Cathepsin G (CG) is expressed at the protein level in B-ALL patient samples. **(A)** Western blots demonstrate CG protein in whole-cell lysates from eight different B-ALL patient samples (**Table 1**). Gels were loaded with 30 μg of protein. U937 and MDA-MB-231 cell lines were used as positive and negative controls, respectively. Tubulin was used as a loading control. The black boxes outlining the U937 and MDA-MB-231 bands indicate that they were transposed from a different gel that was run in parallel. **(B)** Reverse-phase protein array was used to quantify protein levels of CG in blasts from 130 newly diagnosed ALL patients (yellow bars). Controls included healthy donor CD34$^+$ progenitor cells (green bars). **(C)** Box plots show the levels of CG expression in ALL subtypes according to the French–American–British classification. **(D)** The relationship of CG protein levels is demonstrated with both normal and aberrant cytogenetic subgroups across 130 patient samples. Abbreviations: Misc, miscellaneous.

## B-ALL Can Take Up Exogenous CG

Since previous studies (33, 34), including our published data (23–25, 30), have shown that azurophil granules serine proteases, including CG, P3, and NE, can be taken up by tumor cells and since we note variable and at times absent expression of CG in some ALL samples, we next examined whether CG can be taken up by ALL. To differentiate CG uptake from endogenous expression, we selected four ALL cell lines, HMy2.CIR, RS4, SB, and Nalm6, that have low endogenous CG expression (**Figure 3A**). Cells were cocultured overnight with whole PMN, as the source for CG, or PBMC, which have lower expression of CG, at a ratio of 3:1 (PMN:ALL) (**Figure 3A**). Flow cytometry analysis of intracellular CG staining using a gating strategy that included the ALL cell lines and excluded PMN based on forward and side-scatter properties and CD16 negativity (Table S3 and Figure S2 in Supplementary Material) indicates significant uptake of PMN-associated CG by HMy2.CIR, Nalm6, RS4, and SB B-ALL cell lines (**Figure 3A**). We also detected an increase in intracellular CG after coculture of ALL with PBMC, although at a much lower level than what we observed with PMN. We attribute this to the CG that is known to be expressed by monocytes, which are found within the PBMC (16, 35). Further, the degree of uptake differs among the cells lines. Uptake was time-dependent, and increases with the longer durations of coculturing with PMN (**Figure 3B**). Negative controls included RS4 cell line alone or cocultured with irradiated PBMC. PMN and PBMC were used as positive and negative staining controls, respectively.

Furthermore, our data suggest uptake of CG by primary ALL. Specifically, we detected absence of CG transcript in UPN2 and UPN4 (**Figure 2D**) but detected CG protein in these patient

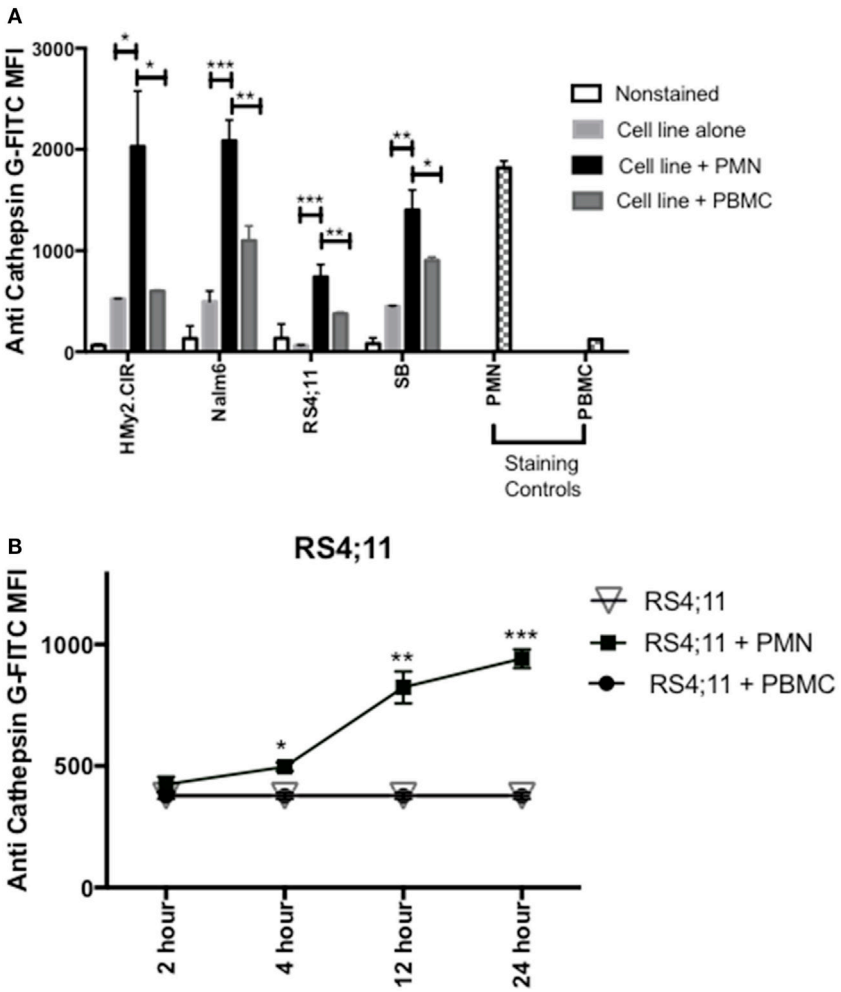


**FIGURE 3** | PMN-associated Cathepsin G (CG) is taken up by B-ALL cell lines. **(A)** Flow cytometry detected intracellular CG in B-ALL cell lines after coculture with whole PMN at a ratio of 3:1 for 12 h. Cells were intracellularly stained with anti-CG antibody. PMN and peripheral blood mononuclear cells (PBMC) were used as positive and negative staining controls, respectively. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. **(B)** RS4;11 cells were cocultured with whole PMN or PBMC at a ratio of 3:1 for 2, 4, 12, and 24 h and analyzed by flow cytometry for intracellular uptake of CG using anti-CG antibody. Cells cultured in the presence or absence of PBMC were used as negative controls. PMN and PBMC were used as positive and negative staining controls, respectively. Flow cytometry indicated that uptake of extracellular CG occurs in HMY2.CIR, Nalm6, and RS4;11 cell lines. Graphs display the mean ± SEM fold increase in median fluorescence intensity (MFI). Data represent triplicate wells from four independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

samples (**Figure 3A**). Together, the data show that B-ALL cell lines lack endogenous CG but can internalize CG from exogenous sources.

## B-ALL Is Susceptible to Killing by CG1-CTLs

Because CG1 has been effectively targeted in myeloid leukemia using CG1-CTLs (17, 18), we investigated whether CG1-CTLs can kill ALL. Based on our data showing that patient sample UPN7 (HLA-A*0201-positive) endogenously expresses CG at the transcript and protein levels (**Figures 1D** and **2A**), UPN7 ALL patient sample was cocultured with CG1-CTLs for 4 h using a standard calcein-AM cytotoxicity assay (28, 29). T2 cells were pulsed with CG1 or E75 (HER2-derived HLA-A2-restricted peptide) (27) as positive and negative controls, respectively. Data indicate a dose-dependent killing of UPN7 by CG1-CTLs (**Figure 4A**). The percent specific lysis reaches a maximum of approximately 30% at higher doses of CTLs and killing is observed at the lowest ratio of 0.625:1 (effector:target) (**Figure 4A**). T2 cells pulsed with CG1 also show dose-dependent killing by CG1-CTLs (positive killing control), whereas T2 cells pulsed with E75 demonstrate no significant target lysis (negative control). Conversely, no target killing of two HLA-A*0201 negative samples (UPN2 and UPN5) was detected, despite low expression of CG protein by these samples. The HLA typing of the samples used in the cytotoxicity assays is shown in Table S4 and Figure S3 in Supplementary Material. Furthermore, we cultured Raji-A2 cells with purified CG protein (Figure S5 in Supplementary Material) and confirmed increased killing of Raji-A2 after coculture with CG1-CTLs (**Figure 4B**).

## CG Expression Is Associated with an Aggressive ALL Phenotype

Although a direct correlation between protein expression by leukemic cells and tumor behavior may not be readily apparent, individual proteins may affect the aggressive phenotype of a malignant cell indirectly through interactions with other cellular proteins. We have correlated the expression of a number of proteins (36–38), including CG (18), and AML aggressiveness. A Pearson correlation coefficient analysis was, therefore, performed in this ALL patient cohort to determine the association between the levels of CG and these proteins. **Figure 5** demonstrates a similar correlation between CG protein expression and the expression of many proteins that have known associations with tumor behavior and response to therapy. Among these, CG-expressing tumor cells were found to concurrently express high levels of adhesion proteins, such as TG2, ITGB3, IGFBP2, IGF1, CD49B, and Galectin 3. Lower expression of stem cell regulating proteins

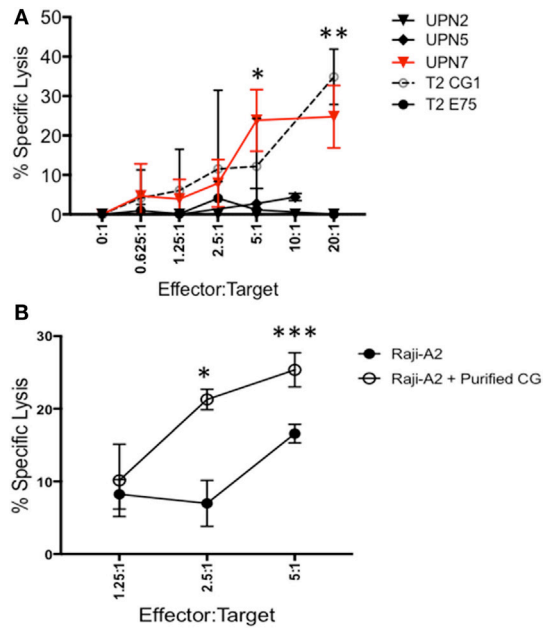


**FIGURE 4** | ALL shows susceptibility to killing by CG1-cytotoxic T lymphocytes (CTLs). Patient samples (UPN2, UPN5 and UPN7), T2 cells, and Raji-A2 cells were loaded with calcein-AM, and then cocultured with CG1-CTLs for 4 h at various E (effector): T (target) ratios. Cytotoxicity was determined by measurement of intracellular calcein-AM in live cells. T2 cell lines were pulsed with CG1 and E75 peptide as positive and negative controls, respectively. **(A)** UPN7, which endogenously expresses Cathepsin G (CG), and **(B)** Raji-A2 pulsed with purified CG protein (see Figure S5 in Supplementary Material) showed increased killing by CG1-CTL. Cytotoxicity data are means ± SEM from triplicate wells from a representative experiment. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

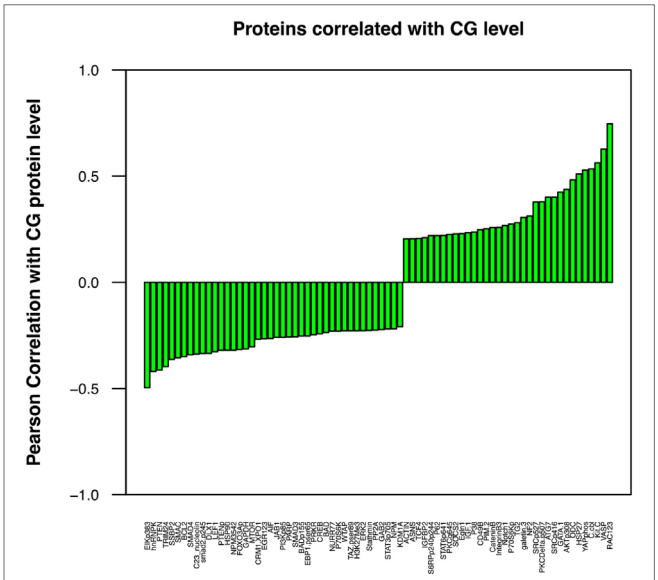


**FIGURE 5** | Cathepsin G (CG) protein expression correlates with the concurrent expression of known leukemia-associated proteins. Pearson correlation coefficient analysis was performed to determine the association between the levels of CG and various selected proteins that are known to contribute to leukemogenesis and progression of ALL. These proteins were identified with a correlation coefficient threshold of 0.2. These proteins were assessed for both co-occurrence and inverse correlation with CG1; cell adhesion molecules TG2 and ITGB3, oncogene C-kit, proliferation-associated protein GATA1, and vascular protein VASP were among the proteins expressed at highest levels in association with CG, while tumor suppressor protein PTEN, apoptotic proteins SMAC and BCL2, and differentiation-promoting protein SSBP2 were expressed at low levels. All proteins were identified with a correlation coefficient threshold of 0.2.

NUR77 and TCF4 and differentiation-promoting proteins SSBP2, along with higher expression of Gata1, Pim2, and Notch 1 were positively associated with CG levels, suggest a loss of stem cell regulation and alteration in differentiation. Increased Hippo pathway regulation *via* increased NF2, and inactivation of YAP by phosphorylation is suggested; however, decreased phosphorylation of TAZ is also observed. CG expression is negatively correlated with apoptosis regulating proteins Smac, BCL2, AIF, and Bad. CG expression is also associated with increased signal transduction and proliferation, evidenced by higher levels of phosphorylated (activated) AKT, PKCδ, SRC, S6RP, and P70S6K, while Stat pathways activity seems to be downregulated as CG is negatively correlated with phosphorylation of Stat3 and 6. Likewise, CG expression is inversely correlated with TGFβ-Smad signaling as levels of pSmad2, Smad3, and Smad4. An increase in pro-vascularization is suggested by a positive association with hypoxia-associated/vascular proteins, such as VASP and EGLN1. Histone methylation/acetylation proteins (hnRNPK, WTAP, H3k4me3, KDM1A) are found to be expressed at lower levels in the presence of CG protein. The actual consequences of modulated CG on each of these pathways require further experimental validation.

In order to assess the correlation between CG expression and clinical outcomes, the ALL patients used in RPPA for whom survival information was available were categorized into Ph-positive ($n = 25$) and Ph-negative ($n = 104$) subgroups. For both patient cohorts, no difference in the duration of survival was noted between patients in the context of CG expression ($P = 0.440$ for Ph-negative ALL; $P = 0.806$ for Ph-positive ALL) (**Figures 6A,B**).

## DISCUSSION

In this manuscript, we demonstrate the expression of the myeloid azurophil granule protease CG by ALL. Specifically, we demonstrate CG expression by B-ALL and T-ALL at the transcript and protein levels. Expression is demonstrated in cell lines and patient samples, and correlates with poor prognosis. In addition to endogenous expression, we show time-dependent uptake of PMN-associated CG by B-ALL. Finally, we demonstrate that patient ALL can be targeted effectively by CG1-CTLs.

Cathepsin G is known to be predominantly expressed by normal myeloid progenitor cells and also in primary AML blasts and leukemic stem cells (39, 40). Our study demonstrates the expression of the myeloid serine protease CG by B- and T-ALL, in cell lines as well as patient samples. This expression may be due to endogenous expression, as is shown in some patient samples and cell lines (**Figure 1**), and is also secondary to CG uptake (**Figure 3**). It may be noteworthy that our analysis not only identifies higher CG expression in B-ALL cells as compared to normal B-cells, but also establishes a hierarchy of expression, wherein CG expression at the transcript level is higher in T-ALL and relapsed B-ALL. The data, therefore, implicate CG as a possible marker of poor prognosis in ALL, similar to what we reported in AML (18), and also validate the uptake of serine proteases as a shared mechanism among tumors.

Furthermore, protein localization within the tumor cell can facilitate peptide presentation by surface class I HLA (24, 41). We have shown that CG, NE, and P3 are aberrantly expressed in AML blasts outside azurophil granules and that the aberrant expression may augment their presentation by AML (17, 24). Since ALL lacks azurophil granules, which normally house serine proteases, ALL may efficiently present CG due to its aberrant localization, irrespective of uptake or endogenous expression. Furthermore, the lack of azurophil granules in ALL could explain the absence of CG protein expression in some patient samples where CG expression at the mRNA level was detected (**Figures 1** and **2**). Specifically, azurophil granules shield proteases, including CG, from intracellular degradation. The absence of these granules could, therefore, facilitate intracellular degradation of serine proteases, including CG (24). In addition to protein expression, we have previously eluted the HLA-A2 restricted peptide CG1 from the surface of ALL. Cytotoxicity data (**Figure 4**) along with a prior report by Fujiwara et al. showing T cell responses to CG in three patients with ALL point to the potential relevance of CG as an immunotherapeutic target in ALL (21). These data showing that CG expression is not limited to the myeloid lineage and is

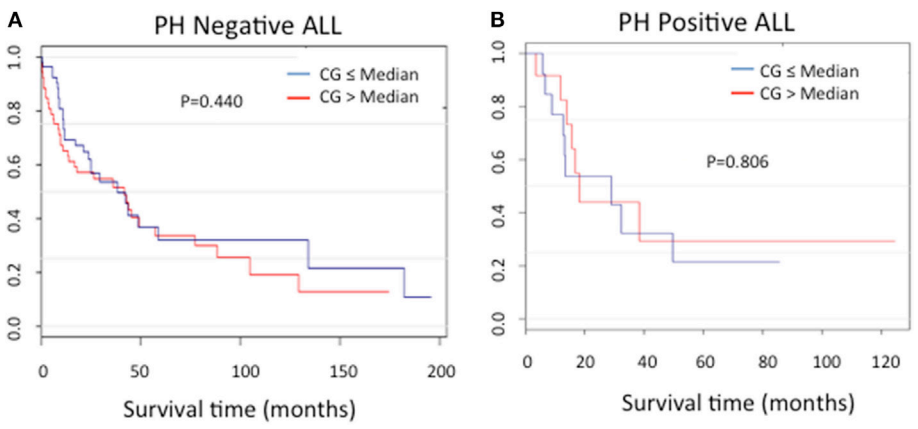


**FIGURE 6** | Cathepsin G (CG) protein expression and ALL survival. Kaplan–Meier curves show no significant differences in survival in patients with **(A)** Ph-negative ($n = 104$; $P = 0.44$) or **(B)** Ph-positive ($n = 25$; $P = 0.806$) ALL according to CG expression.

presented by HLA class I on ALL cell surface warrant further investigation of CG- and CG1-targeting immunotherapies in lymphoid malignancies.

Moreover, we have previously reported that a number of tumor types, including B cell-derived malignancies, take up and cross-present the serine proteases NE and P3, and are consequently rendered susceptible to killing by CTLs targeting the HLA-A2-restricted peptide PR1 (23, 24, 30). Cross-presentation is a well-defined immunologic mechanism whereby exogenous antigens are taken up and cross-presented by HLA class I molecules on the cell surface (42). We now extend these findings to CG. Although other investigators have shown that CG, in addition to NE, can be taken up by lung cancer (34), this is the first report showing CG uptake by a hematologic malignancy. This observation is highly relevant since ALL originates in the BM, where CG expression is higher than that found in other tissues. The uptake and cross-presentation of CG by ALL may be a mechanism that renders ALL susceptible to CG-targeting immunotherapy, thereby expanding the repertoire of ALL-associated immunotherapeutic targets. Nevertheless, we recognize that a significant proportion of ALL lacks CG and, therefore, would not be susceptible to CG-targeting immunotherapy. Furthermore, the ability of ALL to take up and cross-present CG from the BM microenvironment suggests a more widespread process by which antigens that are endogenously absent in ALL can be taken up by the malignant cells, thereby leading to a much broader arsenal of ALL targets.

These data also point to tissue-specificity of CG uptake, since we have shown that breast cancer cells do not take up CG (25), which could be due to a shared receptor between distinct tumor types that facilitates the uptake of serine proteases (43). Our results also indicate that the efficiency and rate of CG uptake varies between different cell lines. Furthermore, previous studies have cited time variations for antigen cross-presentation, with DC cross-presentation occurring as early as 4 h (24, 44), while cross-presentation by solid tumor cells occurred at later time points (24 h) (23, 30). Considering the role of B-lymphocytes as professional APCs, we expect cross-presentation by ALL to occur at early time points, as we have shown that cross-presentation of NE and P3 by the B-ALL cell line HMy2.CIR occurred as early as 6 h. Since priming an immune response requires interactions between co-stimulatory molecules and APCs and their cognate receptors on T cells, our data presented here and published reports suggest that ALL can prime an immune response against CG-derived peptides; however, we recognize that we do not provide data to directly support this hypothesis. Nevertheless, the data presented here highlight the role of CG1 as a target in ALL.

Similar to the results obtained with cell lines, our data show that all B-ALL patient samples express CG to varying extents (**Figures 1** and **2**). Three patient samples (UPN 3, 5, and 7) exhibited endogenous expression of CG at the transcript level (**Figure 1D**). However, western blot analysis shows some degree of expression by all eight patient samples. The discrepancy between transcript and protein expression suggests that CG uptake may play a major role in regulating CG expression by ALL and may prognosticate for poor outcomes in ALL. The poor prognostic role of CG in ALL is not surprising, given that previous studies have correlated higher CG levels with aggressive behavior in breast carcinoma (45) and inferior survival in AML (18). Furthermore, two prior reports have correlated the uptake of serine proteases by lung and breast cancer cells with increased tumor cell proliferation (33, 34). Although the mechanistic studies to explain these observations are scarce, CG has been reported to induce the adhesive action of E-cadherin between tumor cells (46), which may precipitate tumor invasiveness due to E-cadherin-mediated collective metastasis (47) or apoptosis inhibition (48). Furthermore, CG also has been shown paradoxically to suppress monocyte activation, which may contribute additionally to tumor aggressiveness as a result of a downregulated immune response (49). We have shown that CG expression correlated negatively with survival in AML, especially in the presence of intermediate cytogenetics and *FLT3* mutations (18). Similarly, a high level of CG expression is shown to be associated with a higher risk of relapse in ALL (**Figure 1C**), which may be attributed to the associations demonstrated between CG and several other proteins potentially involved in leukemogenesis. The concurrent expression of cell adhesion molecules and oncogenes with CG is shared between AML and ALL cells and has been ascribed to CG-mediated modulation and cleavage; in addition to the phosphorylation of oncogenes, such as SRC, YAP1, and FOXO3, in ALL, this supports their role in the association between CG expression and worse prognosis. Furthermore, higher levels of proliferation-associated proteins in conjunction with lower levels of apoptotic proteins, as observed in CG-expressing cells, is likely to encourage the uncontrolled growth of leukemic cells in the pathogenesis of ALL. While vascular proteins have not been directly implicated in the progression of ALL, EGLN1 is known to negatively regulate hypoxia-inducible factor-1-alpha (HIF-1) and induce a non-hematological malignancy (50). Given the potential of HIF-1 to act as a tumor suppressor in AML (51), a similar effect in ALL may justify its contribution to leukemogenesis. Nevertheless, the observation that higher CG expression correlates with ALL relapse highlights the potential for CG1-CTLs therapy in this patient cohort.

In conclusion, these data demonstrate the expression of a myeloid tumor antigen by ALL. Moreover, our results further highlight the role of cross-presented antigens as targets for immunotherapy. Specifically, our findings expand on the potential to target CG with immunotherapy in ALL.

## AUTHOR CONTRIBUTIONS

MK, SC, and PS performed experiments, analyzed data, and wrote the manuscript. JR performed bioinformatics analyses and wrote parts of the manuscripts. AP, AAP, CK, NQ, MZhang, MZope, JG, MQ, and HJ performed experiments, analyzed data, and assisted with manuscript editing. LJ and KC-D assisted with flow cytometry analyses and edited the manuscript. YQ and SK performed RPPA experiments, analyzed data, and wrote the manuscript. EM and JM provided major contributions to the study design and experimental planning. GA was the principal investigator of this study, designed experiments, analyzed data, and wrote the manuscript and the revisions. MZhang performed experiments, analyzed data, and assisted with manuscript editing.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01975/full#supplementary-material.

**FIGURE S1** | Gating strategy used to determine CG1-CTL frequency following expansion. Cytotoxic T lymphocytes (CTLs) were stained with CG1/human leukocyte antigen (HLA)-A*0201 tetramer in addition to CD3, CD8, lineage (lin) markers CD4, 14, 16, 19, and live/dead Ghost Violet stain. Frequencies of CG1-CTL (CG1/HLA-A*0201 tetramer$^+$) is determined from live, lin$^-$, CD3$^+$, CD8$^+$ cell populations.

**FIGURE S2** | Gating strategy used to determine uptake of cathepsin G (CG) by ALL cell lines. NALM6 cells were surface stained with antibodies including CD10 (BioLegend), CD16 (BioLegend), CD19 (BD), and CD38 (BioLegend). Cells were fixed, permeabilized and intracellularly stained with anti-CG antibody. B-ALL cells were differentiated based on light scatter characteristics as well as established surface phenotype (CD10$^+$/CD16$^-$/CD19$^+$/CD38$^+$).

**FIGURE S3** | Human leukocyte antigen (HLA)-A*0201 status of UPN2. UPN2 ALL was stained with anti-HLA-A*0201 antibody (clone BB7.2) and analyzed using flow cytometry. Data demonstrate UNP2 to be HLA-A2*0201 negative. The HLA-A*0201-positive cell line, T2, was used as a positive control.

**FIGURE S4** | PMN-associated cathepsin G (CG) is taken up by normal B cells. Flow cytometry detected intracellular CG in the B cell population from normal donor peripheral blood mononuclear cells (PBMC) that were cocultured with irradiated whole PMN at a ratio of 3:1 overnight. PBMC were surface stained with lineage antibodies, including CD3, CD14, CD16, and CD19, and intracellularly stained with anti-CG antibody. B cells were identified based on light scatter characteristics as well as being surface CD3$^-$/CD14$^-$/CD16$^-$/CD19$^+$. Median fluorescence intensity (MFI) shown represent CG expression within the gated B-cell population. Non-stained and stained normal PMN were used as negative and positive staining controls, respectively. *$P < 0.05$.

**FIGURE S5** | Raji-A2 cells take up exogenous cathepsin G (CG). Raji-A2 cells were cultured with purified CG (10 μg/mL) for 24 h. Western blot analysis shows uptake of CG by Raji-A2. Western blots demonstrate CG protein in whole-cell lysates from Raji-A2. Gels were loaded with 30 μg of protein. U937 cell line was used as a positive control. Actin was used as a loading control.

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# An Uncoupling of Canonical Phenotypic Markers and Functional Potency of *Ex Vivo*-Expanded Natural Killer Cells

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Recent advances in cellular therapies for patients with cancer, including checkpoint blockade and *ex vivo*-expanded, tumor-specific T cells, have demonstrated that targeting the immune system is a powerful approach to the elimination of tumor cells. Clinical efforts have also demonstrated limitations, however, including the potential for tumor cell antigenic drift and neoantigen formation, which promote tumor escape and recurrence, as well as rapid onset of T cell exhaustion *in vivo.* These findings suggest that antigen unrestricted cells, such as natural killer (NK) cells, may be beneficial for use as an alternative to or in combination with T cell based approaches. Although highly effective in lysing transformed cells, to date, few clinical trials have demonstrated antitumor function or persistence of transferred NK cells. Several recent studies describe methods to expand NK cells for adoptive transfer, although the effects of *ex vivo* expansion are not fully understood. We therefore explored the impact of a clinically validated 12-day expansion protocol using a K562 cell line expressing membrane-bound IL-15 and 4-1BB ligand with high-dose soluble IL-2 on the phenotype and functions of NK cells from healthy donors. Following expansions using this protocol, we found expression of surface proteins that implicate preferential expansion of NK cells that are not fully mature, as is typically associated with highly cytotoxic NK cell subsets. Despite increased expression of markers associated with functional exhaustion in T cells, we found that *ex vivo*-expanded NK cells retained cytokine production capacity and had enhanced tumor cell cytotoxicity. The preferential expansion of an NK cell subset that is phenotypically immature and functionally pleiotropic suggests that adoptively transferred cells may persist better *in vivo* when compared with previous methods using this approach. *Ex vivo* expansion does not quell killer immunoglobulin-like receptor diversity, allowing responsiveness to various factors *in vivo* that may influence activation and inhibition. Collectively, our data suggest that in addition to robust NK cell expansion that has been described using this method, expanded NK cells may represent an ideal cell therapy that is longer lived, highly potent, and responsive to an array of activating and inhibitory signals.

**Keywords: natural killer cells, immunotherapy, *ex vivo* expansion, adoptive transfer, clinical product, phenotypic analysis, functional analysis**

## INTRODUCTION

Natural killer (NK) cells are cytotoxic effector lymphocytes of the innate immune system that are essential for the elimination of various pathogens and transformed cells (1). The role of NK cells in surveillance of transformed cells is supported by observations of an increased risk of cancer in patients with poor NK cell cytotoxic activity (2, 3), and murine studies have directly demonstrated NK cell-mediated tumor elimination (4–8). NK cells may be activated in response to stress-induced ligands, antibodies, or other activating proteins expressed on the surface of target cells, resulting in cytokine production, proliferation, and the release of cytolytic granules containing perforin and granzyme (9).

The interest in using NK cells as a cellular immunotherapy has led to an array of expansion protocols using long-term culture with recombinant cytokines or agonistic antibodies (10). Extended exposure of NK cells to soluble IL-15/IL-15Rα complexes increases in mature murine NK cells exhibiting replicative senescence and diminished cytolytic capabilities after 2 weeks (11). Protocols developed more recently have relied on feeder cell lines in addition to cytokines. A commonly used NK cell expansion clinical protocol uses irradiated K562 cells engineered to express membrane-bound IL-15 and membrane-bound 4-1BBL (K562-mb15-4-1BBL) (12). Studies using these cells demonstrate extensive NK cell expansion, increased activating receptor expression, and pro-inflammatory cytokine production (13, 14). It is not clear, however, whether NK cell subsets are equivalently expanded *ex vivo*, and the relative representation and contributions to antitumor immunity upon administration to patients.

Advances in cellular immunotherapy for patients with cancer using *ex vivo*-expanded T cells have highlighted the importance of their *in vivo* persistence to effectively control disease. NK cell approaches are faced with similar concerns, in particular because they do not generally undergo homeostatic proliferation *in vivo* (15). In addition, the percentages of NK cells that differentiate to memory cells, and the duration of their persistence, are diminished as compared with their T cell counterparts (16, 17). Further, the conditions required to achieve long-term memory in NK cells may not be recapitulated in patients with cancer, raising concerns of long-term persistence following *ex vivo* expansion and adoptive transfer.

Prolonged NK cell stimulation can occur as transformed cells accumulate (18, 19) or during chronic viral infections (20). Although the mechanisms for phenotypic and functional changes in NK cells following chronic stimulation are not fully defined, previous work demonstrates internalization of activating receptors following chronic stimulation (21), uncoupling of signaling adaptor proteins from activating surface receptors (22), and the downregulation of the transcription factor Eomesodermin (Eomes) in NK cells that can no longer control B cell lymphoma tumor growth (23). Consistent with these findings, patients with melanoma have decreased Eomes expression (24), suggesting that this may be a hallmark of NK cells with impaired pro-inflammatory functions. Using chronic stimulation to expand NK cells *ex vivo* may therefore result in a functionally impaired NK cell population, requiring greater numbers of NK cells to achieve efficacy, or subsequent selection of expanded NK cells to improve *in vivo* activation.

Given their demonstrated cytolytic capacity, we hypothesized that K562-mb15-4-1BBL *ex vivo*-expanded NK cells were mature, terminally differentiated, and may therefore have limited capacity for persistence, licensing, and responsiveness to the tumor microenvironment. Surprisingly, however, we find robust proliferation of a canonically less mature NK cell population that express several markers associated with less differentiated NK cells. This population also expresses several genes that enhance NK cells' potential to differentiate and persist *in vivo*, including increased Eomes, granzymes A and K, and decreased granzyme B (GzmB). Together, our data suggest that *ex vivo*-expanded NK cells may have greater longevity *in vivo* than predicted based on both enhanced cytotoxicity following expansion and phenotypic and functional analysis of adoptively transferred T cells.

## MATERIALS AND METHODS

All protocols have been reviewed and approved by the relevant institutional committees, including the Seattle Children's Research Institute Institutional Biosafety Committee (Approval #1211) and Institutional Review Board (Approval #14412).

### Cell Lines, Cell Culture, and Peripheral Blood Mononuclear Cells (PBMCs)

K562 (human erythroblastoid cell line; American Type Culture Collection) and K562-mb15-4-1BBL (12) (a generous gift from Dr. Dario Campana and Dr. Lewis Lanier) were cultured in RPMI-1640 (ThermoFisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Little Chalfont, UK) at 37°C in 5% $CO_2$. Human PBMCs were isolated from healthy donors by centrifugation over a Ficoll gradient per the manufacturer's instruction (STEMCELL Technologies, Vancouver, BC, Canada). PBMCs were stored long term in FBS + 10% DMSO and submerged in liquid nitrogen.

### Expansion of NK Cell Products

Quick-thawed (37°C) bulk PBMC were cultured at a 1:1 ratio with 100 Gy irradiated K562-mb15-4-1BBL in NK cell media containing X-VIVO-10 (Lonza, Basel, Switzerland) supplemented with 10% human AB serum (Corning Cellgro, Inc., Corning, NY, USA) and 1,000 U/mL recombinant human IL-2 (R&D Systems, Minneapolis, MN, USA) and incubated at 37°C and 5% $CO_2$ on day 0. Cultures were expanded in T75 flasks with 10 mL media and IL-2, supplemented with fresh 10 mL media and cytokine on days 4, 7, and 10. All cells in culture were harvested on day 12 for subsequent phenotyping and functional assays. NK cells were donor matched for all measurements before and following expansion.

### NK Cell Isolation

Following thaw of PBMCs (day 0 NK cells) or after 12 days of coculture of bulk PBMCs with K562-mb15-4-1BBL, NK cells were isolated *via* CD14+ cell depletion with the use of a magnetic

bead selection kit (STEMCELL Technologies), followed by NK cell enrichment using a magnetic bead negative selection kit (human NK cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Before functional studies, NK cells were rested in NK media for 24 h.

## Phenotypic Characterization by Flow Cytometry

Peripheral blood mononuclear cells or expanded NK cells were surface stained with panels of monoclonal antibodies used at the manufacturer's recommended concentration. Antibodies from BioLegend (San Diego, CA, USA) included anti-CD3 (clone OKT3), anti-CD14 (clone M5E2), anti-CD56 (clone HCD56), anti-NKG2D (clone 1D11), anti-CD57 (clone HCD57), anti-CD11b (clone M1/70), anti-CD69 (clone FN50), anti-Tim3 (clone F38-2E2), anti-PD-1 (clone EH12.2H7), and anti-CD95 (clone DX2). Antibodies from BD Biosciences (San Jose, CA, USA) included anti-CD16 (clone 3G8) and anti-CD94 (clone HP-3D9). Antibodies from R&D Systems included anti-NKG2A (clone 131411) and anti-KIR2DL2/DL3/DS2 (clone 180704). Antibodies from Beckman Coulter (Indianapolis, IN, USA) included anti-KIR3DL1/DS1 (clone Z27.3.7) and anti-KIR2DL1/DS1 (clone EB6B). All panels also included the Fixable Blue Dead Stain (Life Technologies, Carlsbad, CA, USA) as a viability dye. K562 cells were stained with antibodies to PD-L1 (BioLegend, clone 29E.2A3) and Gal-9 (BioLegend, clone 9M1-3) with DAPI (BioLegend) as a viability dye. After staining, cells were fixed with 2% paraformaldehyde and analyzed using the BD Fortessa instrument (BD, Franklin Lakes, NJ, USA) and FlowJo software (TreeStar, Ashland, OR, USA).

## Calculation of Per-Cell Fold Expansion

The proportion of total live cells belonging to each NK subset was calculated using FlowJo software and then multiplied by the number of PBMCs added to start the culture at day 0 to determine the absolute number of cells in each subset. On day 12, the proportion of total NK cells belonging to each NK subset was calculated and multiplied by the number of NK cells isolated following expansion to give the absolute number of cells in each subset. Fold change for each subset was calculated as the absolute number of cells present following expansion divided by the absolute number of cells in the starting culture. Similar calculations were employed for T cells and monocytes.

## Degranulation/Intracellular Cytokine Staining

Cytokine production and degranulation were analyzed by flow cytometry. NK cells were seeded in a 96-well round bottom plate either alone (control) or together with K562 wild-type (WT) cells at a 1:1 ratio and incubated with anti-CD107a antibody (clone H4A3, BioLegend) for 1 h at 37°C/5% $CO_2$, followed by Brefeldin A (10 μg/mL, Sigma, St. Louis, MO, USA) for an additional 3 h. Cells were then washed and stained with Fixable Blue Dead Stain and surface antibodies to CD3, CD56, and CD16. Cells were then fixed, permeablized with 1× Permwash (BioLegend), and stained intracellularly for GzmB (clone GB11, BD Biosciences), perforin (clone B-D48, BioLegend), and IFNγ (clone 4S.B3, BioLegend). For samples with antibody blockade, NK cells were incubated with antibodies (10 μg/mL in PBS with 2% bovine serum albumin, LEAF purified, same clones as above, BioLegend) for 45 min, washed, then mixed with K562 cells. Samples were analyzed using the BD Fortessa instrument and FlowJo software.

## Chromium Release Cytotoxicity Assays

K562 WT cells (target) were labeled with $^{51}Cr$ (5 mCi/mL; Perkin Elmer, Waltham, MA, USA) for 24 h and washed. NK cells (effector) were cocultured with $^{51}Cr$-labeled K562 cells at 25:1, 10:1, 5:1, and 1:1 Effector:Target (E:T) ratios in 96-well plates for 4 h at 37°C, 5% $CO_2$ as previously described (25). Fifty microliters of supernatant were harvested and dispensed in corresponding wells of a LumaPlate (Perkin Elmer). LumaPlates were allowed to dry overnight at room temperature and were then analyzed on a TopCount NXT (Perkin Elmer) to determine the amount of $^{51}Cr$ released from lysed K562 target cells in counts per minute (cpm). Maximum chromium-51 release was determined by incubation of K562 target cells in 2% SDS solution; spontaneous release was obtained by incubation of target cells in the absence of effectors (media only). The mean percent of cytotoxicity was calculated using the following formula: ((cpm in experimental release − cpm in spontaneous release)/(cpm in maximum release − cpm in spontaneous release)) × 100. Differences between day 0 and day 12 samples were tested for significance using a two-way ANOVA.

## Nanostring (NS) Gene Expression Analysis

Donor-matched NK cells were isolated before or following expansion, rested in NK media overnight, and collected at 10,000 cells/μL in RLT buffer (Qiagen, Germantown, MD, USA) supplemented with beta-mercaptoethanol (Bio-Rad, Hercules, CA, USA). RLT lysate was then used directly in the Human Inflammation Panel (Nanostring, Seattle, WA, USA) per the manufacturer's protocols. Nanostring nSolver Advanced Analysis software was used to normalize gene expression across samples, and *p* values of expression changes between day 0 and day 12 samples were calculated by paired Student's *t*-test with a Benjamini–Yekutieli multiple comparisons adjustment.

## Quantitative PCR of Tbet and Eomes

Total RNA was isolated from excess RLT lysates collected for NS analysis using the RNeasy kit (Qiagen), then cleaned and concentrated using the RNeasy Min-Elute kit (Qiagen). An equivalent amount of total RNA (approximately 500 ng per sample) was reverse transcribed to cDNA with the SuperScript III kit (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed on the cDNA with SYBR Green Master Mix (ThermoFisher) on the Bio-Rad CFX96 thermocycler. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change between day 0 and day 12 samples, including TBP as the housekeeping gene. Primer sequences (written 5′–3′): TBP 5′: GAGCTGTGATGTGAAGTTTCC, TBP 3′: TCTGGGTTTGATCATTCTGTAG, Tbet 5′: GCTCACAA ACAACAAGGGGG, Tbet 3′: TATGCGTGTTGGAAGCGTTG, Eomes 5′: CGCCACCAAACTGAGATGAT, and Eomes 3′: TT GTTGCCCTGCATGTTATTGT.

## Reproducibility and Statistics

All experiments were performed on at least three independent healthy donors. Unless otherwise noted, paired Student's *t*-tests or one-way ANOVAs with Sidak's multiple comparisons test were use to test for significance, as appropriate, using Prism software (GraphPad Software, San Diego, CA, USA).

# RESULTS

Previous studies have demonstrated the ability of expanded NK cells to efficiently kill target cells (12–14, 26, 27), suggesting a mature and terminally differentiated phenotype. However, the degree to which an expanded NK product resembles NK cells found in peripheral circulation is currently unknown. It is likely that the phenotype of NK cells following expansion will at least in part predict patient response, as has been seen in adoptively transferred T cells (28–30). Therefore, we sought to define NK phenotype following expansion in the presence of IL-2 and K562 cells expressing membrane-bound IL-15 and costimulatory 4-1BBL. This feeder line has been used to expand NK cells that have been used to successfully treat patients in clinical trials (31, 32).

We first characterized NK cell subsets by CD16 and CD56 expression, using the gating strategy outlined in Figure S1 in Supplementary Material. NK cells exist on a spectrum of maturation, with less mature cells expressing high levels of CD56 responsible for the majority of cytokine production upon activation. As NK cells mature, they reduce CD56 levels and acquire CD16 expression, enabling efficient antibody-dependent cell cytotoxicity, and are the main cytotoxic population (**Figures 1A,B**). We performed flow cytometry to analyze NK cells from healthy donors both before (day 0) and after (day 12) expansion of NK cells (representative donor, **Figure 1C**; gating strategy in Figure S1 in Supplementary Material). Most notably, in addition to an increased proportion of the total NK cells (from approximately 10% before expansion to >90%) falling in a CD56 bright subset, CD56 staining intensity increases across all NK cell populations. CD56dim cells are concomitantly decreased in the day 12 cells, as illustrated in **Figure 1D**. On a per-cell basis, the four subtypes of NK cells varied in their fold expansion, with total NK cells expanding approximately 65-fold, consistent with previous reports (13, 14), the CD56bright CD16+ subset expanding more than 6,000-fold, and the amount of CD56dim CD16+ after 12 days of culture was 40% less than the number input initially (**Figure 1E**). Furthermore, we observed no monocyte expansion and minimal combined NKT and T cell expansion (average fivefold expansion of total CD3+ cells) (Table S1 in Supplementary Material). Similar expansion of CD3+ cells was seen by other investigators who used a high concentration of IL-2 (14). Because of the low number of certain cell subsets, subsequent analysis delineates expanded NK cell subsets based only on CD56 expression.

High levels of CD56 expression on the surface of NK cells is associated with reduced cytotoxic activity (33). Therefore, we wanted to confirm that the expanded NK cells were functionally similar to those used in previous studies, which have found

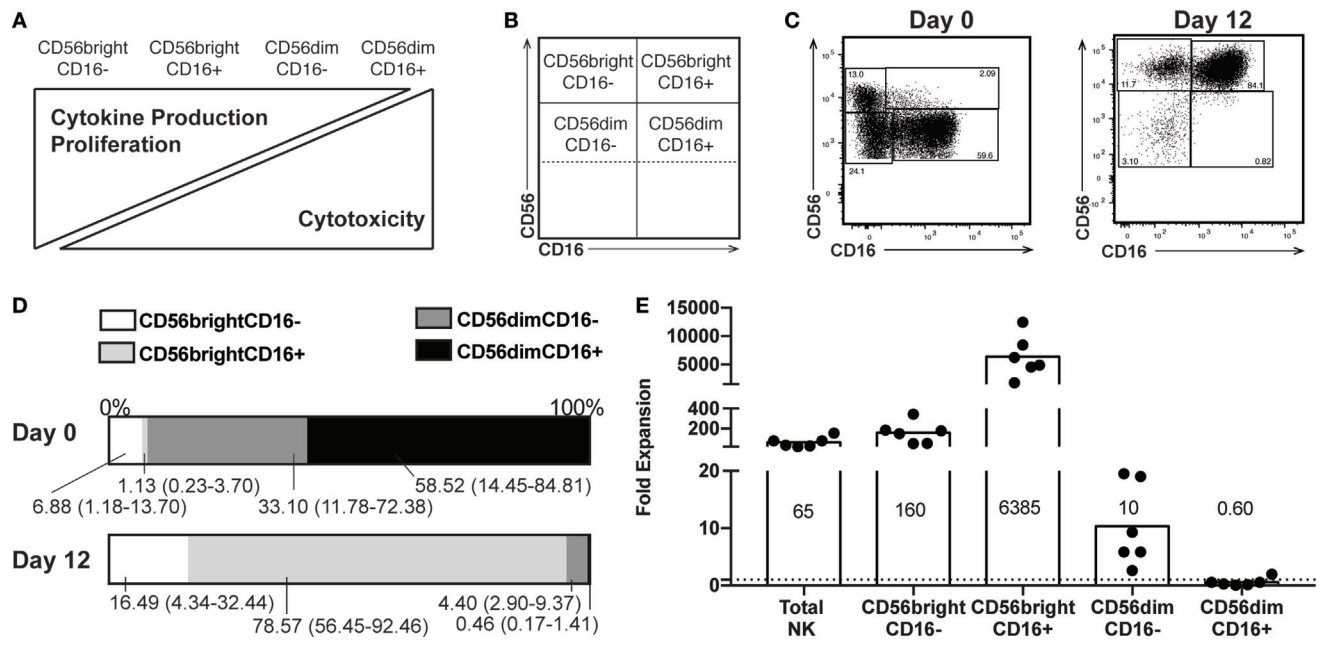


**FIGURE 1** | Proportions of natural killer (NK) subsets following expansion. **(A)** Spectrum of NK functions and maturity based on expression of CD56 and CD16. **(B)** Representation of where subsets appear on flow plots of NK cells. **(C)** Distribution of CD56 and CD16 expression by NK cells from a representative donor before (day 0) and after (day 12) expansion. Percentage of NK cells in each subset is included in each gate. **(D)** Percentages of each subset at day 0 (top) and day 12 (bottom). Labels for each subset indicate a mean and range ($n = 6$). **(E)** Expansion of total NK cells and each subset on a per-cell basis. The mean fold change is indicated for each subset ($n = 6$).

increased cytotoxicity following expansion (12–14, 26, 27, 34, 35). Using a chromium release assay (CRA), we confirmed that by day 12 of expansion, NK cells were more efficient at lysing K562 target cells (representative donor, **Figure 2A**). This suggested that the enriched CD56bright population, generally considered more involved in cytokine production than cytotoxicity, must be playing a significant part in killing the chromium-labeled cells. We therefore performed a degranulation assay, which measures release of cytotoxic granules and externalization of the endosomal protein CD107a. In response to exposure to target cells, both CD56bright and CD56dim subsets externalized CD107a (**Figures 2B,C**). However, in freshly isolated NK cells, the low basal perforin and GzmB content in the CD56bright cells suggest a limited capacity to lyse target cells despite evidence of degranulation, a phenomenon supported by CRA data at lower effector to target ratios. CD56dim cells express high levels of perforin and GzmB; staining is decreased, suggesting degranulation, upon exposure to target cells (**Figures 2D,E**, top). By contrast, at day 12, staining for perforin and GzmB is high in CD56bright cells and decreases following target exposure (**Figures 2D,E**, bottom); CD56dim cells do not appear to be releasing significant cytotoxic granules. Finally, although cytokine production and cytotoxicity are functions performed by different subsets in peripheral blood NK cells, by day 12, the CD56bright subset is still responsible

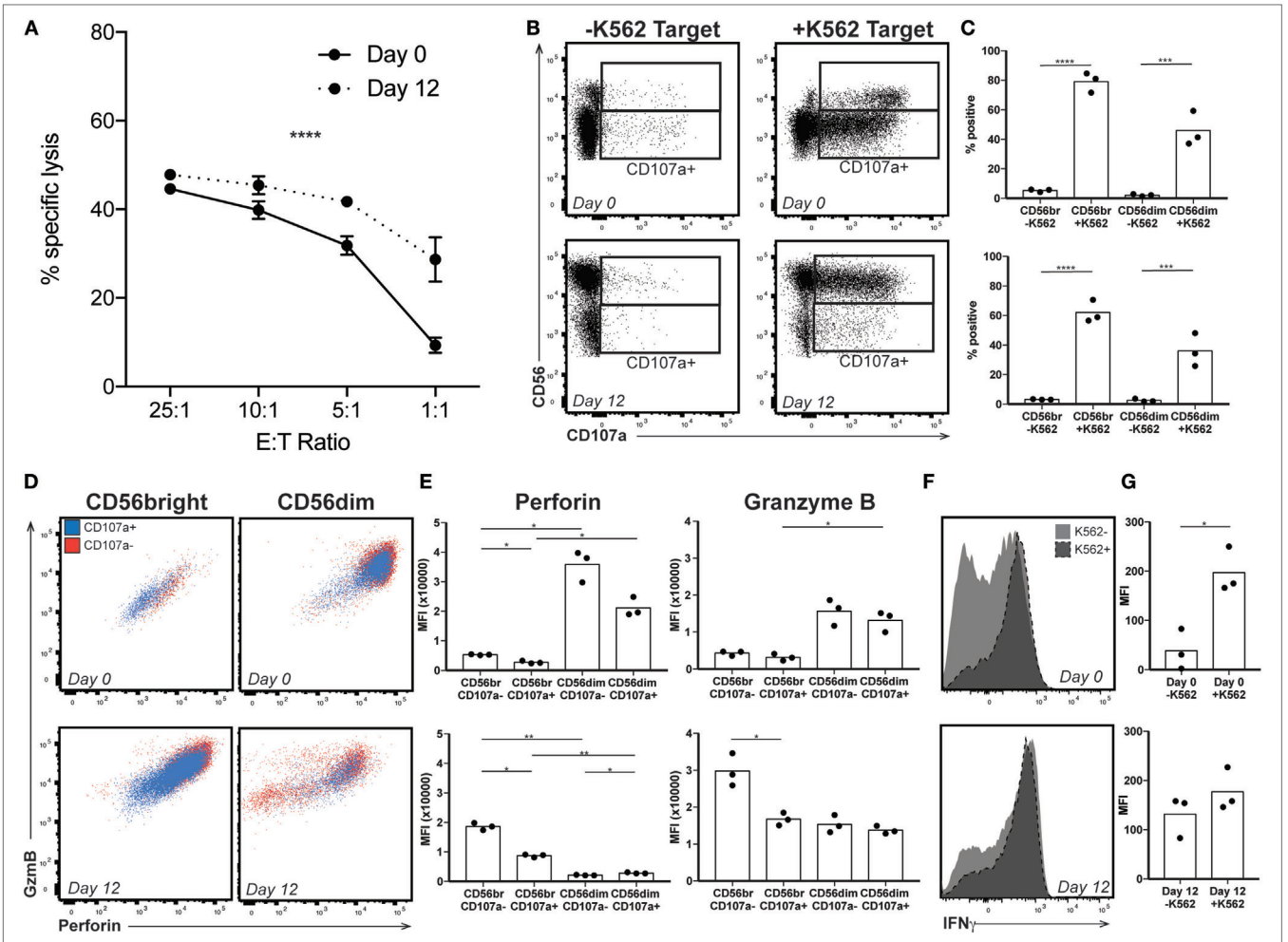


**FIGURE 2** | Expanded natural killer (NK) cell response to target. **(A)** By chromium release assay, NK cells lyse target more efficiently at low E:T ratio following expansion (representative donor). Statistical significance was assessed by two-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). **(B)** Day 0 (top) and day 12 (bottom) NK cells from a representative donor were incubated without (–K562) or with (+K562) target cells and degranulation monitored by staining for the endosomal protein CD107a. Gates indicate CD107a positivity for CD56bright and CD56dim populations. **(C)** Quantification of degranulation by CD56 expression in response to K562 target at day 0 (top) or day 12 after expansion (bottom). Statistical significance was assessed by one-way ANOVA. **(D)** Granzyme B (GzmB) and perforin content by CD56 expression in the presence of K562 target cells in a representative donor. Cells that have released endosomal contents (CD107a+) are represented in blue, while those that did not degranulate (CD107–) are in red. **(E)** Quantification of perforin and GzmB MFI in the presence of K562 target cells. Day 0 NK cells are shown on top, and expanded day 12 NK cells on the bottom. Statistical significance was assessed by one-way ANOVA. **(F)** Interferon-γ expression by total NK cells from a representative donor in the presence or absence of K562 target cells before (top) or after (bottom) expansion. **(G)** Quantification of IFNγ MFI in response to K562 target cells. Day 0 NK cells are shown on top, and expanded day 12 NK cells on the bottom. Statistical significance was assessed by Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

for IFNγ production upon target exposure on top of its role in cytotoxicity (**Figures 2F,G**), suggesting that the expanded NK cell product is comprised of a cell type that phenotypically resembles immature NK cells with high proliferative and cytokine secretory capacity, and functionally retains the properties of an immature NK cell, but with enhanced cytotoxic capability.

Our results so far indicate that by day 12, a highly cytotoxic subset of NK cells has expanded, although its function appears decoupled from its CD56 expression, a phenomenon seen in NK cells cultured in IL-2 and/or IL-15 (12, 36, 37). Given the concern of T cell persistence *in vivo* following *ex vivo* expansion, and the potential for the sustained activation to contribute to exhaustion, we examined canonical markers of T cell activation and exhaustion on the surface of expanded NK cells, all of which were expressed on 5% or fewer NK cells before expansion. Expression of CD95 occurs in T and NK cells upon their activation, and following expansion, nearly 100% of CD56bright and >50% of CD56dim NK cells are CD95+ (**Figures 3A,B**). Similarly, the C-type lectin CD69 serves as a marker of nonspecific activation in response to various stimuli (38) and its upregulation is observed after expansion on both CD56bright and CD56dim NK cells (**Figures 3C,D**). Unlike CD69 expression on CD8+ T cells, however, CD69 expression does not wane following initial upregulation (11), and therefore is not exclusively a marker of early activation. By day 12, the well-characterized T cell exhaustion marker PD-1 increases from about 5% to approximately 50% expression on both CD56bright and CD56dim subsets (**Figures 3E,F**). Tim3, another marker of T cell exhaustion, increases on CD56bright cells from 2% at day 0 to 100% by day 12 (**Figures 3G,H**), and to 14% on CD56dim NK cells. K562 target cells express the PD-1 ligand PD-L1 and the Tim3 ligand Gal-9 (Figure S2 in Supplementary Material). Blockade of either PD-1 or Tim3 did not decrease degranulation, however (**Figures 3I,J**), suggesting that expanded NK cells were not functionally exhausted.

In addition to CD56, several other surface markers and inhibitory receptors have been associated with the state of NK cell maturity. Despite significant CD56 and IFNγ production by the predominant NK subsets at day 12, increased cytotoxicity and expression of exhaustion markers suggest that expanded NK cells would express other proteins present on mature NK cells. Therefore, we expected most expanded NK cells to express CD57, a marker of highly cytotoxic, terminally differentiated NK cells (25). Although there was not a significant increase in CD57 expression by day 12 when comparing CD56bright subsets, comparison of predominant NK cell populations before and after expansion revealed a decrease in the number of NK cells expressing CD57. At day 0, an average of 47% of CD56dim NK cells express CD57, while at day 12, only about 5% of CD56bright cells express CD57 (**Figures 4A,B**). We also looked at expression of NKG2A, an inhibitory receptor for HLA-E expressed by immature NK cells, expecting the opposite trend of CD57 expression, which was lost upon expansion. We were surprised to see retention of NKG2A in the CD56bright subset following expansion, and a concomitant increase in expression by the CD56dim subset (**Figures 4C,D**). The percentage of cells expressing CD94, the co-receptor for NKG2A, also remained constant throughout expansion (**Figures 4E,F**). The integrin CD11b (ITGAM) is generally expressed on most NK cells found in peripheral circulation but absent in immature populations found in particular biological niches in both mice and humans (39, 40). Consistent with these observations, following >95% expression at day 0 in both subsets, CD11b expression after expansion drops to 69 and 31% on CD56bright and CD56dim subsets, respectively (**Figures 4G,H**). Finally, expression of NKG2D, an activating receptor that binds to the stress ligands MICA/B and ULBP1–6, remains near 100% in all NK cell samples (**Figures 4I,J**).

The killer immunoglobulin-like receptors (KIRs) are a family of NK receptors whose activating or inhibitory effect on cytotoxicity is determined by the cytoplasmic domain found on identical surface proteins that are acquired late during NK cell maturation (41). Given our conflicting phenotypic and functional results, we were uncertain whether to expect increased KIR expression, as would be associated with differentiation, or decreased KIR expression, more consistent with a lack of maturity. KIR expression increased in percent positivity (to approximately 8, 15, and 22% positive on KIR3DL1/DS1, KIR2DL1/DS1, and KIR2DL2/DL3/DS2, respectively) on CD56bright cells by day 12 of expansion, reaching expression similar to that found on CD56dim cells in unexpanded cultures at day 0 (**Figures 5A–F**), although increases were not statistically significant. However, even within the expanded population of CD56bright NK cells, there remains at least some degree of maturation heterogeneity: when gated on NKG2A positive or negative cells, which are typically considered less or more mature, respectively, NK cells expressed all three KIR subgroups on an average of fewer than 1% of cells, versus more than 5% of NKG2A- cells (**Figures 6A–C**). A similar trend is seen for the day 0 CD56dim cells, although overall KIR percent triple positive is lower (**Figures 6D–F**). This is consistent with the enhanced cytotoxicity observed in the expanded cells, which appear to have a disparate relationship between maturity and cytolytic capacity than is typically described for NK cells based on CD56 expression. Collectively, these data suggest that CD56 expression is uncoupled from function in *ex vivo*-expanded NK cells.

To assess expression of maturation markers independent of CD56 surface expression, we evaluated the expression of transcription factors critical to NK cell gene expression in different stages of maturation. Differentiation of NK cells in peripheral circulation is driven largely by the reciprocal expression of two transcription factors, Eomes and Tbet. Mature NK cells have a higher ratio of Tbet to Eomes than immature NK cells, and the increase in Tbet throughout maturation is implicated in the acquisition of cytotoxic functions (42). To determine whether *ex vivo* expansion of NK cells impacts the relative expression of Eomes and Tbet, we performed NS analysis of freshly isolated NK cells before or after expansion using a panel of genes associated with inflammation (Table S2 in Supplementary Material). We found a decrease in Tbet (**Figure 7A**) and an increase in Eomes (**Figure 7B**) following expansion, supporting the hypothesis that *ex vivo*-expanded NK cells represent a less differentiated NK cell population with potentially extended longevity and responsiveness to various activating stimuli *in vivo*. This was confirmed by qPCR of Tbet and Eomes (**Figure 7C**). Although the reduction

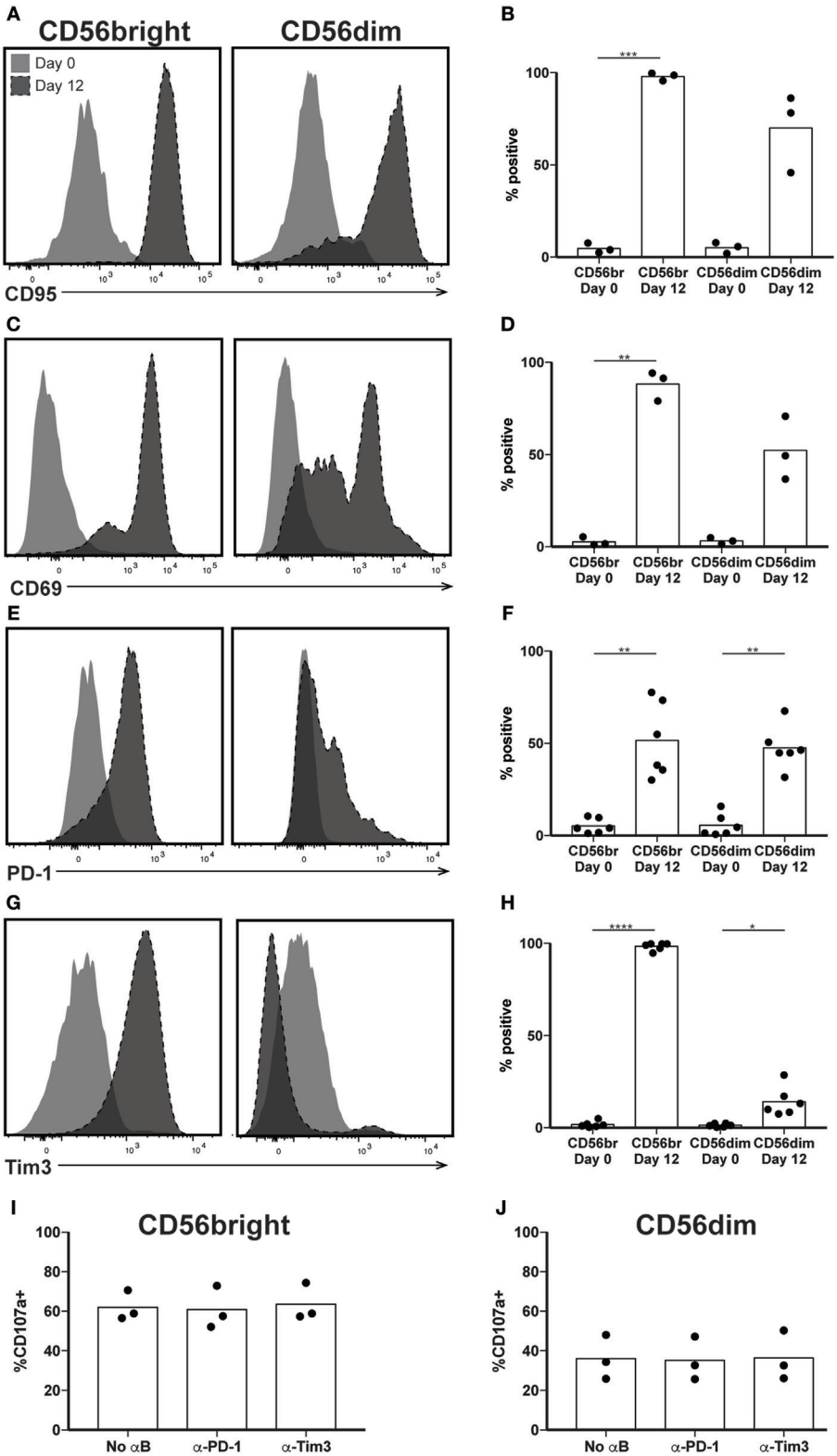


**FIGURE 3** | Expression of exhaustion markers following natural killer (NK) expansion. **(A–H)** Overlays of NK cells from a representative donor before (day 0) and after (day 12) expansion, by CD56bright (left) and CD56dim (right), for CD95 **(A)**, CD69 **(C)**, PD-1 **(E)**, and Tim3 **(G)**. Quantification of percent cells positive at day 0 and day 12 for CD95 **(B)**, CD69 **(D)**, PD-1 **(F)**, and Tim3 **(H)**. Statistical significance was assessed by one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). **(I,J)** Percentage of expanded CD56bright **(I)** or CD56dim **(J)** NK cells that degranulated (CD107a+) in the presence of blocking antibodies to PD-1 or Tim3. Statistical significance was assessed by one-way ANOVA, and no differences seen.

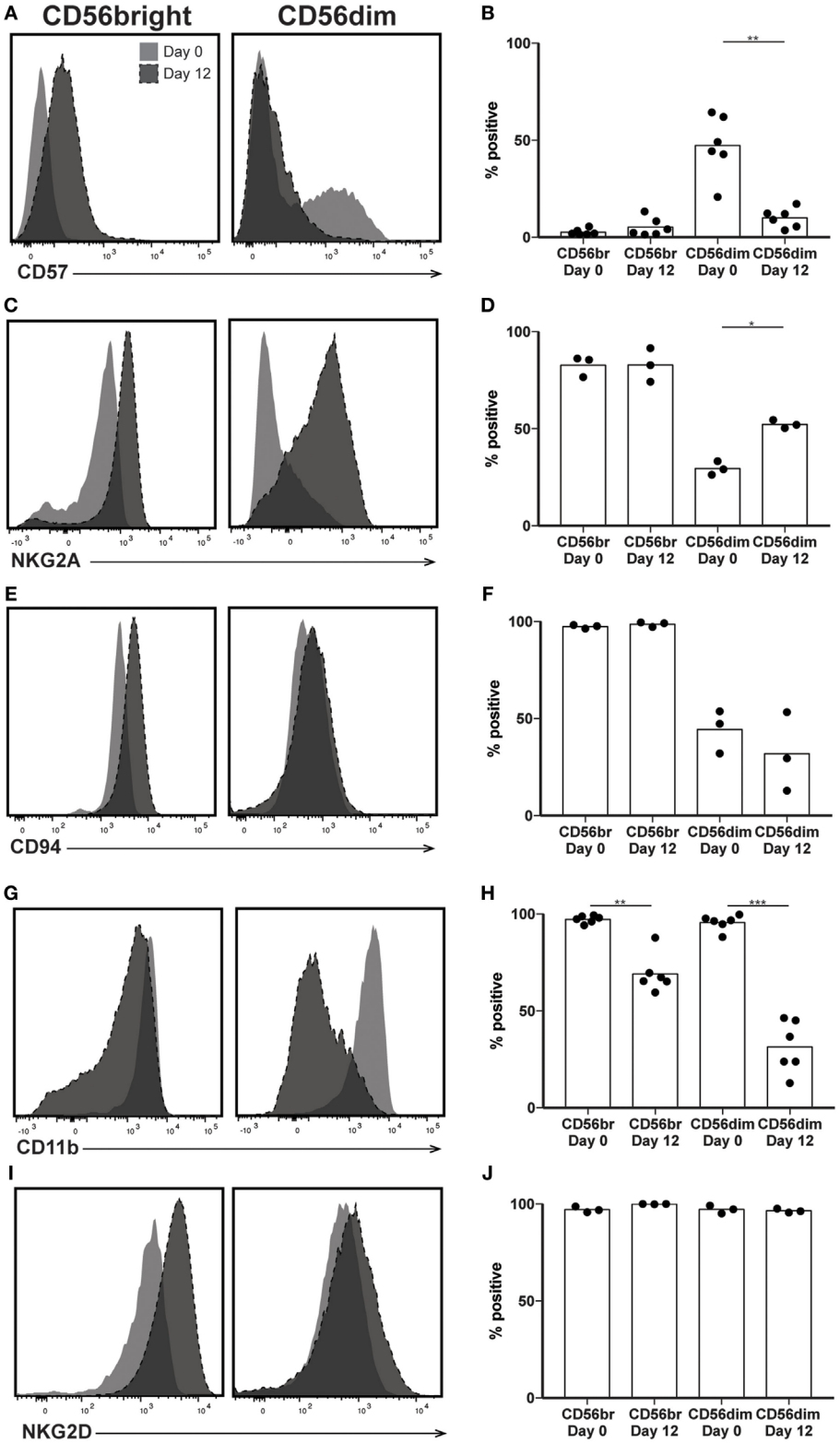


**FIGURE 4** | Expression of maturity markers following natural killer (NK) expansion. **(A–J)** Overlays of NK cells from a representative donor before (day 0) and after (day 12) expansion, by CD56bright (left) and CD56dim (right), for CD57 **(A)**, NKG2A **(C)**, CD94 **(E)**, CD11b **(G)**, and Tim3 **(I)**. Quantification of percent cells positive at day 0 and day 12 for CD57 **(B)**, NKG2A **(D)**, CD94 **(F)**, CD11b **(H)**, and Tim3 **(J)**. Statistical significance was assessed by one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

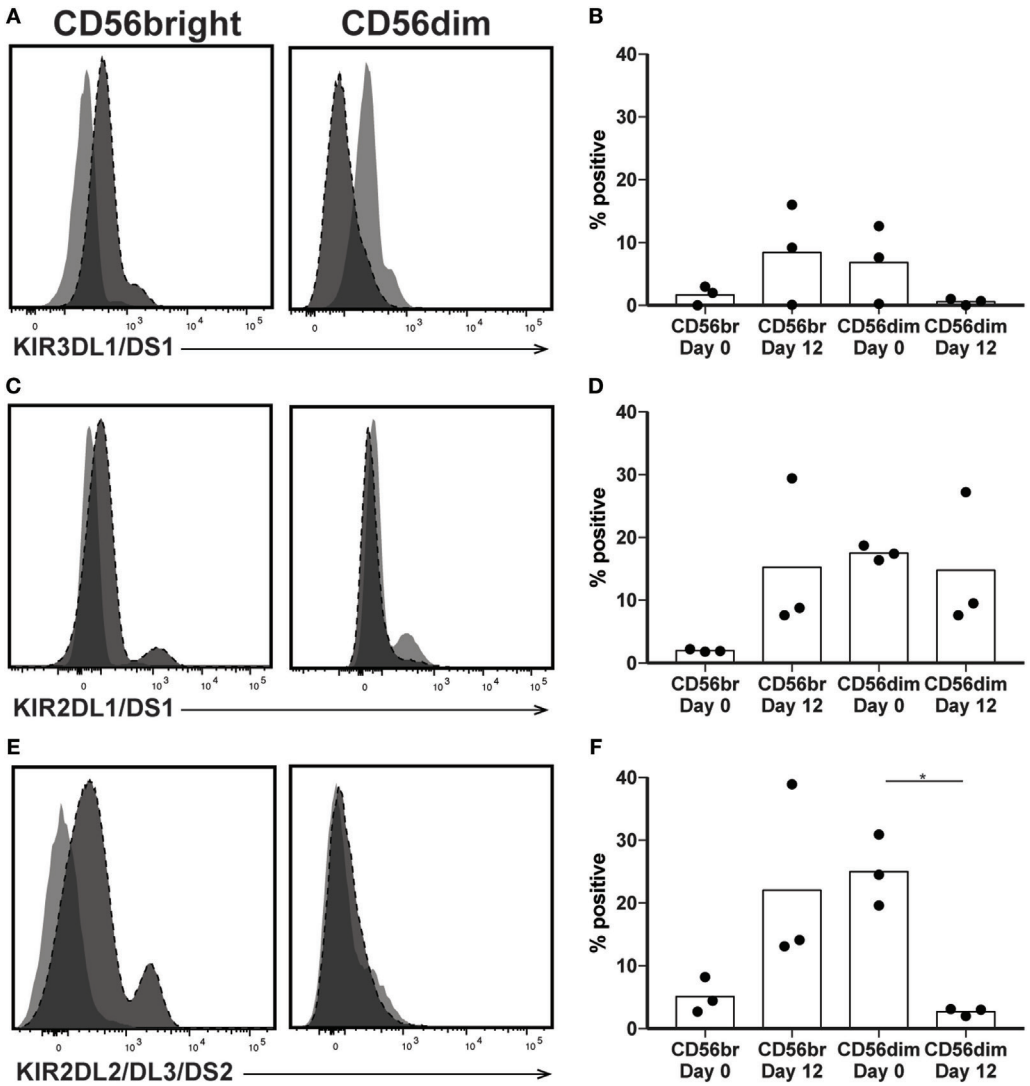


**FIGURE 5** | Expression of killer immunoglobulin receptors following natural killer (NK) expansion. **(A–F)** Overlays of NK cells from a representative donor before (day 0) and after (day 12) expansion, by CD56bright (left) and CD56dim (right), for KIR3DL1/DS1 **(A)**, KIR2DL1/DS1 **(C)**, or KIR2DL2/DL3/DS2 **(E)**. Quantification of percent cells positive at day 0 and day 12 for KIR3DL1/DS1 **(B)**, KIR2DL1/DS1 **(D)**, or KIR2DL2/DL3/DS2 **(F)**. Statistical significance was assessed by one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

in Tbet in day 12 cells was smaller as measured by qPCR relative to NS, the increase in Eomes/Tbet ratio is similar (**Figure 7D**).

In our NS analysis, we confirmed several trends we had seen by flow cytometry, including upregulation of CD56, IFNγ, and specific KIR subgroups following expansion. We also found altered expression of several genes we had not included in our flow cytometry panels, but are consistent with acquisition of highly cytotoxic, yet less traditionally mature phenotype. We saw 54-fold upregulation of Granzyme K and 3.5-fold upregulation of Granzyme A, alongside a 2-fold decrease in GzmB (Table S2 in Supplementary Material), consistent with previous reports of less differentiated CD56bright cells relying on alternative granzymes (43). We also saw a striking increase in several chemokine receptors, CCR1, CCR2, CCR5, CCR6, CXCR3, and CXCR6 (Table S2 in Supplementary Material), suggesting that expanded NK cells may be better able to localize to tumors, many of which overexpress the cognate chemokines (44). Therefore, we propose that the current method for *ex vivo* expansion of NK cells selects for a potent subset capable of proliferation, cytokine production, cytotoxicity, and persistence, making this an ideal adoptively transferred cell population for the treatment of patients with cancer.

## DISCUSSION

Natural killer cells play an important role in immunosurveillance, and their unique ability to eliminate transformed cells without the requirement of a tumor-specific antigen makes them an attractive

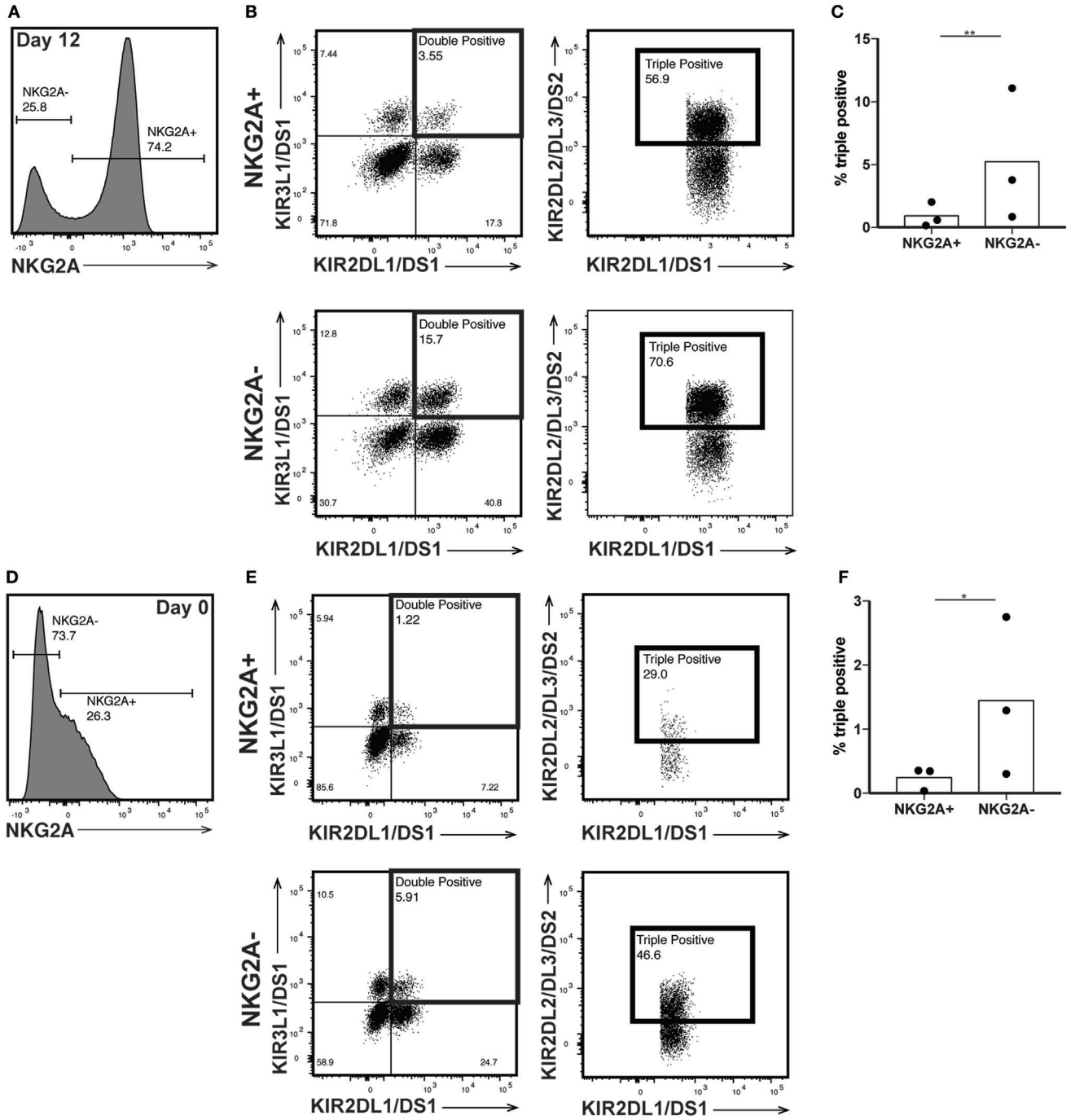


**FIGURE 6** | Killer immunoglobulin-like receptor (KIR) expression is enriched in NKG2A- natural killer (NK) cells. **(A)** Day 12 expanded CD56bright NK cells were gated on NKG2A expression. **(B)** NKG2A+ (top) and NKG2A– (bottom) cells were interrogated for their expression of both KIR2DL1/DS1 and KIR3DL1/DS1 (left). KIR2DL2/DL3/DS2 expression was examined on the double positive cells (right). **(C)** Percent of total NKG2A+ or NKG2A– cells that are positive for all three KIRs. Statistical significance was assessed by Student's *t*-test. **(D)** Day 0 CD56dim NK cells were gated on NKG2A expression. **(E)** NKG2A+ (top) and NKG2A– (bottom) cells were interrogated for their expression of both KIR2DL1/DS1 and KIR3DL1/DS1 (left). KIR2DL2/DL3/DS2 expression was examined on the double positive cells (right). **(F)** Percent of total NKG2A+ or NKG2A– cells that are positive for all three KIRs. Statistical significance was assessed by Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

cell type for the next generation of cancer immunotherapeutics. Following the initial reports of solid tumor regression following high-dose systemic IL-2 as early as 1985 (45), subsequent efforts demonstrated NK cell efficacy in eliminating both solid and hematologic cancers in animal models and clinical trials (31, 32, 46–48). However, like antigen-specific T cells, NK cells have

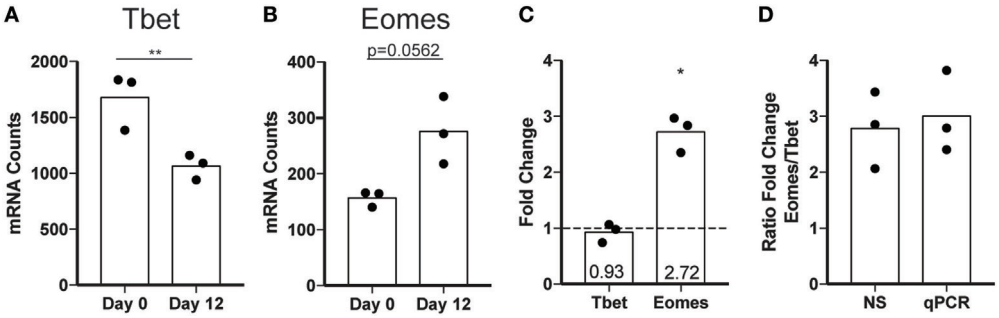


**FIGURE 7** | Transcription factor expression changes following expansion. **(A)** Tbet mRNA expression in total natural killer (NK) cells assessed by nanostring (NS) at day 0 or day 12. Statistical significance was assessed by Student's *t*-test. **(B)** Eomes mRNA expression in total NK cells assessed by NS at day 0 or day 12. **(C)** Fold change by qPCR calculated by the ΔΔCt method for Tbet and Eomes. Statistical significance was assessed by Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). **(D)** Ratio of the fold change for Eomes relative to the fold change in Tbet as calculated for NS and qPCR.

relatively low abundance in peripheral blood, requiring *ex vivo* expansion before adoptive transfer.

As NK immunotherapy is increasingly used in the clinic, an improved evaluation of the expanded cell product with respect to phenotype, functions, and persistence *in vivo* may be of considerable benefit to iterative improvements in selection of patients, cell subsets, and/or *ex vivo* expansion protocols. Recent assessments of CAR T cell products that use well-defined phenotypes to evaluate the ratios of short-lived effectors and persistent memory T cells may be predictive of patient response (29). Phenotypes and functional capacities of *ex vivo*-expanded NK cells, however, are less well understood. To this end, we analyzed the phenotypes and functions of NK cells expanded using a promising method in which PBMCs are cocultured with K562 cells engineered to express the costimulatory molecule 4-1BBL and membrane-bound IL-15, in the presence of high-dose soluble IL-2 developed by Imai et al. (12).

Because several reports (12–14, 26, 27, 34, 35) demonstrated the enhanced cytolytic functions of *ex vivo*-expanded NK cells that are typically associated with more mature, CD56dim NK cells, we expected that *ex vivo*-expanded NK cells would predominantly comprised of a fully differentiated subset characterized by low surface CD56, expression of CD11b and CD57, proteins associated with terminal differentiation, and loss of the inhibitory receptor NKG2A. Relative to donor-matched NK cells that had not undergone *ex vivo* expansion, however, the majority of expanded NK cells had CD56 expression that was higher than was detected on any subset before expansion. This is consistent with previous reports showing increased CD56 on NK cells that have been *ex vivo* expanded (12). Furthermore, the CD56bright population is enriched in patients with chronic infections (49, 50) or autoimmune conditions (51), suggesting increased CD56 expression may be related to chronic stimulation. However, high CD56 expression on expanded NK cells was accompanied by an increased proportion of cells expressing NKG2A, a protein generally associated with immaturity, and negative for markers of terminal differentiation, including CD11b and CD57. Phenotypically, these results suggested that expanded NK cells were not terminally differentiated, a conclusion supported by the increased expression of the Eomes and reduced expression of Tbet, the transcription factors responsible for the spectrum of NK cell differentiation.

Like T cells, immature NK cells are generally less capable of recursive killing than their terminally differentiated counterparts. Since T cells and NK cells share many of the molecular programs that drive terminal differentiation, there were similar concerns for impairment of NK cell functions following extensive *ex vivo* culture and expansion. Paradoxically, we and others found that *ex vivo*-expanded NK cells have an enhanced ability to eliminate tumor cells (12–14, 26, 27, 34, 35). Interestingly, the phenotypically immature, predominant CD56bright population degranulates upon exposure to target cells, a function that is generally associated with terminally differentiated, CD56dim/CD11b+ NK cells. However, like NK cell subsets isolated from patients, the CD56bright population retained its proliferative capacity and ability to produce IFNγ in response to target cells. In contrast to NK cells isolated from peripheral blood, it appears that following *ex vivo* expansion, the delineation of NK cell subsets that are dedicated to cytotoxic function or cytokine production is not associated with the amount of CD56 or other maturation marker expression. Together, these data suggest an uncoupling of phenotypic markers of maturation and canonical functions of NK cell subsets in *ex vivo*-expanded NK cells.

Although NK cells do not express antigen-restricted receptors in the manner of T or B cells, each NK cell does express an array of activating and inhibitory KIRs; the complement of KIRs expressed by any NK cell develops stochastically during maturation and allows significant diversity in the responsiveness of each NK cell to a particular signal (41). Because the expanded NK cells appear more homogeneous than peripheral blood NK cells, as evidenced by both IFNγ production and cytotoxicity occurring in the same cells, we expected that this would be associated with the predominance of a clonal population with uniform KIR expression. By contrast, we found that expanded NK cells had both positive and negative populations of all KIRs evaluated, and that expression of one KIR did not appear to predict expression of another. This is consistent with a report that

culture with IL-2 and IL-15 induced expression of an assortment of KIRs on CD56bright cells (36). Expanded NK cells maintained the KIR expression diversity seen in freshly isolated NK cells, suggesting that the expansion protocol does not enhance NK cell clonal dominance. Furthermore, the increased number of KIRs expressed by any single NKG2A negative NK cell suggests that expanded NK cells retain a spectrum of mechanisms of activation. Retention of KIR diversity may be important because homogeneity in KIR expression could limit efficacy, or exert selective pressure to reduce expression of NK cell activating proteins on the surface of tumor cells, contributing to tumor immune evasion through immunoediting.

T cell expansion protocols that use repeated stimulation and sustained cytokine exposure can result in T cell anergy and replicative senescence of the terminally differentiated effector cells. T cells expanded in these conditions can therefore undergo the phenotypic and functional impairments that are incurred during chronic infections, reducing efficacy in tumor cell elimination following adoptive transfer (52–54). The development of immune checkpoint blockade, which disrupts the interaction between PD-1 and CTLA4 on T cells with their respective ligands to reverse exhaustion caused by chronic antigen exposure, has been an important addition to the arsenal of treatments for several cancers, including melanoma, lung cancer, and bladder cancer (55). The molecular mechanisms underlying NK cell exhaustion are not as clearly defined as they are for T cells, but both PD-1 and Tim3 have been explored as markers of functional NK cell exhaustion. PD-1 expression on NK cell lines can induce exhaustion (56) but Tim3+ NK cells stimulated with IL-12/IL-18 have high cytotoxicity and cytokine production, suggesting Tim3 may be a maturation marker (57). Increased expression of PD-1 and Tim3 has been found on NK cells isolated from cancer patients (24, 56, 58) and patients with chronic viral infections (59). In spite of the increased expression of PD-1 and Tim3 on *ex vivo*-expanded NK cells, expanded NK cells had enhanced cytotoxic activity and maintained the potential for IFNγ production. Similarly, blockade of PD-1 or Tim3 failed to enhance degranulation of NK cells in response to target cells. Given the dissociation of surface marker expression and NK cell functions observed in *ex vivo*-expanded NK cells, it is possible that PD-1 and Tim3 expression on expanded NK cells do not have the same functional consequences as they do in T cells or resting NK cells. It is also possible that expanded NK cells can resist PD-1 mediated functional impairments, which are driven by PI3K/Akt pathway repression (60, 61), and may be overridden by the constitutive PI3K activating IL-15 signal incurred during expansion. Tim3 expression is also driven by IL-15 and cytokine stimulation may override any weak inhibitory effect of Tim3 (57). Furthermore, the observed increase in Eomes expression may play a role in preventing functional exhaustion of expanded NK cells (23).

Because NK cells are rare in peripheral blood, the primary goal of *ex vivo* expansion protocols is to obtain sufficient numbers for use in the clinic. In recent years, expansion of NK cells using only cytokines has been largely supplanted by the use of allogeneic feeder lines, including allogeneic PBMCs, lymphoblastoid cell lines, and K562 [reviewed in Ref. (62)]. K562 cells that have been genetically modified to express cytokines and costimulatory factors for NK cells have shown promise for both their robust expansion and favorable cytotoxicity toward cancer cells. These include the K562-mb15-4-1BBL cells we used in this study and have been successfully employed in the clinic (31, 32), as well K562-4-1BBL cells also expressing membrane-bound IL-21 (63) or the NKG2D ligand MICA (64). In particular, Denman and colleagues' development of an expansion protocol using K562 cells expressing membrane-bound IL-21, a common γ-chain cytokine like IL-2 and IL-15, improved the expansion and cytokine production of NK cells relative to those expanded with K562-mbIL15 while maintaining a similar phenotype and cytotoxic capacity (63). Notably, IL-21-expanded NK cells may represent the latest improvement in the iterative process of developing an effective NK cell expansion protocol, showing efficacy in mouse models of neuroblastoma (65). Although we did not test these cells directly, we expect that because IL-15 and IL-21 share many functional similarities and stimulate overlapping signaling cascades, many of our findings surrounding the uncoupling of function from phenotype will also apply to K562-4-1BBL-mb21-expanded NK cells.

Alongside robust expansion, an important consideration in the development of novel NK cell expansion protocols for use in the clinic is the safety profile of the adoptively transferred product. Because NK expansion protocols frequently include factors such as high-dose IL-2, IL-15, and IL-21 that also stimulate proliferation of T cells, graft-versus-host disease is a particular concern. Therefore, elimination of as many CD3+ T cells as possible is a key step in the certification of NK expansion protocols as GMP compliant. In our hands, CD3+ cells expanded an average of fivefold and represented an average of less than 30% in the final product on day 12, consistent with previous reports that have shown effective elimination of all but trace CD3+ cells by the CliniMACS system (66, 67). There is currently debate in the field about the relative merits of expanding from bulk PBMCs and eliminating contaminating CD3+ cells before infusion, versus expanding from purified NK cells and minimizing concerns about CD3+ contamination at the outset; a recent study found both methods to be functionally equivalent when using K562s modified to express an array of costimulatory molecules, with the advantage of the latter in driving down production costs (63). The presence of monocytes is known to aid in the activation of NK cells (68, 69), as do activated T cells (70). Therefore, development of genetically modified feeder cells that provide the support currently offered by monocytes and T cells may represent an opportunity to improve safety, cost, and fold expansion of NK cells for adoptive transfer.

Collectively, our results suggest that *ex vivo* expansion of NK cells using IL-2 and K562 cells engineered to express 4-1BBL and membrane-bound IL-15 produces a population of NK cells whose phenotype and function do not fit cleanly onto the spectrum of activation and maturity associated with peripheral blood NK cell subsets. Rather, expanded NK cells are capable of both IFNγ production and cytotoxicity and have phenotypic characteristics associated with both mature and immature NK cells. Notably, expanded NK cells have altered the expression of two transcription factors that influence differentiation,

increasing expression of immaturity-associated Eomes while downregulating Tbet, which drives the terminal differentiation program in NK cells (71). It is unclear whether this represents de-differentiation of a mature, cytotoxic subset of NK cells, or the prolific emergence of an immature subset that, through *ex vivo* expansion by 4-1BBL/IL-15, acquires functions that are historically associated with terminally differentiated NK cells. Following administration to multiple myeloma patients, NK cells *ex vivo* expanded with K562-mb15-4-1BBL and high-dose IL-2 exhibit high CD56 expression and proliferate extensively (31). *In vitro*, culture of NK cells with IL-15 causes dilution of CFSE primarily in the immature CD56bright subset (36). This is consistent with reports of persistence of cord blood-derived CD56bright NK cells persisting for up to 2 years (72), as well as a cord. Furthermore, the CD56− CD16+ subset, a unique immature NK population found in cord blood, has also been found to persist following transplant to cancer patients and differentiate into cytotoxic CD56+ antitumor cells (73). Together, these data demonstrate that phenotypically immature NK cells, such as those generated by *ex vivo* expansion using K562-mb15-4-1BBL, can persist long term *in vivo*. Importantly, the resultant NK cell therapy product exhibits both enhanced antitumor activity and a relatively undifferentiated phenotype, which in T cells predicts the most robust and long-term antitumor responses following adoptive transfer. *Ex vivo* expansion with 4-1BBL/IL-15 may therefore generate a potent NK cell product with respect to cytotoxic capacity, versatility in mechanisms of activation, and longevity *in vivo* following adoptive transfer.

## ETHICS STATEMENT

All protocols have been reviewed and approved by the relevant institutional committees, including the Seattle Children's Research Institute Institutional Biosafety Committee (Approval #1211) and Institutional Review Board (Approval #14412).

## AUTHOR CONTRIBUTIONS

NAL, KH, CMC, and CAC conceived the study and designed experiments. MB and CMC developed protocols and provided technical support and troubleshooting. NL performed most experiments with help from KD and KH in particular, as well as HC. KW performed flow cytometry on K562 cells. NL, KH, and KM analyzed data. NL and CAC wrote the manuscript. All authors contributed to final editing.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00150/full#supplementary-material.

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# Chimeric Antigen Receptor Expressing Natural Killer Cells for the Immunotherapy of Cancer

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Adoptive cell therapy has emerged as a powerful treatment for advanced cancers resistant to conventional agents. Most notable are the remarkable responses seen in patients receiving autologous CD19-redirected chimeric antigen receptor (CAR) T cells for the treatment of B lymphoid malignancies; however, the generation of autologous products for each patient is logistically cumbersome and has restricted widespread clinical use. A banked allogeneic product has the potential to overcome these limitations, yet allogeneic T-cells (even if human leukocyte antigen-matched) carry a major risk of graft-versus-host disease (GVHD). Natural killer (NK) cells are bone marrow-derived innate lymphocytes that can eliminate tumors directly, with their activity governed by the integration of signals from activating and inhibitory receptors and from cytokines including IL-15, IL-12, and IL-18. NK cells do not cause GVHD or other alloimmune or autoimmune toxicities and thus, can provide a potential source of allogeneic "off-the-shelf" cellular therapy, mediating major anti-tumor effects without inducing potentially lethal alloreactivity such as GVHD. Given the multiple unique advantages of NK cells, researchers are now exploring the use of CAR-engineered NK cells for the treatment of various hematological and non-hematological malignancies. Herein, we review preclinical data on the development of CAR-NK cells, advantages, disadvantages, and current obstacles to their clinical use.

**Keywords: natural killer cells, chimeric antigen receptor, chimeric antigen receptor, chimeric antigen receptor T, chimeric antigen receptor natural killer, cancer, hematopoietic stem cell transplant**

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## INTRODUCTION

Chimeric antigen receptor (CAR) T cells have gained enormous clinical recognition with remarkable responses reported in patients receiving autologous CD19 (a B cell-specific antigen)-redirected T cells for the treatment of patients with relapsed or refractory B-cell malignancies (1–9). CAR T cells are genetically engineered to express a single chain variable fragment (scFv) derived from an antibody on their surface, which is coupled to a T-cell signaling domain, thus rendering them highly antigen-specific in a non-human leukocyte antigen (HLA)-restricted manner (2, 10, 11). Thus far, the clinical application of CAR T cells has been largely restricted to CD19-expressing B cell malignancies (1–9); however, ongoing studies are testing its applications in other hematological malignancies such as Hodgkin and non-Hodgkin lymphoma, multiple myeloma, and acute myeloid leukemia (12–14). The US Food and Drug Administration recently approved two autologous CD19 CAR T cell products for the treatment of acute lymphoblastic leukemia and certain types of relapsed or refractory large B-cell lymphoma. However, CAR T-cells have several limitations: (i) it is logistically cumbersome to generate an autologous product from patients; (ii) it takes several weeks before

CAR T cells are generated—making it impractical for patients with aggressive disease; and (iii) generation of clinically relevant doses of CAR T-cells can be unfeasible from heavily pretreated lymphopenic patients. An alternative approach is to use previously collected T cells from an allogeneic source; however, even if HLA-matched, T cells pose a risk of serious graft-versus-host disease (GVHD) (15). In contrast to the popularity of CAR T cells, the generation and clinical application of CAR natural killer (NK) cells has lagged behind for various reasons, despite the multiple advantages of NK cells. Herein, we describe these barriers and discuss strategies to overcome them.

## Advantages of NK Cells for CAR Therapy

Among cytolytic lymphocytes, NK cells represent on a per cell basis the most efficient effectors against tumors with a distinct mechanism of action (**Figure 1**) and provide an attractive source of cells for cancer immunotherapy (16, 17). In contrast to other lymphocytes such as T or B cells, NK cells do not express rearranged, antigen-specific receptors. Instead, NK cells express germline-encoded receptors, which are either activating or inhibitory. Upon interaction with their ligands on target cells, the receptors induce a positive or a negative signal, respectively (18). The balance of these signals ultimately govern NK effector function (16, 19) Among the most heavily studied NK cells receptors are the killer-cell immunoglobulin-like receptors (KIRs) are that recognize classical HLA class-I molecules (HLA- A, -B, and -C). Other receptors belong to the C-type lectin family (CD94 and NKG2s, such as NKG2A, -B, -C, -D, -E, and -F) that recognize non-classical HLA class-I molecules (HLA-E and stress-induced MHC-I-related chains—MICA and MICB) [reviewed in Ref. (20–23)]. Healthy cells are protected from NK mediated lysis by the recognition of "self" HLA molecules on their surface by inhibitory NK receptors protects (24–27). On the other hand, tumor or viral infected cells often downregulate or lose their HLA molecules as an escape mechanism against T-cells (28, 29). Loss of HLA class I expression makes them susceptible to lysis by the NK cells due to loss of the inhibitory signal (21, 30–45). Indeed, the clinical significance of NK cell alloreactivity has been demonstrated in multiple studies in the setting of hematopoietic stem cell transplant (HSCT), where patients that received a graft containing alloreactive NK cells had a significantly lower risk of relapse and improved survival (46–55). Adoptive transfer of alloreactive NK cells as a stand-alone therapy (independent of HSCT) also demonstrated encouraging outcomes in a variety of malignancies (56–62). In contrast, several studies of *autologous* NK cell adoptive therapy showed rather disappointing results (63–71).

Thus, NK cells offer an attractive alternative to T-cells for CAR engineering for a number of reasons: (i) allogeneic NK cells should not cause GVHD, as predicted by observations in murine models (72, 73), as well as clinical studies of haploidentical and cord blood (CB)-derived NK cell infusions in patients with hematologic or solid malignancies (56, 59); (ii) mature NK cells have a relatively limited life-span, permitting effective antitumor activity while reducing the probability of long-term adverse events, such as prolonged cytopenias due to on-target/off-tumor toxicity to normal tissues such as B cell aplasia (in the case of CD19 CARs), which can last up to 3 years (74); and (iii) CAR-NK cells retain their intrinsic capacity to recognize and target tumor cells through their native receptors; therefore when compared with the CAR

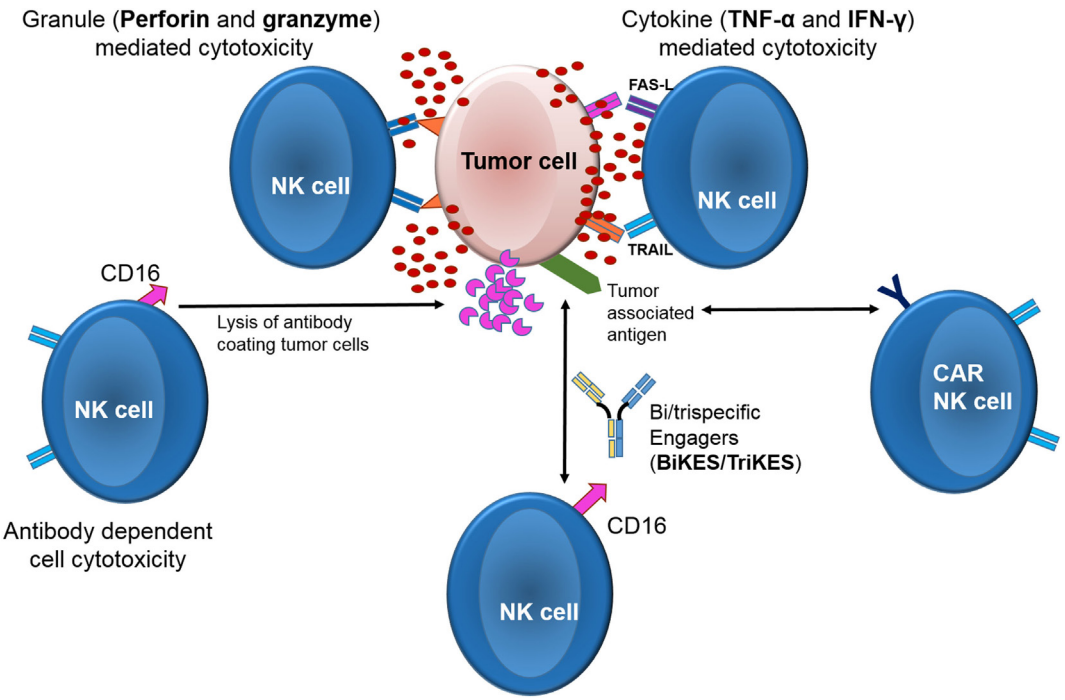


**FIGURE 1** | Mechanisms of action of natural killer cell cytotoxicity.

T cells, it is theoretically less likely for tumor cells to escape NK immunosurveillance even if they downregulate the CAR target antigen (75). This unique property of NK cells could be further exploited for the generation of NK-CARs by selecting donors based on the donor-recipient KIR-ligand mismatch, or based on donor haplotype B *KIR* gene content, as both have been shown to be beneficial in the setting of allogeneic HSCT (48, 50, 55, 76). Thus, allogeneic NK cells offer the potential for an off-the-shelf cellular product for immunotherapy that could be readily available for immediate clinical use, in contrast to the current shortage of CAR T-cell products at many centers (77).

## SOURCE OF NK CELLS FOR ADOPTIVE IMMUNOTHERAPY

Functional NK cells can be generated from numerous sources. Although autologous NK cells can be utilized for adoptive therapy, their efficacy against autologous cancer cells is rather limited (63–71, 78, 79), which we have shown may not be easily overcome by CAR engineering (80). Allogeneic NK cell sources include peripheral blood (PB), bone marrow (BM), human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs) (81–83), umbilical CB, or readily available NK cell lines (84). Obtaining NK cells from the PB by apheresis or from BM by harvesting are both cumbersome and are associated with potential risks to the healthy donors (85–87). NK cell derivation from hESCs or iPSCs (81–83) is a complex process and the field is still evolving. In contrast, NK cell lines such as NK-92 (88–93), KHYG-1 (94), NKL, NKG, and YT, to name a few, provide an easily accessible and homogeneous source of cells for the generation of large numbers of CAR-transduced NK cells. NK-92 is a highly cytotoxic NK cell line that was derived from a patient with NK lymphoma (95) and is characterized as $CD56^{bright}CD16^{neg/low}NKG2A^{positive}$ and $KIR^{negative}$ (except for KIR2DL4) (96, 97). Phase I clinical studies demonstrated the safety of NK-92 cell infusion in cancer patients, even up to doses of $10^{10}$ cells/$m^2$ (98–100). Based on these data, there is great interest in CAR-engineered NK-92 cells for clinical use (**Table 1**) (88–92, 101–115). However, NK-92 cells have a number of disadvantages that need to be taken into account. First and foremost, NK-92 cells are derived from a patient with NK lymphoma (95) and thus have the potential for tumor engraftment following infusion. Moreover, they are EBV-positive and carry multiple cytogenetic abnormalities resembling those of NK lymphoma (116). Thus, as a safety measure, NK-92 cells must be irradiated before infusion into patients to prevent permanent engraftment. This can negatively impact their *in vivo* proliferation and persistence, both factors shown to be crucial for the success of cellular therapy in studies with infusion of tumor-infiltrating lymphocytes (117–119) as well as CAR-T cells (3). Indeed, in a study of NK-92 cells engineered with ErbB2/HER2-CAR, while irradiation had no effect on the *in vitro* cytotoxicity of CAR-transduced NK92 cells, it negatively impacted their *in vivo* replication and persistence, with the cells no longer detectable within 7 days of adoptive infusion (109). Of note, NK-92 cells are CD16 (FCRIIIγ) negative and cannot mediate antibody-dependent cell cytotoxicity (ADCC), unless genetically modified to express CD16 (120).

**TABLE 1** | Clinical trials with NK CAR.

| Clinical trial identifier | NK cell source | Target antigen | Disease | Study location |
|---|---|---|---|---|
| NCT02944162 | NK-92 cell line | CD33 | AML | China |
| NCT02892695 | NK-92 cell line | CD19 | CD19 positive B cell malignancies | China |
| NCT02742727 | NK-92 cell line | CD7 | CD7 positive leukemia or lymphoma | China |
| NCT02839954 | NK-92 cell line | MUC1 | MUC1 positive solid tumors (colorectal, gastric, pancreatic, NSCLC, breast, glioma) | China |
| NCT03056339 | Cord blood | CD19 | CD19 positive leukemia or lymphoma | MDACC, USA |

*AML, acute myeloid leukemia; MDACC, MD Anderson Cancer Center; NK, natural killer; NSCLC, non-small cell lung cancer.*

Cord blood, on the other hand, is a readily available source of allogeneic NK cells with distinct benefits over related or unrelated adult donors, including the speed of availability (especially since it is available as an off-the-shelf frozen product) and tolerance of HLA mismatches, the latter of which expands the donor pool. The frequencies of NK cells in CB (~15–20%) are similar to PB (~10–15%) (121–124). However, until recently the small volume of blood in a CB unit made it challenging to obtain adequate numbers of NK cells for clinical use. Moreover, resting CB NK cells are phenotypically and functionally immature, with higher expression of the inhibitory receptor NKG2A and lower expression of activating and maturation receptors such as NKp46, NKG2C, DNAM-1 (124), and CD57 (124–127). To overcome these limitations, our group has developed a Good Manufacturing Practice (GMP)-compliant procedure, using GMP-grade K562-based artificial antigen-presenting cells (aAPCs) expressing membrane bound IL-21 and 4-1BB ligand, which reliably generates clinically relevant doses of GMP-grade NK cells from a CB unit for adoptive immunotherapy (128). Following *ex vivo* activation and expansion, CB-derived NK cells display the full array of activating and inhibitory receptors, strongly express eomesodermin (Eomes) and T-bet, two factors necessary for NK cell maturation, and exert similar cytotoxicity to PB-NK cells (129, 130). Taken together, these studies support the use of NK cells as a source of cellular therapy in cancer.

### Constituents of CAR

A CAR construct consists of three components: an extracellular antigen-recognition part, a transmembrane domain and an intracellular signaling domain. The extracellular domain is the antigen-recognition site and is generally composed of an scFv derived from the variable regions of both the heavy and light chains of a monoclonal antibody, fused together *via* a flexible linker. Most scFvs studied to date are of murine origin, with the potential to induce a human antimouse antibody (HAMA) or an anti-idiotype immune response. A number of investigators are exploring strategies to humanize scFVs (131–136) to circumvent induction of HAMA; however, this approach will

not prevent the development of anti-idiotype antibodies. The antigen binding domain of a CAR is linked to a "hinge" which imparts flexibility for adequate orientation and binding to the antigen. The hinge binds the extracellular component to a transmembrane domain, which is the link to the intracellular signaling component (137–139). The size of the hinge region has been shown to affect CAR-T cell function, with some studies reporting superior anti-tumor activity of CAR T-cells expressing a shorter hinge (140, 141). The transmembrane domain lies between the hinge and the signaling endodomains. Different types of transmembrane domains have been studied, including the CD3-ζ chain of the T-cell receptor, CD4, CD8, or CD28. The type of transmembrane domain has also been shown to affect the function and stability of the CAR molecule in T cells (142). The endodomain then transmits activation signals to T cells. The "first-generation" CARs used a single intracellular signaling domain (CD3-ζ chain alone) while the second- and third-generation CARs incorporate one or more additional costimulatory signaling domains, such as CD28, CD137, or OX40 to render them more potent (143, 144).

CD3ζ is critical for signaling and activation of both T and NK cells (145). In NK cells, CD3ζ homodimer transmits signals from FcγRIII (CD16), thus aiding in ADCC (146). Although CD28 is one of the most commonly employed costimulatory domains in CAR T cells, except in certain cell lines (147), its role in NK cell function is less clearly defined (148). Nonetheless, its addition to CD3ζ in a second generation ErbB2-specific NK-92 CAR led to improved function compared to a CD3ζ construct alone and was similar to that of CD137-CD3ζ CAR against ErbB2-expressing tumor cells (109). Another study showed that NK-92 cells transduced with a CD19-CAR expressing CD28-CD3ζ had superior cytotoxicity against CD19-positive targets compared to cells expressing a CD137-CD3ζ containing CAR (88). DNAX-activation protein 12 (DAP12) is transmembrane protein involved in signal transduction of several NK cell activating receptors including NKG2C, NKp44, and the activating KIRs (149). One study tested if a CAR against prostate stem cell antigen (PSCA) that used DAP12 as an intracellular signaling domain can provide sufficient signaling to induce NK cell activation when compared to a CD3ζ-containing CAR. The authors transduced YTS-NK cells and primary NK cells with PSCA-DAP12 CAR and noted superior cytotoxicity when compared to NK cells expressing a CD3ζ-based CAR (150). While the importance of incorporating costimulatory molecules in the CAR construct has been clearly shown for CAR T cells (4, 5, 151), additional studies are needed to define the optimal costimulatory molecule and signaling endodomain for NK cells.

## CAR Transduction

The incorporation of a foreign gene into a cell requires the use of a vector, which can be based on viral or non-viral systems. The most commonly used tools for CAR gene delivery include genetically engineered retroviruses [lentiviral (152) and gamma-retroviral (153) vectors]. Lentiviruses have the advantage that they are capable of infecting both dividing and non-dividing cells, while retroviruses only infect dividing cells. Therefore, lentiviral vectors can be used for transduction of a wider variety of cell types including quiescent stem cells (154–156). In addition, lentiviral vectors can accommodate larger transgenes when compared with retroviral vectors (157). Insertional mutagenesis, although extremely rare, remains a concern with viral vectors although its likelihood is influenced by a number of factors such as the specific type of vector used and the site of integration (158). For instance, in earlier trials of gene therapy with $CD34^+$ hematopoietic cells for X-linked severe combined immunodeficiency (SCID), 2 of the 10 treated children developed acute leukemia in one study (159) and 1 of the 4 children in another study (160). However, none of the subsequent trials of gene-modified hematopoietic stem cells in SCID children (161) or studies of adoptive immunotherapy with CAR T-cells (2, 3, 5, 9, 162) have witnessed adverse events related to insertional mutagenesis to date. Yet, to mitigate any theoretical concerns, various non-viral techniques such as the transposon/transposase system (82, 163) or mRNA transfection (113, 115) have also been tested. The transduction efficiency using these techniques varies remarkably from study to study (82, 113, 115, 163, 164) and depends on a number of factors, including the cell source (82, 113, 115, 163–167). In general, the transduction efficiency for CAR T cells is about 50% but can range up to 90% or higher (152, 164, 168).

# CHALLENGES

Despite the many advantages of NK cells, there are several impediments to the successful generation of CAR NK cells for clinical use. Until recently, the genetic engineering of NK cells, even with viral methods, had proved challenging, with reports of <10% transduction efficiency for primary CB or PB derived NK cells (113, 165). However, recent optimization in protocols for viral transduction and electroporation (166, 167) has revived enthusiasm for the genetic engineering of NK cells. While viral methods appear to be largely ineffective for inducing CAR expression in freshly isolated PB NK cells, significantly better transduction efficiency can be achieved when NK cells from PB (12–73%) (113) or CB are activated and expanded (median 69%; range 43–93%) (169) in one study and 80% (range 67–96%) (170) in another study. In contrast to studies with primary NK cells, NK92 cells are easier to transduce with mRNA electroporation (113, 115), with efficiencies averaging from 25 to 50% (171). However, as the mRNA transcript is not incorporated into the genome, expression of the CAR molecule is often short-lived and detectable for only a few days, which may negatively impact the efficacy of the engineered cells following adoptive transfer (115, 166, 167).

Another concern with using allogeneic NK cells is the possibility of infusing contaminating T or B cells in the expanded NK cell product, which can theoretically cause GVHD or posttransplant lymphoproliferative disease, respectively. As some degree of HLA-mismatch after CB transplantation is well tolerated, the risk of clinically significant GVHD may be less with CB-derived CAR-NK cells compared to PB. Plus, with the exception of one study reporting GVHD following adoptive transfer of donor-derived IL-15/4-1BBL-activated NK cells in recipients

of HLA-matched, T-cell-depleted PB HSCT (72), clinical studies of haploidentical and CB NK cell infusions in hundreds of patients with both hematologic and solid malignancies have not reported a higher risk of GVHD (56, 58, 128, 172–174). Rather, experimental evidence obtained in mice have reported reduced risk of GVHD with NK cells *via* multiple mechanisms, including depletion of host antigen-presenting cells and activated alloreactive T cells (46, 72, 175, 176). Another potential limitation of NK cells for immunotherapy is that in contrast to T cells, they are highly sensitive to the freeze and thaw process and they lose activity after thawing. A number of groups are exploring strategies to optimally cryopreserve NK cells and have shown that the activity of frozen NK cells can be restored by overnight incubation with cytokines such as IL-2 (177–182). It is not yet known if a similar strategy can be used to restore function of frozen CAR NK cells for adoptive therapy.

Another characteristic of NK is that they do not persist after adoptive transfer without cytokine support (183). While the shorter life-span of NK cells may be advantageous, allowing for antitumor activity while reducing the probability of long-term adverse events such as prolonged cytopenias caused by on-target/off-tumor toxicity to normal tissues, it may also limit their efficacy. For *in vivo* survival and proliferation, NK cells require continuous cytokine support, without which they are detectable in the circulation for only 1–2 weeks (183). The two most commonly used cytokines to support the persistence of adoptively transferred NK cells are IL-2 and IL-15 (184, 185). The infusion of IL-2 has substantial side effects including fevers, chills, myalgias and capillary leak syndrome (186), and can promote expansion of regulatory T cells ($T_{regs}$) which are suppressive to NK cells (187). IL-15, on the other hand, does not support $T_{reg}$ (188) expansion but when administered as an exogenous bolus to patients with metastatic melanoma and renal carcinoma can result in dose-dependent toxicity, including neutropenia (189). An alternative approach to exogenous administration of cytokines is to treat patients with lymphodepleting chemotherapy such as cyclophosphamide and fludarabine prior to infusion of NK cells, which provides a favorable environment for NK cell expansion by depleting mature lymphocytes (which consume IL-15), resulting in a marked increase in endogenous IL-15 levels (56). Another novel technique is to incorporate genes for IL-2 (104, 190–192) or IL-15 (80, 193–195) within the CAR construct to constantly provide cytokine support to the CAR-transduced cells. We recently showed the feasibility and efficacy of this approach in a mouse model of Raji lymphoma. Although a single infusion of $1 \times 10^7$ CAR.19$^+$ (without IL15) or CAR.19/IL15$^+$ CB-NK cells both improved tumor control and prolonged survival compared to non-transduced CB-NK, CAR.19/IL15$^+$ CB-NK cells controlled tumor expansion and prolonged survival significantly better than the CAR.CD19 construct lacking the *IL-15* gene, which underscores the critical influence of IL-15 in enhancing antitumor activity *in vivo* (80).

## SUICIDE GENES

Given the recent safety concerns such as cytokine release syndrome and neurotoxicity associated with infusion of CAR-modified T cells (196, 197) (and possibly NK cells), careful consideration of whether a suicide system should be incorporated into the construct as a safety measure is needed. One of the most extensively tested safety switches include the herpes simplex virus thymidine kinase gene (198, 199). While a number of studies have tested this approach, the highly immunogenic virus-derived protein can lead to the rejection of cells expressing it, plus it requires administration of ganciclovir—which takes several days to work and leads to cytopenias (200–202). Because of these disadvantages, inducible caspase-9 (iCasp9) has emerged as one of the most commonly used suicide genes in adoptive cell therapy trials (80, 193, 203–206). When exposed to a synthetic bioinert small-molecule dimerizing drug, the iCasp9 becomes activated and leads to rapid apoptosis of cells expressing it. Another suicide gene under investigation is the truncated epidermal growth factor receptor (EGFR), which lacks intracellular tyrosine kinase activity while expressing an intact binding epitope that can be targeted with the anti-EGFR monoclonal antibody cetuximab for the rapid elimination of the transgenic cells (207, 208).

## Preclinical Studies of CAR-NK Cells

Building on the knowledge gained with CAR T-cells, a multitude of preclinical studies have tested the efficacy of CAR NK cells against a variety of target antigens for hematological malignancies such as CD19 (167, 169), CD20 (209, 210), CD138 (211), CS1 (111), CD3 (212), CD5 (101), CD123 (213), as well as solid tumors such as HER-2/Erb-2 (109, 214–216), GD2 (114), EpCAM (195), EGFR and mutant EGFRvIII (89), WT1 (217), and ROR-1 (218) to name a few. An alternative and non-antigen specific approach to engineering NK cells was tested by Chang et al. (170), where the authors induced supra-physiologic expression of NKG2D, a key receptor for NK cell activation and signal transduction *via* DNAX-activating protein 10 (DAP10). *Ex vivo* expanded PB NK cells transduced with a retroviral vector encoding NKG2D-DAP10-CD3ζ showed impressive *in vivo* cytotoxicity in xenogeneic mouse models of hematologic and solid tumors but showed no activity against non-transformed blood or mesenchymal cells (170).

Despite these impressive preclinical data, there are currently only five registered clinical trials testing the safety and efficacy of CAR-NK cells in cancer patients (**Table 1**). Four of these trials are being conducted in China using the CAR-engineered NK92 cells. The only trial using primary NK cells (CB NK cells) is being conducted in the United States by our group at the MD Anderson Cancer Center (NCT03056339). Patients with relapsed or refractory CD19$^+$ B cell lymphoid malignancies are eligible for this trial. All patients receive lymphodepleting chemotherapy with fludarabine and cyclophosphamide, followed by the infusion of allogeneic CB-derived NK cells that are genetically modified with a retroviral vector, iC9-2A-CAR.CD19-CD28-CD3zeta-2A-OhIL-15 (iC9/CAR.19/IL15) (80), which (i) includes CAR.19 gene to redirect specificity to CD19; (ii) produces IL-15 ectopically—a cytokine crucial for NK cell survival and proliferation (219); and (iii) incorporates inducible caspase-9 (*iCasp9*)—a suicide gene, which can be activated pharmacologically to eliminate CAR cells, as needed (203, 219).

## CONCLUSION

We are in an exciting era in the field of cellular therapy. NK cells hold great promise and offer the potential for an off-the-shelf cellular product for immunotherapy that could be readily available for immediate clinical use. Yet, before NK cells can be extended to larger cohorts of patients a number of scientific questions and regulatory hurdles must be addressed. What is the ideal vector, signaling endodomain and costimulatory molecule for NK cells—one with the best response and safety profile? Will CAR NK cells have a different safety and efficacy profile to CAR T cells, given their distinct mechanism of action? Will "off-the-shelf" CAR NK cells be able to sustain the clinical demand, give the shortage of CAR-T cells at many centers and the uncertainty regarding the health economics of this treatment? Can CAR NK cells lead to durable responses, considering their limited *in vivo* life-span or will they be used as a "bridge" to more aggressive treatment such as HSCT? Based on the results of haploidentical and unrelated donor HSCT, should NK cells for CAR modification be selected based on KIR-KIR ligand mismatch or KIR haplotype to harness their native NK cell activity and will this approach reduce the risk of disease escape through downregulation of the CAR target antigen? With the plethora of preclinical studies and clinical research that are underway, it is expected that engineered NK cells will make a significant contribution to the recent paradigm shift in cancer treatment.

## AUTHOR CONTRIBUTIONS

RM and KR reviewed the literature, analyzed data, and wrote the manuscript.

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# Embryonic Fibroblasts Promote Antitumor Cytotoxic Effects of CD8+ T Cells

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Adoptive CD8+ T cell therapy has emerged as an important modality for the treatment of cancers. However, the significant drawback of transfused T cells is their poor survival and functionality in response to tumors. To overcome this limitation, an important consideration is exploring a culture condition to generate superior antitumor cytotoxic T lymphocytes (CTLs) for adoptive therapy. Here, we provide a novel approach to generate potent CTL clones in mouse embryonic fibroblast-conditioned medium (MEF-CM). We found CTLs derived with MEF-CM have higher potential in long-term persistence in tumor bearing and non-tumor-bearing mice. Importantly, adoptive transfer of MEF-CM-cultured CTLs dramatically regressed tumor growth and prolonged mice survival. Characterization of MEF-CM-cultured CTLs (effector molecules, phenotypes, and transcription factors) suggests that MEF-CM enhances the effector functions of CD8+ T cells in part by the upregulation of the T-box transcription factor eomesodermin. Consequently, MEF-CM enhances the intrinsic qualities of effector CD8+ T cells to augment antitumor immunity.

**Keywords: mouse embryonic fibroblast-conditioned medium, CD8+ T cells, cytotoxic T lymphocytes, long-term persistence, adoptive T cell therapy**

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## INTRODUCTION

The transfusion of T lymphocytes, referred to as adoptive T cell therapy, presents a promising approach to treat patients with cancers or infections (1, 2). Early works show that CD8+ T cells are an optimal lymphocyte population for adoptive transfer (3, 4). Especially in recent years, a growing number of engineered T cells with desired antigen specificity have been generated and expanded to overcome the limitation of low frequency of tumor-specific T cells. Examples are T cells engineered to express chimeric antigen receptors or engineered T cell receptors (5). Nevertheless, treatment of *in vitro* expanded CD8+ T cells does not consistently translate into an objective clinical tumor response. This suggests that *in vitro*-generated T cells are less effective in tumor killing or the effector cells have a short life span after infusion by activation-induced cell death (6–10). To overcome the limitations, an important consideration is generation of superior antitumor cytotoxic T lymphocytes (CTLs) by optimizing the *in vitro* culture conditions (7, 11–13).

The plastic culture vessels currently used to expand T cells *in vitro*, however, hardly replicate the *in vivo* environment. Alternatively, a desirable feeder cells could provide T cells a direct contact to mimic *in vivo* environment. Fibroblasts comprise heterogeneous tissue connecting cells that extensively distribute in organs of animals and play a critical role in wound healing through production of extracellular matrix (ECM), matrix metalloproteinase, and cytokine mediators (14, 15). There is evidence that ECM produced by fibroblasts serves as co-stimuli to enhance T cells activation and proliferation (16, 17). In addition, fibroblasts produce many molecules with the potential to modulate T cells functions. For example, fibroblasts derived from human lung tumors or normal

skin can improve the production of interferon-gamma (IFN-γ) and interleukin (IL)-17A by T cells through secretion of soluble factor(s) (18). Another concept is that fibroblasts derived factor(s) also enhance the survival of activated T cells (19). The comprehensive effects of fibroblasts on T cells may potentially allow the alteration of the fate or intrinsic functions of T cells, which could be utilized in an *in vitro* culture system for adoptive cell therapy.

Mouse embryonic fibroblasts (MEFs) are stem cell-like fibroblasts that are widely used as feeder cells, since they secret various growth factors to support embryonic stem cells self-renewal and growth in an undifferentiated state. We were therefore interested in exploring whether MEFs are desirable candidates for facilitating the differentiation of potent effector CTL clones for adoptive cell therapy. Surprisingly, we found that MEFs enhanced effector functions of $CD8^+$ T cells through soluble factor(s). Effector $CD8^+$ T cells generated in mouse embryonic fibroblast-conditioned medium (MEF-CM) persisted long term after adoptive transfer. And in the murine tumor model, transfusion of short-term MEF-CM-cultured CTLs significantly regressed tumor growth.

# MATERIALS AND METHODS

## Mice and Cells

Wild-type (WT) C57BL/6(B6) mice ($Ly5.2^{+/+}$), BALB/c and ovalbumin $(OVA)_{257–264}$-specific TCR (Vα2 and Vβ5) transgenic mice (OT-1) that were maintained on the B6 background were purchased from The Jackson Laboratory. $Ly5.1^{+/-}$ OT-1 mice were obtained from OT-1 that were mated with B6 congenic mice $Ly5.1^{+/+}$. All mice were 7–9 weeks old at the beginning of each experiment, and were raised in a specific pathogen-free environment at Korea University. The experimental protocols adopted in this study were approved by the Institutional Animal Care and Use committee of Korea University.

Primary MEFs were prepared from a pregnant B6 or BALB/c mice at 13 or 14 days post-coitum. MEFs after passage 2 (P2) were collected and maintained as stock cells. EG.7 tumor cells expressing chicken OVA were provided by Dr. M. Mescher (University of Minnesota, Minneapolis, MN, USA). MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin-streptomycin, 10 μg/mL gentamycin, and 50 μM β-mercaptoethanol (Gibco-BRL). Primary MEFs (P3) from B6 or BALB/c were seeded with $1.25 \times 10^5$/ml in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin, 10 μg/mL gentamycin, and 50 μM β-mercaptoethanol (Gibco-BRL) and cultured for 2 days. The culture medium was collected by centrifuging for 5 min at 400 *g* followed by filtration through a 0.22-μm pore size filter and was stored at −85°C (conditioned medium, CM hereafter).

## *In Vitro* Activation of $CD8^+$ T Cells

Splenic $CD8^+$ T cells from OT-1 mouse were purified with a MACS column using anti-mCD8α magnetic beads (Miltenyl Biotec). The purity of the sorted OT-1 cells was >95%. Enriched OT-1 cells were stimulated with $K^b$-OVA beads which consisted of $OVA_{257–264}$ (Genscript) loaded recombinant MHC class I molecules ($H2\text{-}K^b$) and anti-CD28 antibodies coated on magnetic beads. For the preparation of MHC-I beads, 1 μg of biotinylated $H2\text{-}K^b\text{-}OVA_{257–264}$, 0.3 μg of biotinylated anti-CD28 antibodies and 0.05 μg of streptavidin magnetic beads [NEB, S1420S] were incubated for overnight at 4°C with rotation. During cell stimulation, OT-1 cells were co-cultured with or without MEF feeder cells, which were seeded before CTL activation. Otherwise, OT-1 cells were cultured in the presence or absence of CM instead of MEF cells.

For assessment of $CD8^+$ T cell proliferation, purified $1 \times 10^5$ OT-1 $CD8^+$ T cells were labeled with 2.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) and activated by $K^b$-OVA beads. After 2 days of activation, proliferation was assessed by CFSE dilution using fluorescence-activated cell sorting (FACS) analysis.

## Trans-Well Assay

Trans-wells (6.5 mm) with 0.4-μm pores were utilized (Corning). OT-1 $CD8^+$ T cells ($1 \times 10^5$) alone or together with $2.5 \times 10^5$ B6 MEFs in 100 μl RPMI-1640 medium were added to the upper chamber and 600 μl of medium with or without $2.5 \times 10^5$ MEFs were added to the lower chamber. After 3 days of stimulation with $K^b$-OVA beads, IFN-γ from the supernatant in the lower chamber was measured by ELISA, and IFN-γ-producing cells were detected by flow cytometry after intracellular staining.

## Flow Cytometry

The following antibodies were used for flow cytometry for cell surface and intracellular staining: TCRβ-fluorescein isothiocynate (FITC) (H57-597), CD25-FITC (3C7), Ly5.1-FITC (A20), IL-6-FITC (MQ2-13A5), TCRVα2-Phycoerithrin (PE) (B20.1), Ly5.2-PE (104), CD44-PE (IM7), granzyme B-PE (NGZB), Eomes-PE (Dan11mag), IL-1α-PE (ALF-161), IL-2-PE (MQ1-17H12), IL-3-PE (MP2-8F8), IL-4-PE (11B11), IL-13-PE (eBio13A), TNFα-PE (MP6-XT22), GM-CSF-PE (MP1-22E9), MCP-1-PE (2H5), Bcl-6-PE (K112-91), TCF-7/TCF-1-PE (S33-966), TCRVβ5.1,5.2-Allophycocyanin (APC) (MR9-4), CD62L-APC (MEL-14), IFN-γ-APC (XMG1.2), granzyme B-APC (NGZB), IL-17A-APC (ebio17B7), IL-5-APC (TRFK5), IL-10-APC (JES5-16E3), Perforin-APC (ebioOMAK-D), T-bet-APC (4B10), Blimp-1-Alexa Fluor® 647 (5E7), CD8a-PerCP-Cyanine5.5 (53-6.7), B220-PerCP-Cyanine5.5 (RA3-6B2), streptavidin-APC.Cy7, and CD122-biotin (5H4). Flow cytometry was performed using a FACSVerse or FACSCalibur device (BD Biosciences) and data were analyzed using FlowJo_V10 (FlowJo LLC).

## *In Vitro* Cytotoxicity Assay

The ability of effector OT-1 $CD8^+$ T cells to kill target cell EG.7 was evaluated by a CFSE/7-AAD-based flow cytometry assay as previously described (20). Briefly, effector cells (splenic $CD8^+$ T cells) were sorted from OT-1 mice and were activated by $K^b$-OVA beads for 3 days in the presence or absence of MEF-CM (50%, v/v) were labeled with CFSE. CFSE-labeled effector cells were incubated 4–5 h with target cells at an effector:target (E:T) ratio of 8:1, 4:1, 2:1, 1:1, and 0:1. Following a further wash, cells were stained with 7-AAD to identify dead cells. The cells were

then analyzed *via* flow cytometer. Cytotoxicity was calculated on CFSE-negative cells as follows: %lysis = 100 × (% sample lysis − % basal lysis)/(100 − % basal lysis).

## Adoptive Transfer Studies

### Cell Persistence Assay

For non-tumor-bearing mice, congenic Ly5.1$^{+/-}$ effector CD8$^+$ OT-1 cells ($5 \times 10^4$) generated in the presence or absence of B6 MEF-CM (50%, v/v) were intravenously transferred to Ly5.2$^{+/+}$ B6 WT mice. After 1 month, transferred OT-1 cells were restimulated with of $OVA_{257-264}$ peptides (100 μg/mouse) and the frequencies of OT-1 cells were measured in peripheral blood, spleen, and lymph node 5 days after peptide treatment. For tumor-bearing mice, OVA-expressing EG.7 tumor cells were transferred subcutaneously to WT B6 mice ($1.5 \times 10^5$ cells/mouse). When tumor size was 40–50 mm$^3$ (10 days after tumor inoculation), medium cultured Ly5.1$^{+/+}$effector CD8$^+$ OT-1 cells ($0.75 \times 10^6$) and B6 MEF-CM (50%, v/v) cultured Ly5.1$^+$Ly5.2$^+$ effector CD8$^+$ OT-1 cells were co-transferred to Ly5.2$^{+/+}$ B6 WT mice. The persistence of transferred cells was analyzed after 7 and 14 days of T cell transfer, respectively.

### Tumor Rejection Assay

For tumor rejection experiment, EG.7 tumor cells were transferred subcutaneously to WT B6 mice ($5 \times 10^4$ cells/mouse). When tumors were detectable (usually between 11 or 12 days after tumor inoculation), $1 \times 10^6$ *in vitro*-generated effector cells were intravenously injected, and tumor sizes were measured every 2–4 days. Tumor sizes were calculated by determining the length of short ($l$) and long ($L$) diameters (tumor volume = $l^2 \times L/2$). Experimental endpoints were reached when tumor volume exceeded 2,500 mm$^3$.

## Statistical Analysis

Prism software (GraphPad Prism6.0) was used for statistical analysis. Statistically significant differences were determined by unpaired *T*-test and one- or two-way ANOVA with Bonferroni's multiple comparisons tests. Log-rank (Mantel–Cox) tests were used to analyze mouse survival curves. The bars in all graphs were expressed as mean ± SEM. In all figures, ns denotes no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$.

# RESULTS

## MEFs Significantly Promote CD8$^+$ T Cells Activation

In order to determine whether MEFs influence the activities of antigen-recognized CD8$^+$ T cells, isolated splenic CD8$^+$ T cells from OT-1 mice were stimulated by K$^b$-OVA beads in the presence of different numbers of autologous MEF feeders. Following 3 days of stimulation, IFN-γ production measured by ELISA positively correlated with the number of MEF feeders (**Figure 1A**). About a twofold enhancement of IFN-γ-producing cells for MEF-based cultures was confirmed by intracellular flow cytometry analysis (**Figure 1B**). Next, we evaluated the proliferative potential of CTLs in the presence of MEF feeders by the CFSE dilution assay. Following 48 h of stimulation, the CD8$^+$ T cells in division zones 2 and 3 were increased in the presence of MEF feeders (**Figure 1C**), which indicated that MEF feeders also enhanced CTL proliferation.

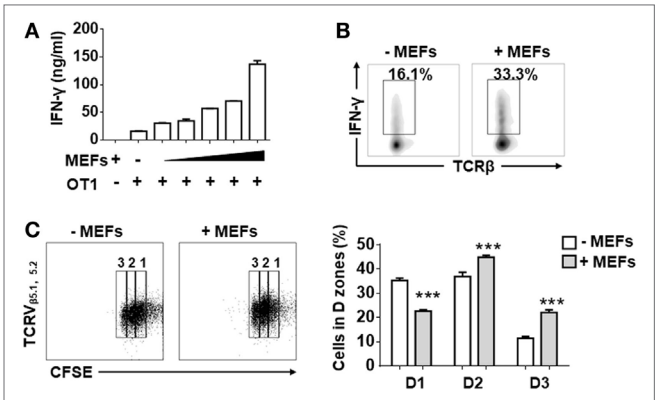


**FIGURE 1** | Mouse embryonic fibroblasts (MEFs) promote activation of CD8$^+$ T cells activation. **(A)** Sorted CD8$^+$ T cells from OT-1 splenocytes were activated by K$^b$-OVA beads in the presence of different numbers of B6 MEF feeders [0, 0.3, 0.6, 1.3, 2.5, 5 × 10$^4$/well (96 well, 200 μl culture medium)]. After 3 days of TCR stimulation, the level of interferon-gamma (IFN-γ) production was detected by ELISA. **(B)** On day 3, flow cytometry analysis of IFN-γ production in OT-1 CD8$^+$ T cells in the presence or absence of B6 MEF feeders (2.5 × 10$^4$/well) was determined. **(C)** Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-1 CD8$^+$ T cells were stimulated in the presence or absence or B6 MEF feeders (2.5 × 10$^4$/well), and after 2 days of TCR stimulation, the proliferative capacity of CD8$^+$ T cells was determined by CFSE dilution. Statistical significance was analyzed by two-way ANOVA with Bonferroni's multiple comparisons tests (***$P \leq 0.001$). All data represent at least two independent experiments.

## MEFs Enhance CD8$^+$ T Cell Differentiation to Effector Cells Through Soluble Factor(s)

To determine whether MEFs could promote CTL activation through direct cell–cell contact or through soluble factor(s), the Trans-well co-culture system was utilized. After 3 days of TCR stimulation, IFN-γ production was enhanced in both direct and indirect contact (**Figures 2A,B**). In terms of IFN-γ production by OT-1 cells, indirect condition was not inferior to direct cell–cell contact condition. Therefore, it was very likely that MEFs promoted CD8$^+$ T cell activation through soluble factor(s). In addition, to exclude the effect of direct cell–cell contact, CD8$^+$ T cells were stimulated in different volumes of MEF-CM. Following 3 days stimulation, IFN-γ and granzyme B levels were proportional to the volume of treated MEF-CM (**Figure 2C**). In these experiments, we used MEF-CM produced from autologous C57/BL6 (B6, H-2$^b$) MEF cells. To further analyze whether allogenic MEF-CM also has the same effects on CD8$^+$ T cells, MEF-CM-mediated enhancement in OT-1 CD8$^+$ T cells was compared between allogenic BALB/c (H-2$^d$) MEF-CM and autologous B6 MEF-CM. Increased levels of IFN-γ and granzyme B were also observed in BALB/c MEF-CM treatment, which was similar with the effects of B6 MEF-CM (**Figure 2D**). Cytokine expression profile between conventional medium and MEF-CM-cultured CTLs was further compared.

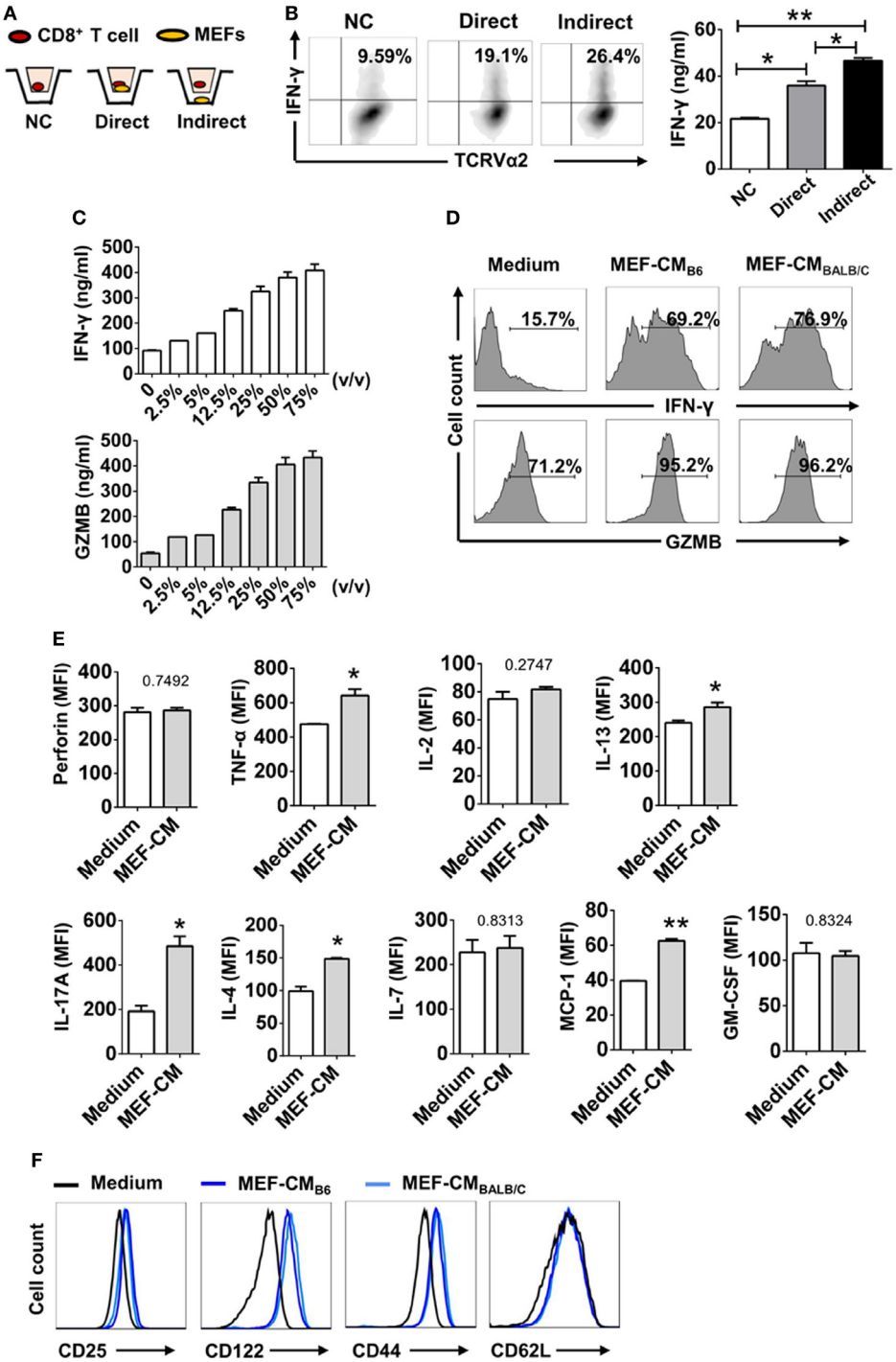


**FIGURE 2** | Mouse embryonic fibroblasts (MEFs) promote activation of CD8+ T cells through soluble factor(s). **(A)** Schematic diagram of trans-well co-culture system. **(B)** OT-1 CD8+ T cells ($1 \times 10^5$) were incubated alone (No MEFs) or together with $2.5 \times 10^5$ B6 MEFs in the upper chamber (Direct), or CD8+ T cells in the upper chamber were incubated with $2.5 \times 10^5$ MEFs in the lower chamber (Indirect). After 3 days of TCR stimulation, interferon-gamma (IFN-γ) production was evaluated by intracellular flow cytometry analysis. **(C)** OT-1 CD8+ T cells ($1 \times 10^5$) were stimulated with different volumes of B6 mouse embryonic fibroblast-conditioned medium (MEF-CM) for 3 days. IFN-γ and granzyme B (GZMB) levels were quantified by ELISA. **(D)** OT-1 CD8+ T cells were stimulated in the presence (50%, v/v) or absence of B6 MEF-CM or BALB/c MEF-CM. 2 days later, the level of IFN-γ and GZMB was determined by flow cytometry analysis. Statistical significance was analyzed by one-way ANOVA with Bonferroni's multiple comparison test (*$P \leq 0.05$; **$P \leq 0.01$). **(E)** OT-1 CD8+ T cells were stimulated in the presence (50%, v/v) or absence of B6 MEF-CM for 2 days, the level of cytokines and chemokines was determined by flow cytometry analysis. Statistical significance was analyzed by unpaired *t*-test (*$P \leq 0.05$; **$P \leq 0.01$). **(F)** Surface markers were analyzed following 3 days stimulation by flow cytometry analysis. All data represent three independent experiments.

We observed MEF-CM significantly elevated IL-17A and also slightly elevated TNF-α, IL-4, IL-13, and GM-CSF expression. However, there were no significant differences in the expression of perforin, IL-7, and MCP-1 between the two cultures (**Figure 2E**; Figure S1 in Supplementary Material). IL-5, IL-6, and IL-10 were undetectable in both of the two cultures (data not shown). Collectively, these results indicated that MEF-CM promoted the enhanced production of some effector molecules by CTLs following TCR engagement.

Since typical surface markers are another hallmarks for effector cell differentiation, surface markers of CTLs were analyzed following 3 days of TCR stimulation in the presence or absence of MEF-CM (50%, v/v). Effector markers CD25 (IL2Rα) and CD122 (IL2Rβ), responsible for IL-2-mediated proliferation, were dramatically upregulated in the presence of either B6 or BALB/c MEF-CM (**Figure 2E**). Another effector marker, CD44, a glycoprotein involved in cell adhesion and migration, was also significantly induced by MEF-CM. With respect to naïve marker CD62L, there was no significant difference between medium cultured and MEF-CM-cultured CTLs (**Figure 2F**). Taken together, these results support the idea that MEF-CM augments antigen-induced differentiation of $CD8^+$ T cells into effector $CD8^+$ T cells, and may be capable of enhancing the intrinsic functions of effector $CD8^+$ T cells.

## MEF-CM Strongly Induces Expression of Eomesodermin

The T-box transcription factors T-box expressed in T cells (T-bet) and Eomesodermin (Eomes) have been implicated as master regulators of $CD8^+$ T cell differentiation and function (21, 22). To assess the effects of MEF-CM on transcriptional regulation of effector $CD8^+$ T cell differentiation, intracellular levels of T-bet and Eomes of $CD8^+$ T cells were measured in the presence or absence of MEF-CM following antigen priming. As shown in **Figure 3A**, Eomes was significantly elevated only in MEF-CM-cultured CTLs, whereas Eomes was always expressed in a low level in the absence of MEF-CM during 72 h stimulation (**Figure 3A**, left panel). With respect to T-bet, both of medium

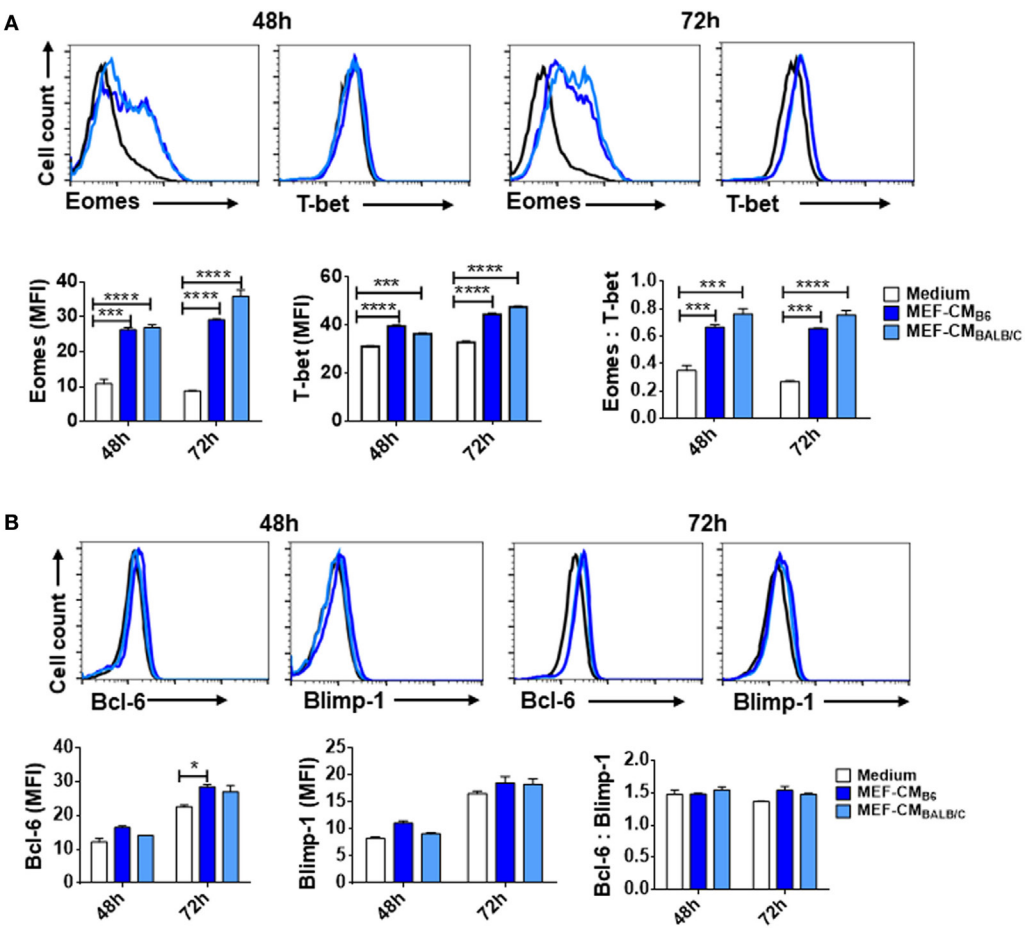


**FIGURE 3** | Mouse embryonic fibroblast-conditioned medium (MEF-CM) strongly induces eomesodermin expression. OT-1 $CD8^+$ T cells were stimulated in the presence or absence of MEF-CM (50%, v/v). **(A,B)** T-bet, Eomes, Bcl-6, and Blimp-1 were examined after 48 and 72 h stimulation using intracellular fluorescence-activated cell sorting analysis. Histograms represented the mean fluorescence intensity (MFI) values of the transcription factors (left and middle), and the ratio of MFI for Eomes relative to T-bet or Bcl-6 to Blimp-1 (right). Data represent at least three independent experiments with similar results. Statistical significance was analyzed by two-way ANOVA with Bonferroni's multiple comparison test (*$P \leq 0.05$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

cultured and MEF-CM-cultured CTLs expressed high level of T-bet (**Figure 3A**, middle panel). Consequently, the ratio of Eomes:T-bet, which is critical for the regulation of memory cell differentiation, was dramatically increased in MEF-CM culture conditions (**Figure 3A**, right panel). In addition, another antagonistic transcription factors Bcl-6 and Blimp-1 that also control effector and memory cell differentiation were also examined (**Figure 3B**). Although a modest increase of Bcl-6 was observed in MEF-CM treatment, there was no significant difference in the expression of Blimp-1 as well as the ratio of Bcl-6:Blimp-1 that is critical for conversion to memory cells (23, 24). Given the notion that T-bet and Eomes cooperatively or alternatively promote cytotoxic lymphocyte formation by inducing the expression of IFN-γ and cytolytic molecules (21, 22), these data suggest that MEF-CM-mediated upregulation of IFN-γ and granzyme B could be a result of strongly induced Eomes.

## Antibody Array Analysis of Cytokines Secretion From MEFs

The cytokine environment is a key factor governing the direction of T cell differentiation (25–27). For example, IL-2 and IL-4 are capable of inducing Eomes expression to modulate the fate and function of cells (12, 28, 29). In order to investigate whether cytokines secreted by MEFs were responsible for $CD8^+$ T cell modulation, MEF-CM (B6 and BALB/c) cytokine arrays were used. We could not observe cytokines that are known to modulate T-cell functions such as IL-2, IL-4, and IL-12 on the array (Figure S2 in Supplementary Material). The result indicates that at least IL-2 and IL-4 were not the major factors for the MEF-CM-mediated enhancement of T cell activation. Rather, there may be another factor(s) produced by MEF that improves CTLs effector functions. Furthermore, a recent report proposed that MEF feeders enhance hESCs differentiation to cardiomyocytes through the co-activation of Wnt3 and Eomes (30). It was also validated that induction of Wnt3-β-catenin signaling promotes memory $CD8^+$ T cell differentiation (31, 32). To know whether there were Wnt ligands in MEF-CM to modulate T cell functions, we examined whether MEF-CM enhanced the level of TCF-7/TCF-1 (transcription factor-7, also known as T cell factor 1) that is downstream of Wnt-β-catenin pathway. We observed MEF-CM did not influence $CD8^+$ T cells in the expression of TCF-7/TCF-1 (Figure S3 in Supplementary Material). Therefore, the MEF-CM effects we observed may not dependent on Wnt-β-catenin signals.

## MEF-CM Enhances CTLs Cytotoxicity

As elevated IFN-γ and granzyme B production by CTLs in the presence of MEF-CM was observed (**Figure 2**), we further examined the ability of CTL-mediated lysis to target tumors *in vitro*. The flow cytometry-based cytotoxicity assay was performed using EG.7 OVA-expressing tumor cells as targets. As expected, the cytotoxic capacity of CTLs derived with MEF-CM was much higher than that of conventional medium cultured CTLs (**Figure 4**). Collectively, the data concerning the production of effector molecules, expression of phenotypic cell surface makers, and *in vitro* cytotoxicities indicate that MEF-CM has potential to modulate $CD8^+$ T cell activation toward more potent CTLs.

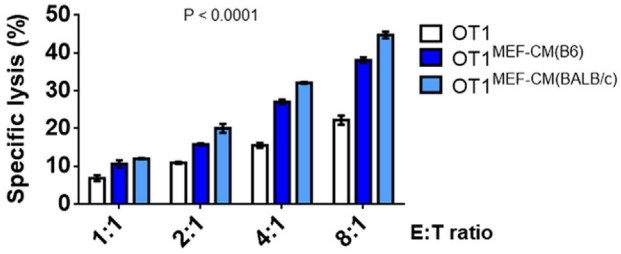


**FIGURE 4** | Specific lysis of EG.7 targets by effector OT-1 cytotoxic T lymphocytes (CTLs), effector OT-1 $CD8^+$ T cells were prepared by the stimulation for 3 days in the presence or absence of mouse embryonic fibroblast-conditioned medium (MEF-CM) (50%, v/v). EG.7 cells which expression OVA was used as target cells. Effector cells and target cells were incubated 4–5 h in E:T ratios ranging from 1:1 to 8:1. To prevent the contamination of effector cells from the analysis of target cell lysis OT-1 CTLs were labeled with carboxyfluorescein diacetate succinimidyl ester and excluded in fluorescence-activated cell sorting analysis. The cell death was measured by 7-AAD incorporation. Data represent at least three independent experiments with similar results. Statistical significance was analyzed by two-way ANOVA with Bonferroni's multiple comparison test.

## CTLs Derived With MEF-CM Are Very Likely to Survive After Adoptive Transfer

Mouse embryonic fibroblast-derived factor(s) strongly induced transcription factor Eomes (**Figure 3A**), which is considered necessary for memory $CD8^+$ T cell differentiation (33–35). Therefore, we hypothesized that MEF-CM-cultured CTLs may acquire the potential for long-term survival after primary activation. To validate this assumption, *in vitro*-generated effector $Ly5.1^{+/-}$ OT-1 in the presence or absence of MEF-CM were adoptively transferred to $Ly5.2^{+/+}$ B6 mice. One month later, the sustained population of transferred cells in tissues (peripheral blood, spleen, and lymph node) was analyzed. The frequency of $Ly5.1^{+/-}$ OT-1 cells was significantly increased in the group transferred with MEF-CM-educated OT-1 cells (**Figures 5A,B**). To further analyze whether MEF-CM improves CTLs survival in tumor-bearing mice, medium cultured $Ly5.1^{+/+}$ OT-1 and MEF-CM culture $Ly5.1^+Ly5.2^+$ OT-1 with ratio of 1:1 were co-transferred to tumor bearing $Ly5.2^{+/+}$ B6 mice. After 11 days T cell transfer, tumor was totally rejected. The persistence of donor cells was examined in peripheral blood on day 7 and day 14, respectively. As expected, the frequency of MEF-CM-cultured CTLs was gradually increased, whereas medium cultured CTLs was decreased (**Figure 6A**). The persisted cells in other tissues (spleen and lymph node) were also compared on day 14. Notably, higher frequency of MEF-CM-cultured CTLs was homing to secondary lymphoid organs comparing to that of medium cultured CTLs (**Figure 6B**). The effector function of the transferred cells was compared by *in vitro* re-stimulation. The producing level of IFN-γ and granzyme B was comparable between MEF-CM and conventional medium cultured CTLs (**Figure 6C**). To analyze the ability of transferred cells in tumor infiltrating, tumor-infiltrating cells were examined on day 7, consistence with the results of other tissue migration, higher frequency of MEF-CM-cultured CTLs infiltrated to tumors (**Figure 6D**).

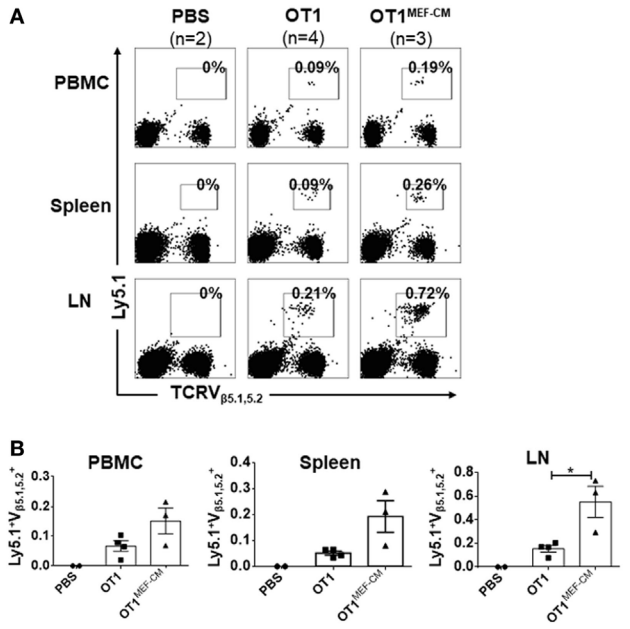


**FIGURE 5** | High potential of mouse embryonic fibroblast-conditioned medium (MEF-CM) cultured cytotoxic T lymphocytes in long-term persistence and proliferation after adoptive transfer. **(A,B)** Ly5.1$^{+/-}$ OT-1 cells ($5 \times 10^4$) that were generated in the presence or absence of MEF-CM (50%, v/v) were transferred to Ly5.2$^{+/+}$ B6 wild-type mice. After 1 month, mice were subcutaneously administrated with $OVA_{257-264}$ peptide antigen and the number of Ly5.1$^{+/-}$ OT-1 cells were determined from blood, spleen, and lymph node 5 days later. **(A)** A representative fluorescence-activated cell sorting result. **(B)** Summary of result **(A)** with recipient groups transferred with PBS alone (two mice), OT-1 cells (four mice), and OT-1 cells cultured with MEF-CM (three mice). Data are representative of two independent replicates. Statistical significance was analyzed by two-way ANOVA with Bonferroni's multiple comparison test (*$P \leq 0.05$).

## CTLs Derived With MEF-CM Effectively Regress Tumors

The ability of T cells to proliferate and survive for a long term after adoptive transfer is associated with effective antitumor immunity. Consequently, we investigated whether MEF-CM-educated CD8$^+$ T cells were superior in the ability of tumor regression using solid EG.7 tumor model. *In vitro*-activated OT-1 cells generated in the presence or absence of MEF-CM ($K^b$-OVA beads stimulation) were adoptively transferred into tumor bearing B6 WT mice. WT CD8$^+$ T cells that lacked antigen specificity (anti-CD3/CD28 stimulation) were used as a negative control. Monitoring of tumor size demonstrated that MEF-CM-educated OT-1 CTLs had superior potential to regress the tumor growth compared with OT-1 cells cultured without MEF-CM (**Figure 7A**). In addition, improved survival was also observed in mice that received MEF-CM-educated OT-1 cells (**Figure 7B**).

## DISCUSSION

Successful adoptive cell therapy for cancer is the outcome of multi-steps that depend on sufficient numbers of antigen-specific CTLs, survival and proliferation of CTLs after transfusion, and successful infiltration of CTLs to target sites. A deficiency at any step along this chain of events will reduce or even invalidate antitumor efficiency for infused CTLs (2, 3, 10, 36). Therefore, each step should be optimized to develop a curative antitumor therapy. Here, we provide a novel method to produce long-lived, functionally potent CTL clones using MEF-CM treatment cultures. Compared with CTLs cultured solely in medium, MEF-CM-cultured CTLs displayed greater potential for long-term survival and exerted superior antitumor immune response after adoptive transfer.

The original role of MEF cells in this study was as feeder cells to support CTLs *ex vivo* expansion, which provides CTLs a contact with "tissues." Unexpectedly, the Trans-well experiment showed that Mouse embryonic fibroblasts dramatically enhanced the levels of IFN-γ and granzyme B of CTLs through the production of soluble factor(s) (**Figures 2A–D**). Hence, we further extended this investigation. We assessed that MEF-CM enhanced antigen-induced acquisition of cytolytic CD8$^+$ T cell characteristics at the levels of phenotype expression (**Figure 2F**), transcriptional regulation (**Figure 3**), and cytolytic function (**Figure 4**). These results support the notion that fibroblasts have the capacity to modulate the function of T lymphocytes (18, 19, 37).

The activation of CD8$^+$ T cells has largely been determined by expression of effector molecules, such as IFN-γ and granzyme B. Here, we observed these expression levels were dramatically enhanced in the presence of MEF-CM (**Figure 2**). T-bet and Eomes are key transcription factors in cytotoxic lymphocyte lineage differentiation through the regulation of IFN-γ, granzyme B, and perforin expression (21, 22, 35, 38). During 72-h stimulation, MEF-CM-cultured CTLs expressed high levels of Eomes, contrary to the medium alone cultured CTLs, which always displayed a low level (**Figure 3A**). Although T-bet was slightly enhanced in the presence of MEF-CM, it was true also in the culture without MEF-CM. In addition, Blimp-1 has also been reported to involved in granzyme B expression (39). However, there was no significant difference in the expression of Blimp-1 between MEF-CM- and medium cultured CTLs (**Figure 3B**). Collectively, it implies MEF-CM-mediated enhancement of IFN-γ and granzyme B is mainly influenced by the upregulation of Eomes. In addition, evidence supports the view that T-bet and Eomes are important for differentiation and maintenance of effector and central memory cells. T-bet directs effector cell differentiation, whereas Eomes is responsible for memory CD8$^+$ T cells differentiation (33–35). Therefore, we hypothesized that strongly induced Eomes in MEF-CM-cultured CTLs enhances the survival of CTLs following *in vivo* transfer. To assess that, a low dose of congenic effector OT-1 CD8$^+$ T cells generated in the presence or absence of MEF-CM were transferred to WT mice. MEF-CM-educated CD8$^+$ T cells displayed more potential to differentiate to long-lived memory cells (**Figures 5A,B**). Very few CD8$^+$ T cells cultured in the absence of MEF-CM with a low level of Eomes persisted after infusion. This result provides further support back up the viewpoint that effector CD8$^+$ T cells with high level of Eomes lean to memory cell differentiation. In addition, co-adoptive transfer experiment in tumor-bearing mice further validated the superior persistence of MEF-CM-cultured

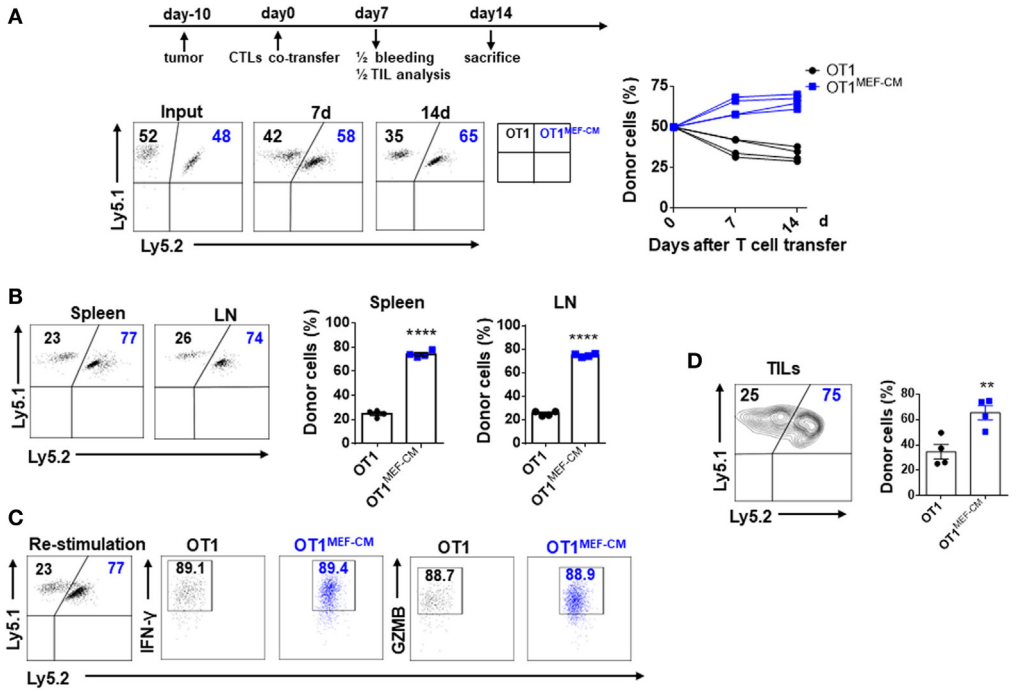


**FIGURE 6** | Mouse embryonic fibroblast-conditioned medium (MEF-CM)-educated cytotoxic T lymphocytes (CTLs) exert higher potential of persistence in tumor-bearing mice. Medium cultured Ly5.1$^{+/+}$ OT-1 ($0.75 \times 10^6$) and MEF-CM culture Ly5.1$^{+}$Ly5.2$^{+}$ OT-1 ($0.75 \times 10^6$) were co-transferred to tumor bearing (40–50 mm$^3$) Ly5.2$^{+/+}$ B6 mice ($n = 8$). The frequency of OT-1 cells were determined in peripheral blood **(A)** on days 7 and 14 of T cell transfer, and spleen and lymph node **(B)** on day 14 of T cell transfer. A representative fluorescence-activated cell sorting result showing percentages in plots indicate percent of donor cells among Ly5.1$^{+}$ CD8$^{+}$ T cells. Graphs are the summary of the dot plots on the left with each dot representing for a recipient ($n = 4$). **(C)** Whole splenocytes were restimulated with OVA$_{257–264}$ peptides for 2 days *in vitro*, interferon-gamma, and granzyme B-producing levels were examined. **(D)** Transferred cells in the tumors were analyzed after 7 days of T cell transfer. Half of the recipients ($n = 4$) were analyzed for panels **(A–C)** and the remaining were analyzed for panel **(D)**. Statistical significance was analyzed by unpaired *t*-test (**$P \leq 0.01$; ****$P \leq 0.0001$). Two independent biological experiments were proceeded with similar results.

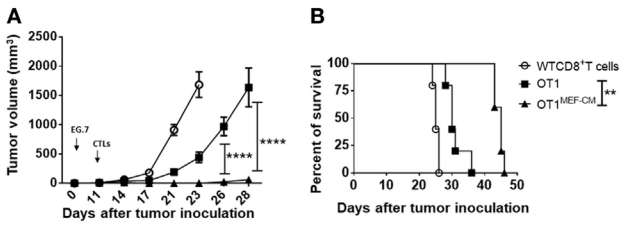


**FIGURE 7** | Potent *in vivo* antitumor immunity of mouse embryonic fibroblast-conditioned medium (MEF-CM)-educated cytotoxic T lymphocytes (CTLs). **(A,B)** EG.7 tumor cells were subcutaneously inoculated in B6 WT mice. After 10–11 days, tumors were detectable. Adoptive transferred CD8$^{+}$ T cells which were generated in the presence or absence of MEF-CM (50%, v/v). Tumor size and survival were monitored regularly. Serial tumor measurements were obtained. One representative experiment of three with $n = 6$ mice per group. Comparison of tumor size by two-way ANOVA with Bonferroni's multiple comparison test. Comparison of survival curves with Log-rank (Mantel–Cox) test (**$P \leq 0.01$; ****$P \leq 0.0001$).

CD8$^{+}$ T cells (**Figure 6**). And as shown in **Figure 7**, MEF-CM-cultured CD8$^{+}$ T cells significantly regressed tumor growth and prolonged mice survival following adoptive transfer. These results support the notion that T-box transcription factors, especially Eomes, are critical for CTL antitumor immunity (40, 41).

Mouse embryonic fibroblast was known to secrete diverse chemokines, cytokines, and growth factors. Forty cytokines arrays of MEF-CM revealed that the mainly detectable molecules were chemokines in both B6 and BALB/c (Figure S2 in Supplementary Material). Although cytokines such as IL-2 and IL-4 enhance Eomes expression in CD8$^{+}$ T cells (12, 28, 29), we did not found any evidence of IL-2 and IL-4 expression through the antibody array. This result was consistent with an early investigation on MEF (42). It can be considered that Eomes upregulation is not due to IL-2 or IL-4. As a recent report proposed that MEF feeders enhance hESCs differentiation to cardiomyocytes through the co-activation of Wnt3 and Eomes (30), although MEFs work on different cells, this finding is partially consistent with our results in terms of Eomes induction.

In conclusion, we provide a novel method to convert the fate of differentiated T cells from exhaustion to vigor, which overcomes the need for repetitive infusion of high dose of CD8$^{+}$ T cells due to most of *in vitro* expanded CTLs loss of survival and functions. To our knowledge, this is the first study to investigate whether embryonic fibroblasts have the ability to enhance the intrinsic qualities of effector CD8$^{+}$ T cells with promising outcomes in adoptive cancer therapy. Here, we achieved strong antitumor activity and prolonged survival of antigen-specific CTLs in just 3 days of culture. Our findings provide a clue to overcome the limitation

of T cell based adoptive therapy, such as inferior cytotoxicity and a short life span, of *in vitro*-cultured CTLs. Identifying molecular factor(s) of MEF-CM responsible for the CTL potentiation will further shed light to anti-cancer T cell therapy.

## ETHICS STATEMENT

The experimental protocols adopted in this study were approved by the Institutional Animal Care and Use committee of Korea University.

## AUTHOR CONTRIBUTIONS

YQ and S-HP designed the experiments; YQ performed the experiments; YQ and S-HP undertook the analysis of data. JS and J-HY reviewed data and manuscript preparation. YQ and S-HP wrote the paper.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00685/full#supplementary-material.

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