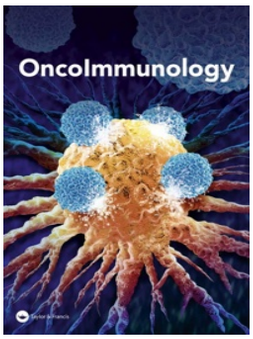

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IL-21-reprogrammed Vδ1 T cells exert killing against solid tumors which is enhanced by CAR arming for off-the-shelf immunotherapy

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IL-21-reprogrammed Vδ1 T cells exert killing against solid tumors which is enhanced by CAR arming for off-the-shelf immunotherapy

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ABSTRACT

Cancer cell therapies have primarily focused on engineering autologous αβ T cells with chimeric antigen receptors (CARs), achieving clinical success against hematologic malignancies. However, their effectiveness against solid tumors is limited by challenges such as antigen escape, suppression by the metabolically hostile tumor microenvironment (TME), and manufacturing difficulties. γδ T cells are unconventional T cells with innate tumor-targeting capabilities independent of MHC class I, making them an emerging candidate for allogeneic cell therapy. While the Vδ1 T cell subset has shown promising anti-tumor killing their clinical application has been hindered by difficulties in achieving robust expansion for therapeutic use. Here, we evaluated the potential of K562 feeder cells expressing membrane-bound IL-21 (K562-mb-IL-21) to expand and activate γδ T cells from peripheral blood. Our findings show that this method preferentially expands Vδ1 T cells, resulting in an activated phenotype characterized by enhanced expression of NK cell activation receptors, innate cytotoxicity against breast and ovarian cancer cells, and sustained metabolic function in patient-derived ascites TME. When engineered with a CAR, Vδ1 T cells exhibited further enhanced anti-tumor efficacy in an immunodeficient NRG xenograft model of human ovarian cancer. These findings highlight K562-mb-IL-21 expanded peripheral blood Vδ1 T cells as a promising 'off-the-shelf' allogeneic therapy for solid tumors.

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Introduction

Autologous chimeric antigen receptor (CAR)-expressing αβ T cell therapies have achieved unprecedented complete responses in hematologic malignancies including refractory leukemia and lymphoma. Despite this clinical success, their widespread implementation has been hindered by challenges in manufacturing personalized cell products including increased time for production and donor variability. In addition, their efficacy against solid tumors remains limited in part due to the nutrient deficient and immunosuppressive solid tumor microenvironment (TME), loss or heterogenous expression of the CAR-targeted antigen, and potential on-target off-tumor toxicities. Hence, the development of safe and 'off-the-shelf' allogeneic adoptive cell therapies that can target tumors with heterogenous antigen expression while remaining functional in the TME is essential to target solid tumors. Innate cells, which directly sense and kill tumor cells through recognition of stress associated antigens, offer a promising alternative to CAR-expressing αβ T cell therapies.

γδ T cells are innate-like T cells that account for approximately 0.5–5% of peripheral blood lymphocytes.[1] Although low in frequency, γδ T cells contribute to cancer immune surveillance and have been identified as the tumor infiltrating immune cell subset most significantly associated with improved overall patient survival across 39 different cancers.[2] There are three main human γδ T cell subsets which are classified

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based on their expression of the variable δ (Vδ) chain of the T cell receptor (TCR). The most prevalent subset in adult peripheral blood expresses the Vδ2 chain which most commonly associates with the Vγ9 chain.[3] The Vδ1 T cells are the tissue-resident subset representing only approximately 10–20% of γδ T cells in peripheral blood and commonly reside in the mucosa and subcutaneous tissues.[4,5] Similarly to natural killer (NK) cells, γδ T cells recognize tumor cells independent of antigen presentation via major histocompatibility complex (MHC) class I, allowing them to target tumor cells that downregulate MHC and demonstrate lower risk for graft-vs-host disease (GvHD).[6] The varying γδ T cell subsets can be activated through their TCR in response to different ligands. Vγ9 Vδ2 T cells recognize phosphoantigens (pAgs) which are upregulated on tumor cells in response to stress,[7] while ligands recognized by Vδ1 T cells are still not fully characterized but include the CD1 family of proteins.[8] Independently of their TCR, Vδ1 T cells recognize stress ligands through NK cell receptors including NKp44, NKp30, DNAM-1, and NKG2D.[9–14] Activated γδ T cells exert anti-tumor killing through degranulation of perforin and granzymes, and Fas and TRAIL ligands, which trigger target cell apoptosis through death receptor signaling.[6] Hence, γδ T cells could be used as an 'off-the-shelf' allogeneic cellular therapy to overcome current challenges with CAR-expressing αβ T cell therapies.

While γδ T cell immunotherapy has been largely focused on expanding and activating Vγ9 Vδ2 T cells, their clinical efficacy has been limited.[15] Harnessing Vδ1 T cells for solid tumors has grown in interest as tumor infiltrating Vδ1, but not Vδ2, T cells correlate with improved outcomes in various solid tumors including triple negative breast cancer and non-small cell lung cancer.[9,16,17] However, large-scale expansion of Vδ1 T cells has been a challenge since they are limited in the peripheral blood and are not stimulated by pAgs. Protocols to expand Vδ1 T cells have used mitogens such as phytohemagglutinin or concanavalin A which have limited clinical translation, or cytokine cocktails and anti-CD3 or anti-TCR stimulation.[10,11] Artificial antigen presenting cells (APCs) including K562 feeder cells expressing CD19, CD64, CD86, 4-1BBL and membrane bound IL-15 (K562-mb-IL-15) have been used to expand polyclonal γδ T cells from CD56-depleted peripheral blood mononuclear cells (PBMCs).[18,19]

Despite demonstrating anti-tumor capacity, strategies that robustly expand and activate Vδ1 T cells are needed for their clinical translation. K562 feeder cells expressing membrane-bound IL-21 and 4-1BBL (K562-mb-IL-21) have been used for the large-scale expansion of highly potent anti-tumor human NK cells for clinical use.[20,21] The high yields driven by this method has been attributed to increased immune cell telomere length and telomerase reverse transcriptase expression in expanded NK cells, enabling sustained long-term proliferative capacity.[22,23] Further, we have shown that IL-21 based expansion metabolically reprograms human NK cells, allowing them to sustain their metabolism and potent anti-tumor functions in the TME.[24] Therefore, we propose that K562-mb-IL-21 cells may show benefits over mb-IL-15 APCs for expanding other innate-like immune cells such as γδ T cells.

In this study, we evaluated the anti-tumor functions of peripheral blood-derived γδ T cells expanded with K562-mb-IL-21 feeder cells for use as an 'off-the-shelf' cell therapy. We found that expansion of PBMCs long-term with K562-mb-IL-21 cells gives rise to activated γδ T cells primarily comprising of Vδ1 T cells. Expanded γδ T cells display innate cytotoxicity against breast and ovarian cancer cells *in vitro*, control tumor growth *in vivo*, and retain their metabolic function in an immunosuppressive patient-derived ovarian cancer model of the TME. The anti-tumor capacity of these expanded Vδ1 T cells against epithelial-derived tumors is further enhanced through stable expression of a human epidermal growth factor receptor 2 (HER2)-specific CAR. These results support the potential of expanded Vδ1 T cells as an 'off-the-shelf' therapy for solid tumors and provide a foundation for further modifications of expanded Vδ1 T cells to enhance their clinical efficacy.

Materials and methods

Ethics

The use of human samples was approved by the Hamilton Integrated Research Ethics Board (13-813T-Ashkar) located in Hamilton, Ontario. Peripheral blood was collected from healthy donors at McMaster University and malignant ascites fluid was collected from patients with high-grade serous ovarian cancer undergoing paracentesis at the Juravinski Cancer Centre located in Hamilton, Ontario. Written informed

consent was provided by all donors prior to sample collection. Experiments involving mice were conducted according to protocols, and in compliance with regulations, approved by the McMaster Animal Research Ethics Board (AUP# 21–04–12) located in Hamilton, Ontario and the study adhered to ARRIVE guidelines.

Cell lines

The adherent human ovarian cancer OVCAR8 and SKOV3 cells, the triple negative breast cancer MDA-MB-231 cells and the breast cancer SKBR3 cells were generously obtained from Dr. Karen Mossman (McMaster University). The OVCAR8 and SKOV3 cells engineered to express a luciferase gene were previously generated as previously described.[24,25] The suspension K562 feeder cells engineered to express mb-IL-21 or mb-IL-15 as previously described were kindly obtained from Dr. Dean A. Lee (Nationwide Children's Hospital).[22,26] Adherent cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin and 1% HEPES (complete) at 37°C and 5% CO2. Suspension cell lines were cultured in complete RPMI 1640 media at 37°C and 5% CO2.

γδ T cell and NK cell expansion

PBMCs were isolated from whole blood via density gradient centrifugation by layering blood diluted in PBS on Lymphoprep at a final 1:1:1 ratio (STEMCELL Technologies). To expand γδ T cells, either bulk PBMCs or CD3+ T cells isolated from PBMCs using the EasySep™ Human CD3 Positive Selection Kit II (STEMCELL Technologies) were co-cultured with K562 mb-IL-21 cells at a 1:2 ratio. For some experiments, bulk PBMCs were expanded using K562 mb-IL-15 at 1:2 ratio. The EasySep™ Human CD3 Positive Selection Kit II (STEMCELL Technologies) was used to isolate expanded γδ T cells from the bulk PBMC expanded cultures. CD3+ isolated cells were re-expanded with K562 mb-IL-21 cells and γδ T cells were expanded for a minimum of 5 weeks prior to use in functional assays. Cells were cryopreserved in 10% DMSO, 20% FBS, and 70% complete RPMI 1640 media. The phenotype of expanded γδ T cells used in functional assays is found in Table 1.

Table 1. Phenotypic characteristics of expanded γδ T cells used in functional assays.

Donor	% CD3+	% CD3-CD56+	% αβ TCR+	% γδ TCR+	% Vδ1+	% Vδ2+	% Vδ1-Vδ2-
1	98.7	1.1	0	99.4	97.3	0	2.72
2	99.4	0.31	5.33	91.7	99.0	0	1.0
3	96.5	2.51	0.15	97.7	22.5	0	77.4
4	98.1	0.82	1.87	97.7	99.2	0	0.68
5	98.8	0.72	0.24	93.6	99.3	0	0.24
6	99.4	0.3	11.5	87.3	40.8	0	59.1
7	97.9	1.71	1.59	95.9	85.2	0.4	14.3

Similarly, NK cells were expanded from PBMCs using K562-mb-IL-21 cells at a ratio of 1:2. All expanded γδ T cell and NK cell cultures were maintained in complete RPMI 1640 media supplemented with human IL-2 (100 U/mL) and cultured at 37°C and 5% CO_2. All cultures were replenished with irradiated feeder cells on a weekly basis and media and IL-2 was replaced every two to three days.

γδ T cell isolation

Fresh unexpanded human γδ T cells were isolated from PBMCs using a TCRγ/δ+ T cell isolation kit (Miltenyi Biotec) or a EasySep™ Human Gamma/Delta T Cell Isolation Kit (STEMCELL Technologies) and were used immediately in functional assays.

Vδ2 T cell expansion

Vγ9 Vδ2 T cells were activated with 10 mM Zoledronic acid (Sigma-Aldrich) and 4 ng/mL IL-2 (Cellgenix) from bulk PBMCs. PBMCs were seeded at $2x10^6$ cells/cm^2 in a 24-well G-Rex plate (Wilson Wolf). Cells were maintained in complete RPMI 1640 supplemented with 1 mM sodium pyruvate, 1 mM non-essential

amino acids, and 55 μM β-mercaptoethanol for the first 4 days of culture. On day 4 of culture, cells were collected, rinsed with PBS and resuspended in CTS OpTmizer T cell Expansion SFM (Gibco) supplemented with 1X Glutamax Supplement (Gibco) and 5% CTS Immune Cell Serum Replacement (Gibco). A 75% media replacement was performed every 3–4 days with 4 ng/mL IL-2 added every 2–3 days and 5 ng/mL IL-15 (BioLegend) added every 2–3 days starting on day 7 of culture. Cells were harvested on day 14 of culture, depleted of αβ T cells using CD4 and CD8 Microbeads (Miltenyi Biotec). Purified cells were cryopreserved in CryoStor CS10 (StemCell Technologies), thawed, and rested in IL-2 (1.5 ng/mL) and IL-15 (5 ng/mL) for 24 h prior to use in experiments.

Incubation in ascites fluid

Cell-free ascites fluid was obtained by passing fluid through a 100 μm and 40 μm nylon filter mesh followed by centrifugation to remove cells as previously described.[24] γδ T cells were incubated in complete RPMI media only or in 90% cell-free ascites fluid. A low-dose of IL-15 (10 ng/mL) was added to both conditions to sustain their viability for three days prior to cytotoxicity, nutrient receptor expression, or extracellular flux assessment.

Generation of HER2 CAR/AAVS1 KO Vδ1 T cells

The protocol to generate CAR-expressing expanded Vδ1 T cells was adapted from a previously described protocol used for human NK cells.[27] Briefly, expanded Vδ1 T cells were electroporated with HiFi CRISPR/Cas9 Nuclease V3 (Integrated DNA Technologies) and guide RNA targeting the AAVS1 safe harbor locus. After electroporation, cells were transduced with ssAAV6 encoding the HER2 CAR construct at a 300K multiplicity of infection (MOI). Wild type control Vδ1 T cells were generated by electroporating cells with CRISPR/Cas9 without AAV transduction (WT-AAVS1 KO) or only electroporating cells without CRISPR/Cas9 (WT-Electroporated). The CAR construct contains a HER2-specific DARPin28z binding domain, a CD28 co-stimulation domain, and the cytoplasmic domain of CD3ξ for signaling as previously described,[28] with the modification of an IgG heavy chain for the hinge domain. The transduced cells were replenished with irradiated K562 mb-IL-21 cells 48 h after electroporation and cryopreserved after 7 days. Cells were replenished with irradiated K562 mb-IL-21 cells weekly post-thaw to expand cells for functional assessment.

In vitro cytotoxicity and degranulation assays

Cytotoxicity and degranulation assays were performed as previously described.[24,28] Briefly, OVCAR8, SKBR3, or MDA-MB-231 tumor cells were stained with carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich) for 15 min at 37°C prior to co-culture with effector cells. CFSE+ target cells were plated with effector cells in a 96-well plate at indicated effector:target (E:T) ratios. After 5 h, cells were stained with Fixable Viability Dye eFluor 780 (APC-Cy7; Thermo Fisher) for tumor cell death assessment. For transwell cytotoxicity assays, effector cells were seeded in the apical surface of the 0.4 μm transwell (Sarstedt) at 4:1 E:T ratio and CFSE+ OVCAR8 cells were seeded in the basolateral surface. After 5 h, cells in the basolateral surface were collected and stained with Fixable Viability Dye eFluor 780. Tumor cell death was determined by gating on CFSE+/APC-Cy7+ cell events. The formula used to calculate % specific lysis was: ((% tumor cell death – % basal tumor cell death)/(100- % basal tumor cell death)) x 100.

To evaluate the degranulation, CFSE+ target cells were co-incubated with effector cells at a 1:1 E:T ratio in a 96-well plate in the presence of an anti-CD107a antibody. After 1 h, GolgiStop (2.0 μM;BD Biosciences) was added and the plate was incubated for an additional 4 h at 37°C until staining with extracellular antibodies. Cells were also stained for intracellular IFN-γ expression. For all assays, 50, 000–200,000 target cells were added per well and all conditions were performed in duplicates.

Cytokine quantification after cell stimulation

In some experiments, expanded γδ T cells were stimulated for 24 h with IL-15 (20 ng/mL), IL-12 (10 ng/mL), IL-18 and IL-2 (100 IU/mL) or media only with IL-2 (100 IU/mL). GolgiStop was added during the last 20 h of the incubation. Cells were counted and washed followed by intracellular staining for IFN-γ

expression. To assess cytokine expression after PMA/Ionomycin stimulation, freshly isolated and expanded γδ T cells were incubated with the Leukocyte Activation Cocktail containing BD GolgiPlug (BD Biosciences) for 4 h according to manufacturer's instructions followed by intracellular staining with anti-IFN-γ and anti-TNF-α antibodies.

Assessment of tumor cell apoptosis

OVCAR8 or MDA-MB-231 cells were labeled with the cell membrane-labeling DiD dye (APC-Cy7, Invitrogen) according to manufacturer's instructions. To evaluate the level of cleaved caspase-3 expression induced by γδ T cells, DiD-labeled OVCAR8 or MDA-MB-231 cells were co-cultured with the effector cells in a 96-well plate at the indicated E:T ratios for 5 h at 37°C. After the incubation, cells were washed with PBS and stained with Fixable Viability Dye BV510. The cells were then fixed and permeabilized and incubated with a Cleaved Caspase-3 Rabbit monoclonal antibody (1:400; Cell Signalling Technology) for 30 min at room temperature (RT). Cells were washed and then incubated with an Alexa 488-conjugated anti-Rabbit IgG (H+L), F(ab')2 Fragment (1:500; Cell Signaling Technology) secondary antibody for 30 min at RT. DiD + cells were gated on APC-Cy7+and Alexa 488+ events to detect tumor cells having undergone apoptosis.

Receptor blocking assays

Expanded γδ T cells were incubated for 1 h at 37°C and 5% CO_2 with blocking antibodies and then co-cultured with CFSE-labeled OVCAR8 or MDA-MB-231 cells in duplicates at a 4:1 E:T ratio in a 5 h cytotoxicity assay. The antibodies used were anti-NKG2D (3 μg/mL, BioLegend, Clone 1D11), anti-γδ TCR (3 μg/mL, Invitrogen™, Clone 5A6.E9), anti-DNAM1 (5 μg/mL, BD Biosciences, Clone DX11), anti-FasL/TNFSF6 (2 μg/mL, R&D systems, Clone 100,419), and anti-human TRAIL/TNFSF10 (1 μg/mL, R&D systems, Clone 75,411). For all experiments, expanded γδ T cells were also incubated with matched concentrations of the matched isotype controls. The isotype controls used were mouse IgG1 isotype control (R&D systems) and mouse IgG2B isotype control (R&D systems).

Seahorse extracellular flux assays

Real-time analysis of expanded γδ T cell extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) was assessed using the Seahorse XF Glycolytic Stress Test (GST, Agilent Technologies) or the Seahorse XF Mito Stress Test (MST, Agilent Technologies), respectively. Assays were performed as previously described for expanded NK cells.[24] Briefly, expanded γδ T cells were washed and resuspended in GST or MST assay medium and 200,000 cells/well were plated in triplicates on 96-well plates that were pre-coated with Poly-L-Lysine (Sigma-Aldrich). ECAR and OCAR was measured using a Seahorse XFe96 Analyzer and Wave Software (Agilent Technologies) according to manufacturer's instructions.

Mice

NOD-Rag1$^{-/-}$*Il2rg*$^{-/-}$ (NRG) mice were obtained from The Jackson Laboratory and bred and housed at McMaster's Central Animal Facility in specific pathogen-free conditions with 12 h light/12 h dark daily cycles. Only female mice at an age of 7–20 weeks were used at no more than 5 mice per cage and the control and treated mice were age-matched.

In vivo xenograft tumor models and adoptive transfer

For the OVCAR8 model, NRG mice were injected intraperitoneally with 2.5x10^5 OVCAR8-luciferase cells at day zero. Two days after tumor engraftment, 20 x10^6 expanded γδ T cells were injected intraperitoneally at the indicated time points. For experiments assessing the efficacy of CAR-engineered Vδ1 T cells, NRG mice were injected intraperitoneally with 5x10^5 SKOV3-luciferase cells. Two days after tumor engraftment, 10x10^6 CAR-engineered Vδ1 T cells or control cells were injected intraperitoneally. Human IL-2 (20,000 IU/mouse) was administered intraperitoneally every two to three days in both experiments. To quantify

tumor burden, D-Luciferin (Perkin Elmer) was injected intraperitoneally into mice, and bioluminescence (radiance) was measured after 14 min using an IVIS Spectrum Imaging System. Radiance (photons/sec/cm^2/sr) was quantified using the Living Image Software (Perkin Elmer). The tumor burden was averaged across experimental conditions prior to expanded γδ T cell administration. The investigators were not blinded in the study. Weight and body condition was tracked twice a week for endpoint assessment. Percent change in weights was calculated as: [(weight – weight before adoptive transfer)/weight before adoptive transfer) *100%]. Mice were anesthetized under gaseous anesthesia using isoflurane and humanely euthanized following procedures based on guidelines by the Canadian Council on Animal Care and approved by the McMaster's Animal Research Ethics Board.

Flow cytometry

Cells were first stained for viability for 30 min at 4°C in the dark. Cells were then washed in PBS and stained with fluorescently labeled antibodies in FACS buffer (0.2% bovine serum albumin in PBS). To assess HER2-CAR expression, cells were incubated with a HER2 Fc-chimeric protein (R&D systems) at RT for 30 min, followed by staining with an anti-human IgG Fc antibody as previously described.[28] To measure DNAM1, cells were first incubated with anti-DNAM1 antibody (BD Biosciences, clone DX11), followed by staining with a rat anti-mouse IgG1 antibody. Stained cells were washed with FACS buffer and fixed with 2% Paraformaldehyde at 4°C for 30 min if no intracellular staining was performed. For intracellular staining, cells were first fixed and permeabilized using the Fixation/Permeabilization Solution Kit (BD Biosciences) then incubated with intracellular antibodies. All antibody staining steps were performed for 30 min at 4°C in the dark. The antibodies and stains used for flow cytometry can be found in Table 2. A BD LSRFortessa (BD

Table 2. List of antibodies and stains used for flow cytometry.

Antibody	Clone	Company
CD56 BV421	NCAM16.2	BD Biosciences
CD56 PE-CF594	NCAM16.2	BD Biosciences
TIGIT AlexaFlour 647	A15153G	BioLegend
Perforin PE-Dazzle549	DG9	BioLegend
NKp30 APC	P30–15	BioLegend
NKp44 PE	P44–8	BioLegend
CD3 APCH7	SK7	BD Biosciences
CD3 PerCp-Cy5.5	UCHT1	BioLegend
αβ TCR AlexaFluor 488	IP26	BioLegend
αβ TCR APC	IP26	BioLegend
γδ TCR BV650	B1	BD Biosciences
Vδ2 PerCp-Cy5.5	B6	BioLegend
Vδ1 PE	T58.2	ThermoFisher
CD107a APC	H4A3	BD Biosciences
IFN-γ BV421	4S.B3	BD Biosciences
TNF-α	Mab11	BioLegend
NKp46 BV786	9-E2	BD Biosciences
TIM-3 BV786	7D3	BD Biosciences
CD14 PE-Cy7	M5E2	BD Biosciences
CD71 BV711	M-A712	BD Biosciences
CD98 PE	UM7F8	BD Biosciences
CD69 PE-CF594	FN50	BD Biosciences
CD158a APC	HP3E4	BD Biosciences
CD158b PE	DX27	BioLegend
CD158e1 AlexaFluor 700	DX9	BioLegend
Granzyme A APC	CB9	BioLegend
Granzyme B AlexaFluor 700	QA18A28	BioLegend
NKG2A PE-Vio770	REA110	Miltenyi Biotec
Glut1 FITC	202915	R&D Systems
NKG2D PerCP-Cy5.5	1D11	BD Biosciences
CD69 PE-CF594	FN50	BD Biosciences
FasL PE	NOK-1	BioLegend
TRAIL APC	RIK-2	BioLegend
IgG Fc PE	QA19A42	BioLegend
IgG1 PE Cy7	RMG1–1	BioLegend
Cell stain		**Company**
Fixable Viability Stain 510		BD Biosciences
eFluor780 Fixable Viability Dye		eBioscience
Vybrant DiD Cell-Labeling Solution		Invitrogen

Bioscience) or 5 L Cytek Aurora (Cytek Biosciences) was used for sample acquisition and FlowJo software was used for data analysis.

Statistical analysis

All data are expressed as mean ± Standard Error of the Mean (SEM) unless otherwise described. Differences in means between two groups were analyzed using a two-tailed Student's t-test. An analysis of variance (ANOVA) was used to determine differences between three or more groups, and a two-way ANOVA was used when there were two independent variables. The specific post hoc tests used for multiple comparisons are provided in the figure legends. The statistical tests were conducted using GraphPad Prism version 10. The level of statistical significance is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). The number of replicates for each experiment is provided in the figure legends. Sample size was not determined using a priori sample size calculation.

Results

Vδ1 T cells preferentially expand long-term using membrane-bound IL-21 feeder cells

Robust and scalable methods are still needed to generate large quantities of functional γδ T cells, particularly for the Vδ1 subset, from accessible sources to enable their development as an off-the-shelf therapy. Here, we assessed the potential of K562 mb-IL-21 feeder cells to expand γδ T cells from healthy donor peripheral blood. We first co-cultured the irradiated feeder cells with bulk PBMCs or isolated CD3+ T cells every seven days with 100 U/mL of IL-2 and tracked the proportion and fold expansion of NK, αβ T, and γδ T cells in the cultures weekly (Figure 1A). The proportion of γδ T cells in the CD3+ population increased after six weeks of expansion in both co-culture conditions compared to baseline (Figure 1B). When tracking fold expansion, both γδ T cells and αβ T cells in the isolated CD3+ T cell cultures had greater fold expansion compared to the bulk PBMC co-cultures (Figure 1C,D). This was expected, as majority of expanded live cells in the bulk PBMC co-cultures were NK cells (Figure 1E). However, there was a trend toward a higher proportion of γδ T cells and lower frequency of αβ T cells in the CD3+ population within the bulk PBMC co-cultures compared to CD3+ T cell isolated co-cultures (Figure 1F-H). Hence, expanded γδ T cells were isolated from bulk PBMC expanded co-cultures using a CD3+ immunomagnetic positive selection kit before further expansion prior to use in functional experiments. We also assessed the impact of cryopreservation and thawing on the viability and proportion of cell subsets within the bulk PBMC expanded cultures. We did not detect a decrease in the viability or change in the proportion of cell subsets post-thaw (Figs. S1A-D). We further tracked the proportion of the Vδ1+, Vδ2+, and Vδ1-Vδ2- γδ T cell subsets throughout eight weeks of expansion in the bulk PBMC co-cultures (Figure 1I). The percentage of Vδ1 T cells significantly increased from mean 6.14 ± 3.99% at pre-expansion to mean 70.20 ± 11.37% after eight weeks (Figure 1J). The Vδ2 T cells became almost completely absent after the expansion, significantly deceasing from mean 81.50 ± 7.60% to mean 3.79 ± 2.91%. Lastly, the percentage of Vδ1-Vδ2- T cells did not significantly increase post-expansion. We also assessed the expansion of γδ T cells from bulk PMBCs using K562-mb-IL-15 feeder cells due to previous studies showing their ability to expand polyclonal γδ T cells.[18,29] We found there was a trend toward a lower proportion of γδ T cells within the CD3+ population (mean 58.02 ± 30.30) after five weeks compared to cultures expanded with K562-mb-IL-21 cells (mean 76.85 ± 15.13) (Figs. S2A,B). Further, there was also a lower proportion of Vδ1 T cells five weeks post-expansion (Fig. S2C). Overall, these results demonstrate the potential of K562-mb-IL-21 feeder cells to drive efficient expansion of human γδ T cells, particularly the Vδ1 T cell subset, from healthy donor blood.

Expanded γδ T cells exhibit heighted activation marker expression and innate cytotoxicity against solid tumor cell lines

Having achieved robust expansion from PBMCs, we moved to assessing expanded γδ T cell anti-tumor functions against solid malignancies. We first measured expression of activation and inhibitory markers involved in mediating γδ T cell anti-tumor killing. We found that expansion significantly increased γδ T cell

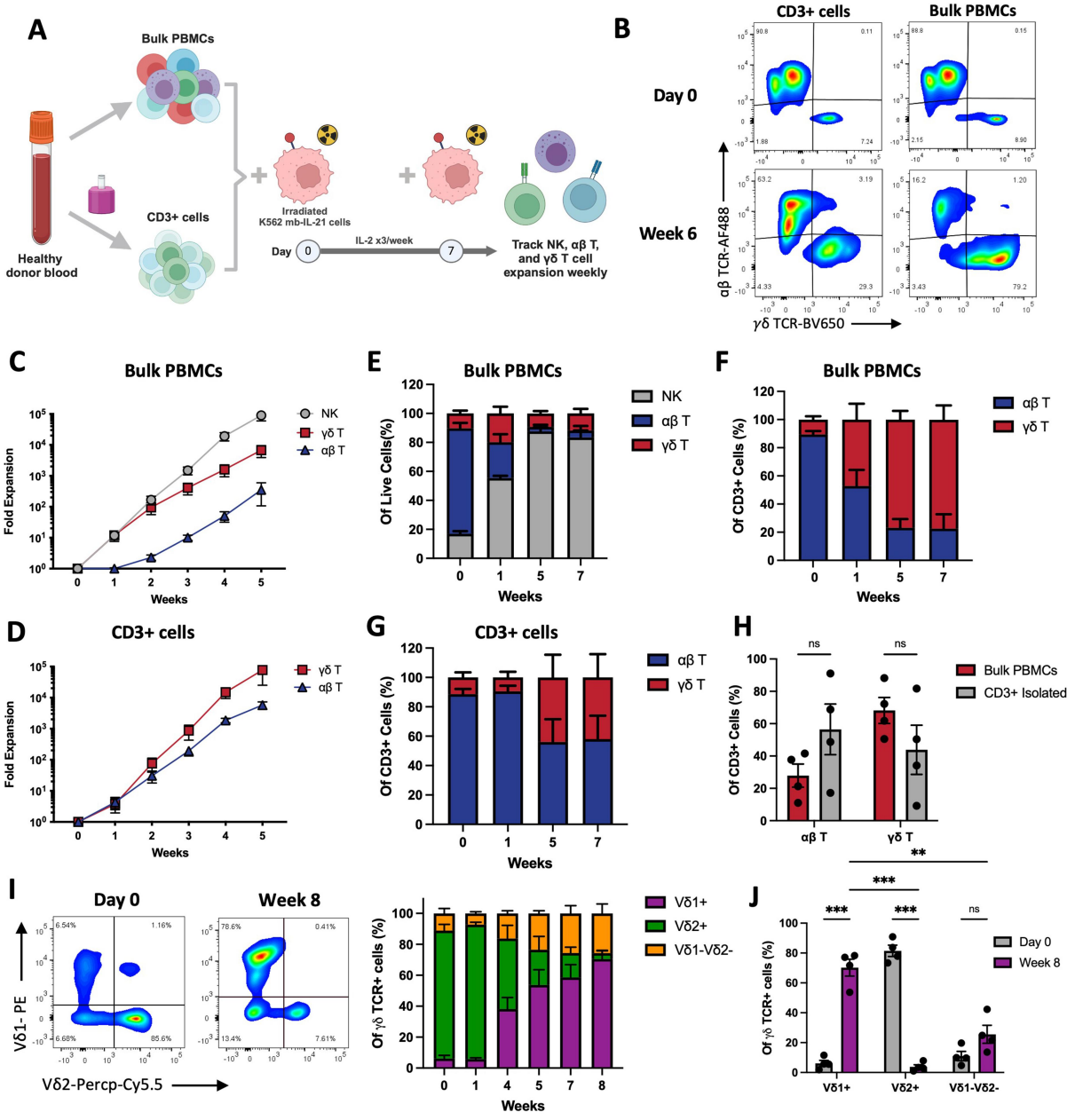

Figure 1. K562 mb-IL-21 feeder cells lead to Vδ1 γδ T cell expansion from PBMCs long-term. (A) Bulk PBMCs or CD3+ T cells were isolated from healthy donor peripheral blood and expanded using K562-mb-IL-21 feeder cells and IL-2 (100 U/mL) weekly. The expansion of NK cells, αβ T cells, and γδ T cell from each co-culture was tracked weekly. (B) Representative flow plots showing the percent αβ TCR+ and γδ TCR+ events of CD3+ T cells pre-expansion and at six weeks post-expansion. (C) Fold expansion of NK cells (CD3-CD56+), αβ T cells and γδ T cells in bulk PBMC co-cultures. (D) Fold expansion of αβ T cells and γδ T cells in CD3+ isolated co-cultures. (E) The proportion of NK cells, αβ T cells, and γδ T cells out of total live cells in co-cultures expanded from bulk PBMCs (F,G) the proportion of αβ TCR+ and γδ TCR+ cells out of CD3+ T cells in co-cultures expanded from bulk PBMCs (F) or from CD3+ T cells (G). (H) The percent αβ TCR+ and γδ TCR+ cells of CD3+ T cells five weeks post-expansion in bulk PBMC or CD3+ isolated T cell co-cultures. I) Representative flow plots showing the percent Vδ1, Vδ2, and Vδ1- Vδ2- γδ T cells expanded from bulk PBMCs pre-expansion and eight weeks post-expansion. The proportion of Vδ1, Vδ2, and Vδ1-Vδ2- γδ T cells in co-cultures over eight weeks of expansion was graphed. J) The percentage of Vδ1, Vδ2, and Vδ1-Vδ2- γδ T cells pre-expansion and eight weeks post-expansion. Data represent mean ± SEM of four to six biological replicates per condition. $^{**}p < 0.01$, $^{***}p < 0.001$, ns, not significant (H; two-way ANOVA with Sidak's post hoc tests, J; two-way ANOVA with Tukey's post hoc tests).

surface expression of the NKG2D, NKp30, and NKp44 NK cell activation markers, and the CD69 activation marker (Figure 2A,B). Meanwhile there were no significant differences in surface expression of NKG2A and the killer immunoglobulin-like receptors (KIRs) CD159a, CD158b, and CD158e. Expression of the

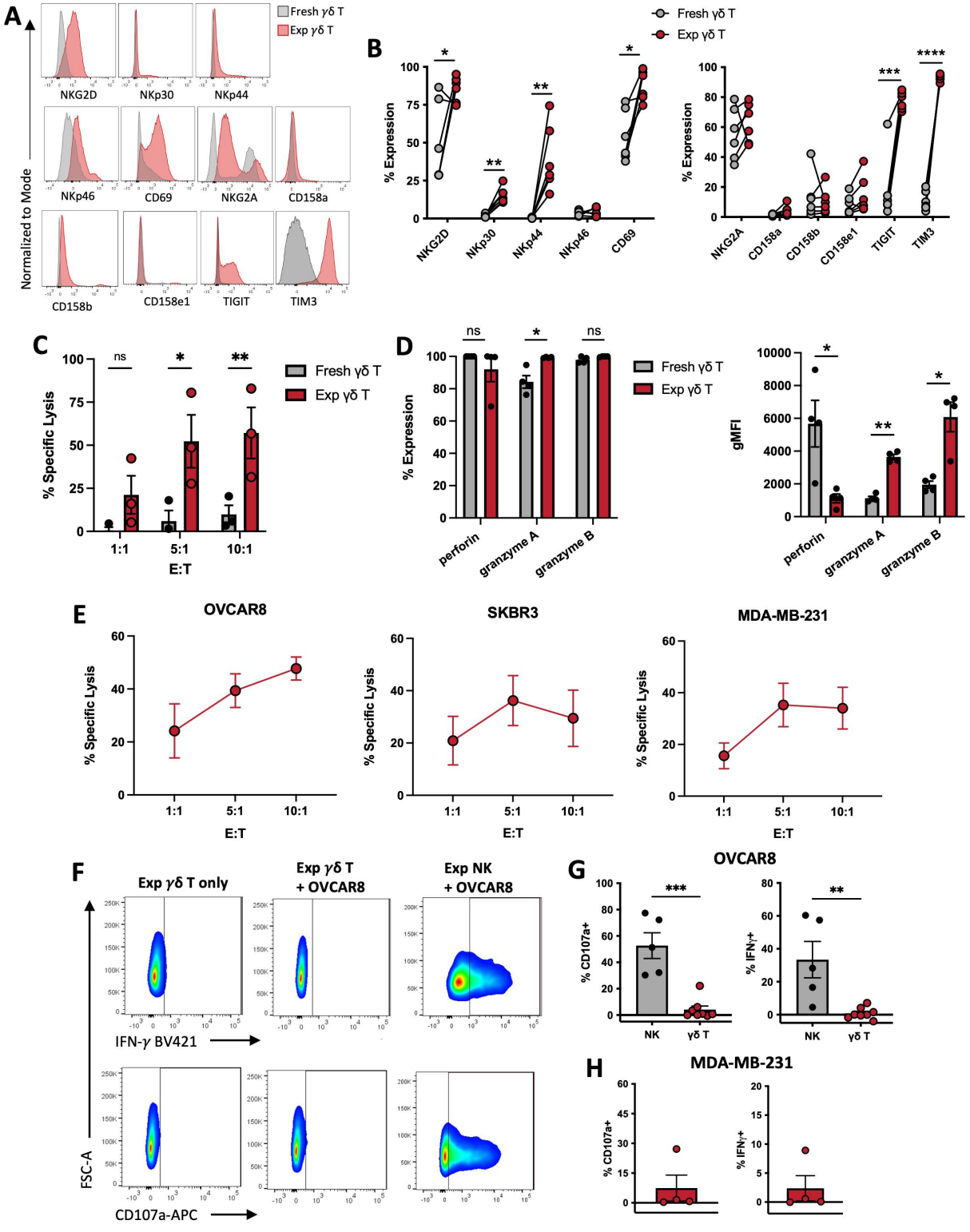

Figure 2. Expanded γδ T cells have enhanced expression of activation markers and exhibit innate cytotoxicity against various solid tumor cell lines. (A) Representative flow plots showing expression of activation and inhibitory markers in freshly isolated or expanded γδ T cells. (B) The percent expression of activation markers and inhibitory markers was quantified by flow cytometry ($n = 6$). (C) Cell-mediated cytotoxicity of freshly isolated and expanded γδ T cells against OVCAR8 cells at different effector: target (E:T) ratios. The percent-specific lysis was graphed. D) The percent expression and MFI of perforin, granzyme A, and granzyme B was measured intracellularly in freshly isolated and expanded γδ T cells. (E) Cell-mediated cytotoxicity of expanded γδ T cells against OVCAR8, SKBR3, and MDA-MB-231 tumor cell lines. Graphs show the percent-specific lysis. (F-H) Expanded NK cells or γδ T cells were co-cultured with OVCAR8 or MDA-MB-231 cells at a 1:1 E:T ratio for 5 h. F) Representative flow plots of IFN-γ and CD107a expression by expanded γδ T cells and NK cells co-cultured

exhaustion/co-inhibitory receptors TIGIT and TIM3 was significantly upregulated in expanded γδ T cells compared to unexpanded γδ T cells (Figure 2A,B). We also found that expansion significantly enhanced γδ T cell expression of CD56 (Fig. S3A). Although CD56 is known as the prototypical maker for human NK cells, CD56 is also expressed on γδ T cells and has been correlated with enhanced anti-tumor activity.[30,31] CD56 expressing expanded γδ T cells showed significantly higher expression of NK cell activation markers: NKG2D, NKp30, NKp44, and CD69 and inhibitory markers: NKG2A, CD158a, CD158b, TIGIT, and TIM3 compared to the CD56-negative expanded γδ T cells (Figs. S3B,C). Overall, these results suggest that expanded γδ T cells have a higher activation status than peripheral blood γδ T cells.

We then compared the cytotoxicity of freshly isolated γδ T cells against OVCAR8 human ovarian cancer cells and found that expanded γδ T cells exerted significantly higher tumor killing at higher effector-to-target (E:T) ratios (Figure 2C). When assessing intracellular expression of perforin and granzymes, which are cytotoxic molecules that mediate tumor cell death, we detected significantly higher expression of granzyme A and MFI of granzyme A and B in expanded γδ T cells compared to unexpanded γδ T cells (Figure 2D). Surprisingly, a significantly lower MFI of perforin was seen in expanded γδ T cells. We also compared the cytotoxicity of zoledronate activated Vδ2 T cells to donor-matched expanded Vδ1 T cells against OVCAR8 cells (Figs. S4A,B). Expanded Vδ1 T cells exhibited a trend for greater killing at the 2.5:1 and 5:1 E:T ratios compared to Vδ2 T cells (Fig. S4C). To see whether expanded γδ T cells have broad antigen-independent anti-tumor functions we assessed their killing against SKBR3 HER2+ breast cancer cells and MDA-MB-231 human triple negative breast cancer cells. Expanded γδ T cells exerted cytotoxicity against all three cell lines, with OVCAR8 cells being the most sensitive to expanded γδ T cell killing (Figure 2E). However, there was an overall lack of improvement in cytotoxicity at the 10:1 E:T from the 5:1 E:T ratio. We next assessed degranulation (by measuring extracellular CD107a expression) and IFN-γ expression of the expanded γδ T cells in response to OVCAR8 and MDA-MB-231 tumor targets (Figure 2F). We included donor-matched expanded NK cells as an internal control for some experiments. We found that expanded γδ T cells exhibited significantly less degranulation and IFN-γ expression in response to OVCAR8 tumor cell stimulation compared to expanded NK cells (Figure 2G). Similarly, expanded γδ T cells had low levels of degranulation and IFN-γ expression against MDA-MB-231 cells (Figure 2H). To confirm that expanded γδ T cells could induce apoptosis in tumor targets, we assessed the expression of cleaved caspase-3 after co-incubation with OVCAR8 or MDA-MB-231 target cells for five hours. Indeed, cleaved caspase-3 expression in apoptotic tumor cells increased with increasing effector: target (E:T) ratios in both cell lines (Figs. S5A,B).

Due to minimal IFN-γ expression detected in response to tumor targets, we asked whether stimulation with PMA/Ionomycin or cytokines could induce pro-inflammatory cytokine expression by expanded γδ T cells. Stimulation using PMA/Ionomycin resulted in robust expression of IFN-γ and TNF-α which was comparable to freshly isolated γδ T cells (Fig. S6A). We then tested stimulation with IL-12, IL-18, and IL-15, as this cocktail has been demonstrated to enhance γδ T cell IFN-γ expression independent of TCR signaling.[32] We found that stimulation with these cytokines could induce IFN-γ expression by expanded γδ T cells in both Vδ1+ and Vδ1-Vδ2- T cell subsets, with the extent of IFN-γ expression being donor-dependent (Figs. S6B,C). These results suggest that expanded γδ T cells have the capacity to express IFN-γ but it is not triggered by the recognition of the tumor cells tested in this study. Overall, these results suggest that the innate cytolytic function of expanded γδ T cells may occur independently of degranulation events measured through CD107a.

Expanded γδ T cells control tumor burden in a xenograft ovarian cancer model

To further evaluate the anti-tumor capacity of expanded γδ T cell we assessed their ability to reduce tumor burden using a preclinical xenograft model of human ovarian cancer. NRG mice were injected intraperitoneally with OVCAR8-Luciferase cells (OVCAR8-Luc) and then administered either PBS (tumor only) or

with OVCAR8 cells. G,H) The percent expression of IFN-γ and CD107a in the effector cells after incubation with OVCAR8 (G) or MDA-MB-231(H) cells was quantified by flow cytometry. Data represent mean ± SEM of three to nine biological replicates per condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ ns, not significant (B,D; paired two-tailed t-test, C; two-way ANOVA with Sidak's post hoc tests, G; unpaired two-tailed t-test).

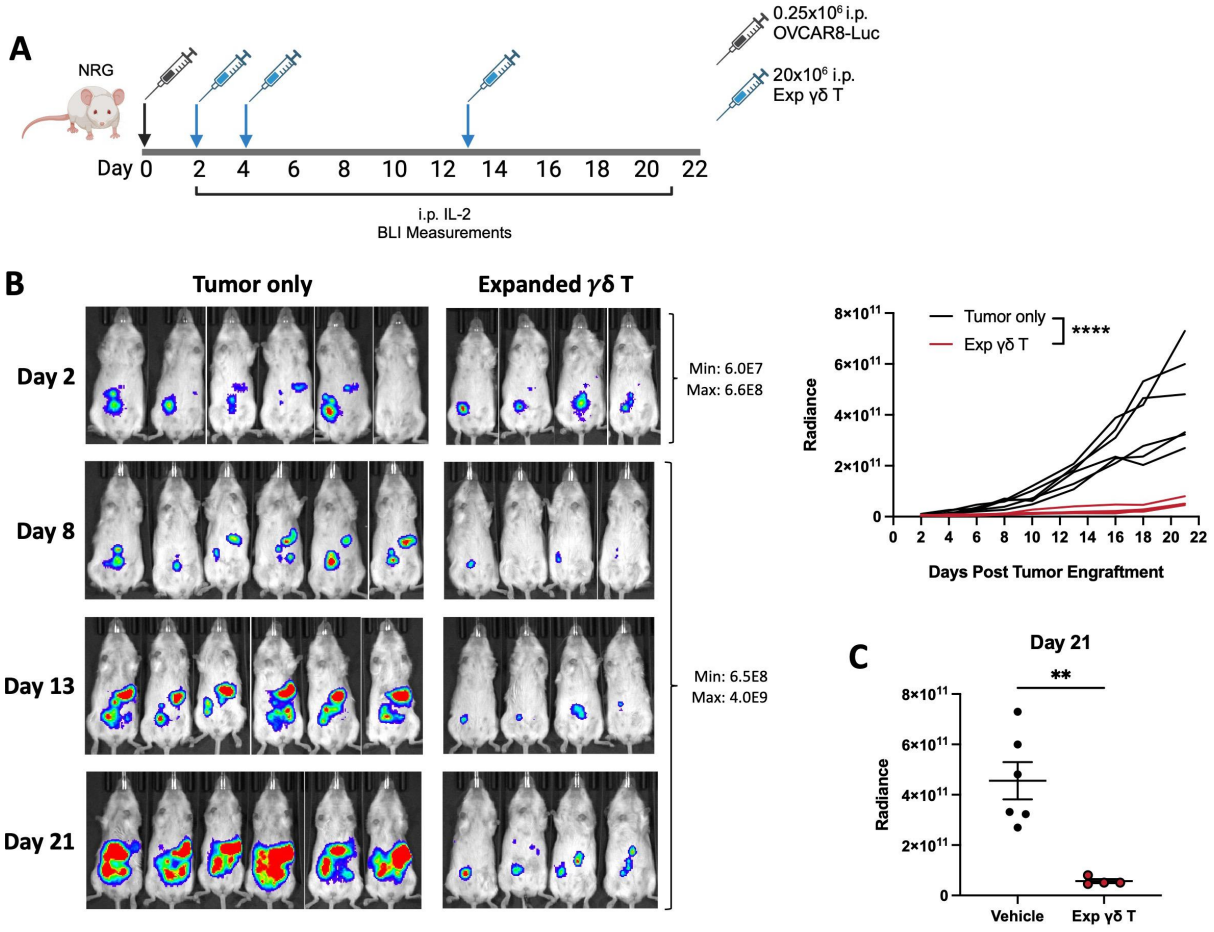

Figure 3. Expanded Vδ1 T cells reduce tumor burden in an OVCAR8 human ovarian cancer xenograft model. A) Expanded γδ T cells were adoptively transferred intraperitoneally (i.p.) to NRG mice two days post-injection with OVCAR8-luciferase cells. IL-2 (20,000 U/mouse) was administered every two to three days to support γδ T cell survival *in vivo*. Schematic shows experimental procedure. B) The tumor burden was quantified via bioluminescence at different time points. C) Tumor burden at day 21 post tumor engraftment. Radiance units are photons/sec/cm2/sr. Data represent mean ± SEM of four to six mice per group. $^{**}p < 0.01$, $^{****}p < 0.0001$ (B; two-way ANOVA with Sidak's post hoc tests, C; unpaired two-tailed t test).

$20x10^6$ expanded γδ T cells from a donor with > 95% Vδ1 T cells at multiple time points (Figure 3A). Tumor burden was assessed by measuring bioluminescence over 21-days post-tumor engraftment in both groups. Administration of expanded Vδ1 T cells significantly controlled tumor growth compared to mice that did not receive adoptively transferred cells (Figure 3B,C). These results demonstrate that K562-mb-IL-21 expanded Vδ1 T cells exert *in vivo* anti-tumor efficacy despite low levels of degranulation detected *in vitro*.

Blocking γδ TCR, NKG2D, and DNAM-1 does not abrogate expanded γδ T cell cytotoxicity

After confirming *in vitro* and *in vivo* anti-tumor activity of expanded γδ T cells, we next sought to determine the ligand interactions mediating their cytotoxicity against tumor cell lines used in this study. We first confirmed that expanded γδ T cell-mediated killing was contact dependent as we detected no tumor cell death when γδ T cells were placed in the apical side of a transwell chamber (Figure 4A). We then used blocking antibodies against the γδ TCR and NK cell activation receptors known to mediate γδ T cell engagement and assessed the effect on expanded γδ T cell killing (Figure 4B).[33] We found no significant difference in the killing of OVCAR8 and MDA-MB-231 cells by expanded γδ T cells when NKG2D or the γδ TCR was blocked in isolation or in combination compared to isotype controls (Figure 4C-E and Figs. S7A-C). As DNAM-1 has also been shown to play a role in γδ T cell tumor cell recognition we also measured DNAM-1 expression and assessed its involvement in mediating expanded γδ T cell cytotoxicity.[34] Expanded

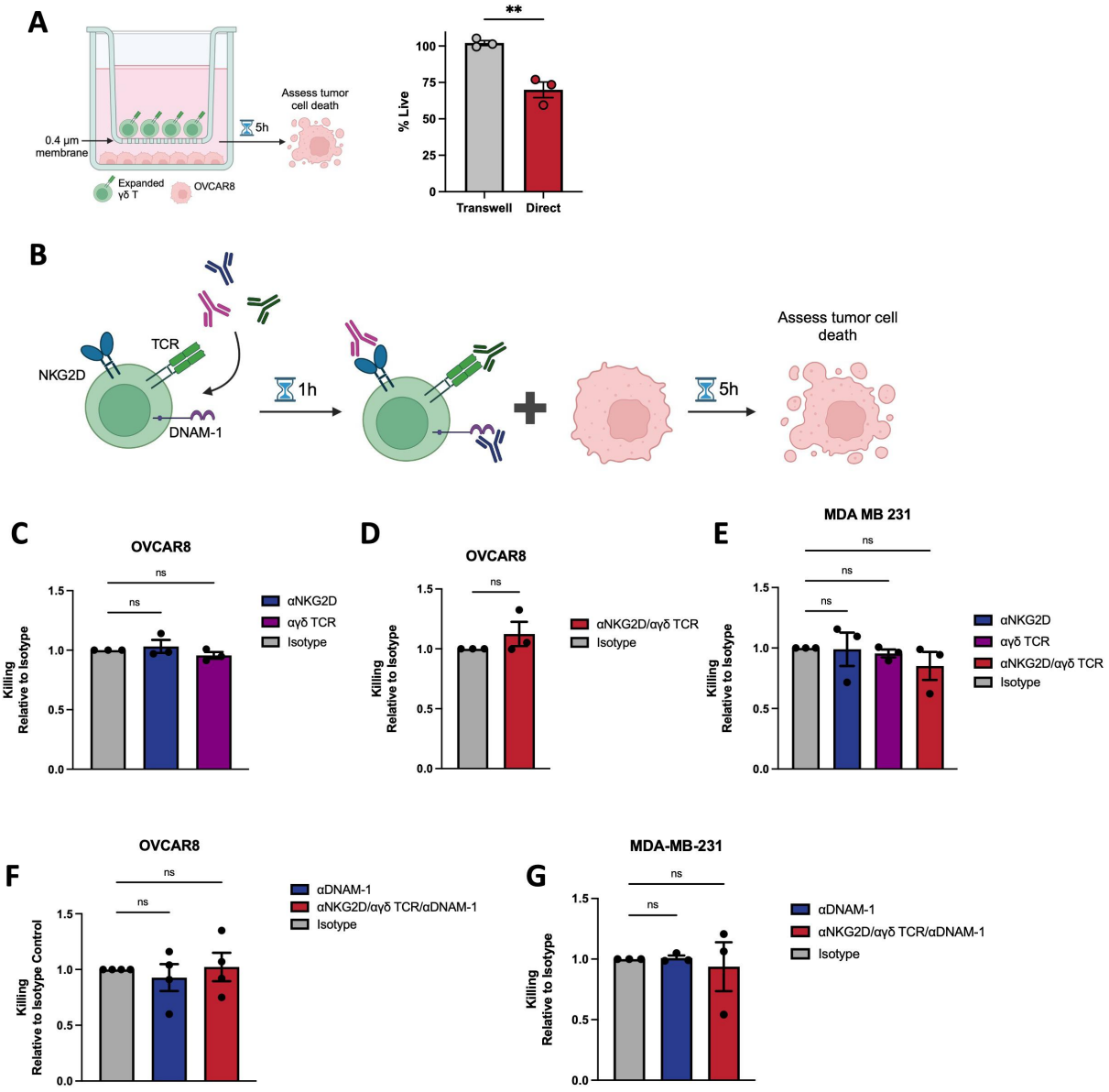

Figure 4. Expanded γδ T cell cytotoxicity is not abrogated by blocking NKG2D, DNAM-1, or the γδ T cell receptor. (A) Schematic of transwell killing assay: OVCAR8 cells were seeded in the basolateral side and expanded γδ T cells were added to the apical side of a 0.4 µm transwell. After five hours, the percent live tumor cells seeded in the transwell or after direct contact with expanded γδ T cells was calculated. (B) Schematic: expanded γδ T cells were incubated with blocking antibodies for one hour followed by co-culture with tumor target cells for an additional five hours at a 4:1 effector-to-target ratio. (C, D) Relative change in specific lysis against OVCAR8 cells in the presence of blocking antibodies against NKG2D and the γδ TCR either individually (C) or in combination (D) was calculated compared to isotype controls. E) Relative change in specific lysis against MDA-MB-231 cells in the presence of blocking antibodies against NKG2D and the γδ TCR was calculated compared to isotype controls. F,G) Relative change in specific lysis against OVCAR8 (F) or MDA-MB-231 (G) cells in the presence of blocking antibodies against DNAM-1, NKG2D, and the γδ TCR was calculated compared to isotype controls. Data represent mean ± SEM of three to four biological replicates per condition. $^{**}p < 0.01$, ns, not significant (A,D; unpaired two-tailed t-test, C,E-G; one-way ANOVA with Dunnett's post hoc tests).

γδ T cells indeed expressed high levels of DNAM-1 (Fig. S7D). However, there was no significant difference in cell-mediated killing of OVCAR8 or MDA-MB-231 cells by blocking DNAM-1 alone or in combination with NKG2D or γδ TCR compared to the isotype controls (Figure 4F,G and Figs. S7E,F).

We also aimed to determine whether death ligand-induced apoptosis could be leading to tumor killing as expanded γδ T cells did not upregulate surface expression of CD107a upon tumor co-culture. We first measured extracellular expression of FasL and TRAIL on expanded γδ T cells. Extracellular expression of

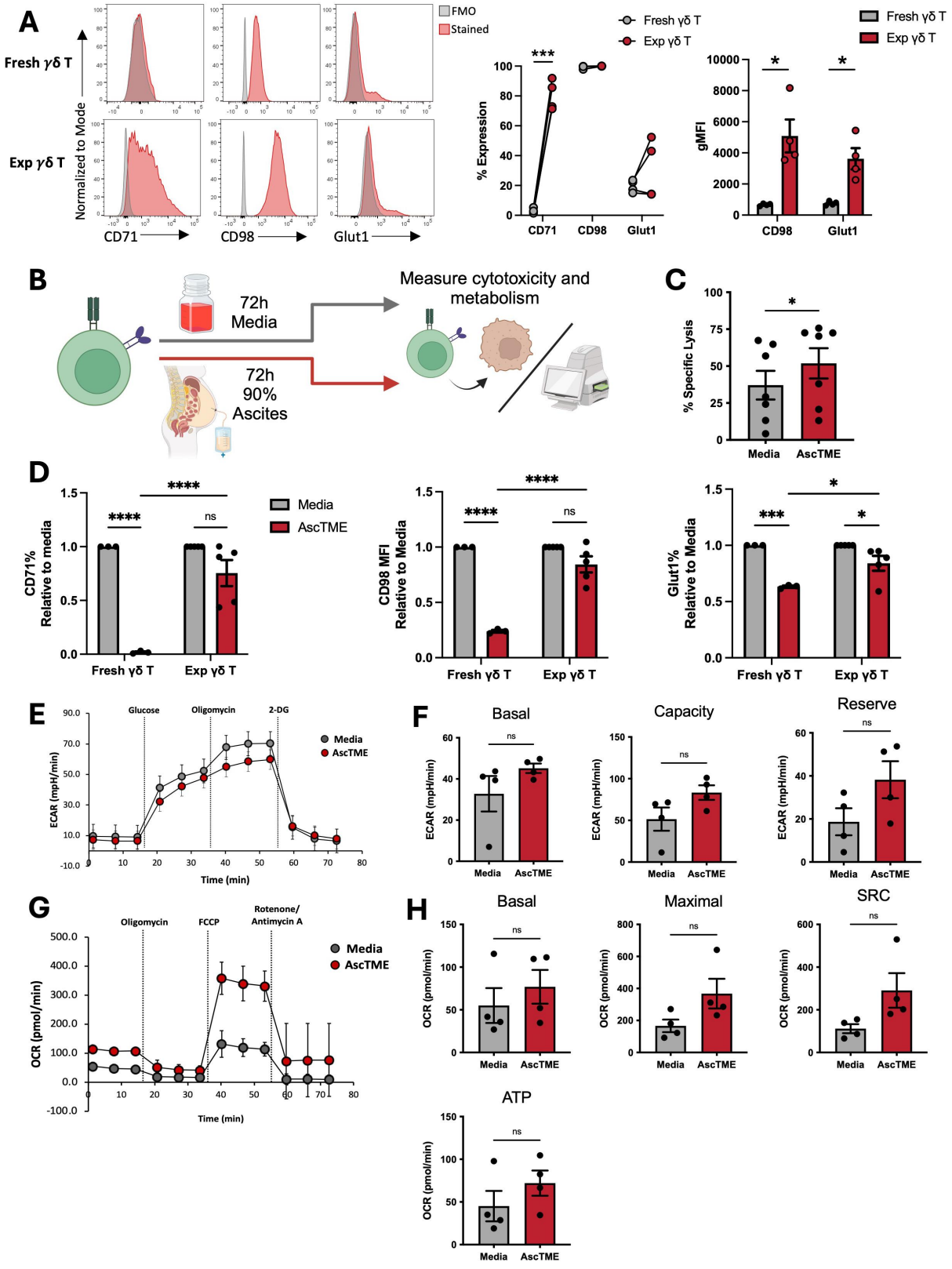

Figure 5. Expanded γδ T cells sustain their cytotoxicity and metabolic function in the malignant ascites tumor microenvironment. A) Representative flow plots showing expression of CD71, CD98 and Glut1 in fresh and expanded γδ T cells. The percent and MFI expression of CD71, CD98 and Glut1 was measured by flow cytometry. B) Schematic showing incubation of expanded γδ T cells in media or the ascites tumor microenvironment (AscTME) for three days prior to cytotoxicity and metabolism assessment. C) Expanded γδ T cell-mediated cytotoxicity against OVCAR8 cells was measured at a 1.25 E:T ratio after incubation in media or AscTME. D) Cell surface expression of CD71, CD98 and Glut1 on expanded and freshly isolated

FasL and TRAIL was low with TRAIL expressed to a higher extent (Fig. S7G). To determine the contribution of FasL and TRAIL engagement for expanded γδ T cell killing we assessed their cytotoxicity against MDA-MB-231 cells in the presence of FasL and TRAIL blocking antibodies. There was no change in cytotoxicity by γδ T cells when FasL and TRAIL were blocked alone or in combination compared to isotype control (Figs. S7H). Overall, these results suggest that FasL or TRAIL are not involved in mediating tumor cell apoptosis by expanded γδ T cells. Further, tumor killing is not dependent on activation through the γδ TCR and although expanded γδ T cells highly express NKG2D and DNAM-1, blocking these receptors does not abrogate their cytotoxicity.

Expanded γδ T cells resist the immunosuppressive patient-derived ovarian cancer ascites

Maintaining cellular metabolic fitness in the immunosuppressive and metabolically hostile TME is a critical factor to achieving durable anti-tumor responses against solid tumors. However, few studies to date have evaluated the metabolic profile of pre-clinical γδ T cell products. To assess the metabolic function of expanded γδ T cells, we first measured their expression of the transferrin receptor (CD71), a component of the amino acid transporter (CD98) and the glucose transporter (Glut1). Surface expression of CD71 and the MFI of CD98 and Glut1 nutrient receptors was significantly enhanced after expansion pointing toward an enhanced metabolic profile (Figure 5A). To evaluate whether expanded γδ T cells would be supressed by the TME we assessed their cytotoxicity and metabolic function after exposure to patient-derived human ovarian cancer ascites fluid (ascTME) (Figure 5B). Ascites fluid is known to suppress critical functions of anti-tumor immune cells including NK cells, due to its enrichment in immunosuppressive factors, high acidity, and depletion of nutrients.[35] We found that extended incubation in the ascTME did not abolish the cytotoxicity of expanded γδ T cells against ovarian cancer OVCAR8 cells (Figure 5C). When comparing nutrient receptor expression after incubation in the ascTME, freshly isolated γδ T cells almost lost complete expression of the transferrin receptor CD71 and had significantly lower levels of the amino transporter CD98 (Figure 5D and Figs. S8, S9A,B). However, expanded γδ T cells retained expression of both transporters in the ascTME (Figure 5D and Figs. S9C,D). Further, although Glut1 expression was decreased on both freshly isolated and expanded γδ T cells in the ascTME, expanded γδ T cells expressed significantly higher Glut1 in these suppressive conditions compared to freshly isolated γδ T cells (Figure 5D and Fig. S9E, F). When comparing to zoledronate activated Vδ2 T cells, expanded γδ T cells also showed greater expression of CD71 and Glut1 in the ascTME (Figs S10A,B,E,F). Meanwhile, the MFI of CD98 after exposure to the ascTME was similar on both expanded γδ T cells and Vδ2 cells (Figs. S10C,D). Next, we measured the ability of expanded γδ T cells to perform glycolysis and oxidative phosphorylation (OxPhos) after ascites exposure. Expanded γδ T cell maintained their basal glycolytic function, glycolytic capacity, and glycolytic reserve (Figure 5E,F). A similar trend was seen with OxPhos, with expanded γδ T cells sustaining their basal and maximal OxPhos, as well as spare respiratory capacity and ATP production in the ascTME (Figure 5G,H). These results show that expansion with K562 mb-IL-21 enables sustained cytotoxicity and metabolic fitness of expanded γδ T cells in the TME.

Expanded Vδ1 T cells are amenable to stable CAR expression

Having tested the innate tumor killing capacity of expanded γδ T cells, we investigated whether introducing a CAR would further enhance their anti-tumor functions. Clinical application of 'off-the-shelf' CAR-expressing γδ T cells requires stable transgene CAR expression for successful scale-up. However, achieving this with lentiviral systems has been a challenge for innate cells like human NK cells. Here, we assessed whether we could adopt the CRISPR/Cas9 and adeno-associated viral (AAV) vector system for stable CAR

γδ T cells was measured by flow cytometry after incubation in media or the AscTME. Relative change in expression compared to media only controls was quantified. E) Representative measures of ECAR in one donor. F) Quantified basal glycolysis, glycolytic capacity, and glycolytic reserve. G) Representative measures of OCR in one donor. H) Quantified basal oxidative phosphorylation (OxPhos), maximal OxPhos, spare respiratory capacity (SRC), and mitochondrial linked-ATP production. Data represent mean ± SEM of four to seven biological replicates per condition. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. ns, not significant (A,C,F,H; paired two-tailed t-test, D; two-way ANOVA with Sidak's post hoc tests).

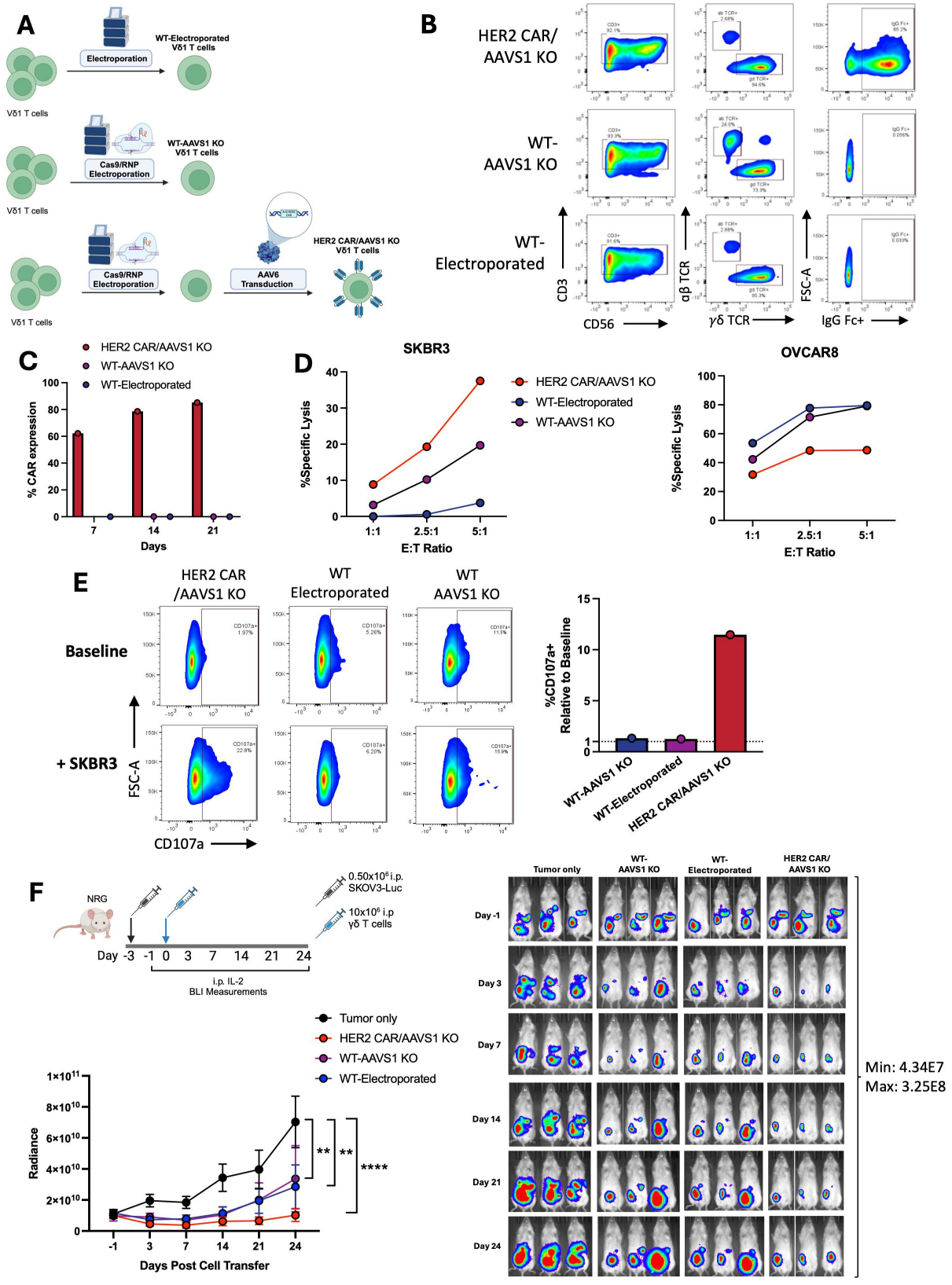

Figure 6. Expanded Vδ1 T cells expressing a HER2-specific CAR show enhanced anti-tumor functions against HER2-overexpressing tumors. A) Schematic depicting the generation of HER2 CAR/AAVS1 KO Vδ1 T cells and wild type Vδ1 T cell controls using CRISPR/Cas9 electroporation and AAV transduction. B) Flow cytometry plots showing expression of the HER2 CAR on expanded γδ T cells. C) The percent expression of the HER2 CAR was measured every seven days by flow cytometry over 21-days of expansion. D) Cell-mediated cytotoxicity of HER2 CAR/AAVS1 KO-, WT-Electroporated-, or WT-AAVS1 KO- Vδ1 T cells against HER2+ SKBR3 or HER2- OVCAR8 tumor cells at the indicated effector-to-target (E:T) ratios. The

expression in expanded Vδ1 T cells. This method can achieve high transduction efficiencies and stable, long-term transgene expression in human K562-mb-IL-21 expanded NK cells.[27] This approach involves electroporating cells with a Cas9/ribonucleoprotein (RNP) complex which introduces a double-stranded break at a specific genomic locus. This is followed by delivery of AAVs containing a DNA template encoding the transgene of interest, which is incorporated via homologous recombination during the cell's intrinsic DNA-repair process.[27] For this study, we chose a HER2-specific second-generation CAR with a CD28 co-stimulatory domain and CD3ξ that was previously shown to enhance tumor targeting of human expanded NK cells and αβ T cells.[28,36] Using this method, the HER2 CAR was integrated at the AAVS1 safe harbor locus of expanded Vδ1 T cells producing HER2 CAR/AAVS1 KO-Vδ1 T cells with 62% CAR expression (Figure 6A). To determine whether the CAR remained stable, we assessed CAR expression over three weeks of expansion after HER2 CAR/AAVS1 KO-Vδ1 T cell generation. We found that CAR expression was not reduced over this expansion period (Figure 6B,C) and HER2 CAR/AAVSI KO-Vδ1 T cells exhibited similar growth to control cells (Fig. S11). These results demonstrate that expanded Vδ1 T cells are amenable to stable CAR transgene expression during our expansion protocol, exemplifying their potential as an 'off-the-shelf' therapy.

Arming Vδ1 T cells with CAR increases their anti-tumor activity against HER2+ cancers

To determine whether CAR expression enhanced the anti-tumor functions of expanded Vδ1 T cells we first assessed their cytotoxicity against HER2 overexpressing-SKBR3 and HER2 low-OVCAR8 cells compared to wild type Vδ1 T cells. HER2 CAR/AAVS1 KO-Vδ1 T cells led to the most efficient cell death of SKBR3 cells but did not show enhanced killing against OVCAR8 cells (Figure 6D and Fig. S12). Since we did not detect high levels of degranulation against tumor cells in non-engineered γδ T cells we asked whether CAR-mediated signaling would lead to CD107a surface expression. Activation of Vδ1 T cells through the CAR led to an increase in CD107a after co-culture with HER2+ SKBR3 tumor cells compared to baseline (Figure 6E). We next evaluated the *in vivo* anti-tumor capacity of HER2 CAR/AAVS1 KO-Vδ1 T cells in a human ovarian cancer model. NRG mice were intraperitoneally engrafted with HER2+ SKOV3-Luciferase cells (SKOV3-Luc) and allowed to establish. Mice were then randomized into tumor only (PBS), HER2 CAR/AAVS1 KO-Vδ1 T cells, wild type-AAVS1 KO Vδ1 T cells (WT-AAVS1 KO), and WT-Electroporated Vδ1 T cells groups based on bioluminescence. Mice in the treatment groups received one injection of $10x10^6$ cells to discern anti-tumor differences between the control Vδ1 T cells and CAR-expressing Vδ1 T cells (Figure 6F). After 24 days following adoptive cell transfer, HER2 CAR/AAVS1 KO-Vδ1 T cells led to the greatest significant reduction in tumor growth compared to tumor only mice. Of note, there was no treatment related toxicity by HER2 CAR/AAVS1 KO-Vδ1 T cells as assessed through change in body weight over the study period (Fig. S13). Overall, these results demonstrate that Vδ1 T cells expressing CAR can effectively control tumor burden in an ovarian cancer xenograft mouse model.

Discussion

γδ T cells are a promising allogeneic cell therapy alternative to traditional autologous αβ T cells due to their innate anti-tumor activity against a broad range of tumors without the reliance on MHC-I antigen presentation. While the Vγ9 Vδ2 T cell subset has been the most investigated in preclinical and clinical studies, Vδ1 T cells have been widely understudied due to their low prevalence in the peripheral blood, their

percent specific lysis was graphed. E) The cell surface expression of CD107a (degranulation) on HER2 CAR/AAVS1 KO-, WT-Electroporated-, or WT-AAVS1 KO- Vδ1 T cells were measured at baseline and after co-culture with HER2+ SKBR3 tumor cells after 5 hours at a 1:1 E:T ratio. The relative expression of CD107a compared to baseline was calculated and graphed. Data represent mean of technical replicates from one biological donor. F) NRG mice were implanted intraperitoneally (i.P.) with $0.50x10^6$ SKOV3-luciferase cells followed by $10x10^6$ HER2 CAR/AAVS1 KO-, WT-Electroporated-, or WT-AAVS1 KO- Vδ1 T cells two days after tumor engraftment. IL-2 (20,000 U/mouse) was given every two to three days to support γδ T cell survival. The tumor burden was quantified via bioluminescence at different time points and graphed. Data represent mean ± SEM of three to seven mice per group. $^{**}p < 0.01$, $^{****}p < 0.0001$. ns, not significant (F; two-way ANOVA with Tukey's post hoc tests).

relatively unknown activating ligands, and challenges with long-term *ex vivo* expansion. However, Vδ1 T cells show promise for targeting solid tumors as their tumor infiltration correlates with a positive prognosis across various solid tumors including lung and breast cancer, and infiltrating Vδ1 T cells in colon cancer have high capacity to exert cytotoxicity.[9,17,37] In this study we show that K562-mb-IL-21 feeder cells can be used to obtain clinically relevant numbers of anti-tumor Vδ1 T cells from healthy donor PBMCs without additional stimulation through the TCR via activating antibodies and phytohemagglutinin or mitogens like Concanavalin A. Importantly, these expanded Vδ1 T cells sustain their metabolic fitness and cytotoxicity in the immunosuppressive TME and can be engineered to express a CAR leading to enhanced tumor control in a xenograft model of human ovarian cancer.

Previous studies have utilized feeder cells engineered with co-stimulatory molecules including CD80, CD83, 4-1BBL, and IL-15 in combination with zoledronic acid or exogenous IL-2 and IL-21 to expand Vδ2 or polyclonal γδ T cells from PBMCs.[18,38] Given that our group and others have shown STAT3 signaling via mb-IL-21 enhances proliferation, anti-tumor functions, and metabolic fitness in NK cells, we assessed whether these IL-21 driven effects also extend to γδ T cells.[24,39] Since K562-mb-IL-15 feeder cells successfully expand γδ T cells without TCR stimulation, and addition of an anti-γδ TCR agonist did not increase γδ T cell yield across all donors early in expansion,[29] we postulated that K562-mb-IL-21 cells could also expand γδ T cells in the absence of TCR stimulation. Using this method, we observed an over 500-fold and 26,000-fold expansion of γδ T cells from bulk PBMCs or isolated CD3+ T cells starting cell populations, respectively. Despite a greater fold-expansion rate seen in the CD3+ T cell isolated cultures, there was a trend for a higher purity of γδ T cells when expanded from bulk PBMCs. It is possible that NK cells that co-expand provide stimulatory signals to support γδ T cell proliferation as it has been shown that NK cells can form stable interactions with γδ T cells.[40] The observed preferential expansion of Vδ1 T cells over Vδ2 T cells over the culture period suggests that expanded Vδ1 T cells may be more resistant to activation-induced cell death or exhaustion, however, follow up studies are needed to confirm this. Alternatively, differences in the expression of 41BB between activated Vδ1 and Vδ2 T cells could also be a contributing factor since the interaction between 41BB and 41BBL is known to be a main driver of γδ T cell expansion.[18,41] While we observed an enrichment in γδ T cells across donors, a low level of αβ T cells persisted which may have contributed to observed effects in this study and increase the risk of GvHD. Therefore, incorporating an αβ T cell depletion step either prior to or during the expansion may be necessary for clinical application. We also observed a donor-dependent enrichment of Vδ1 T cells, with two donors exhibiting a prominent Vδ1-Vδ2- population. While factors such as exercise and the baseline Vδ1/Vδ2 ratio prior to expansion have been correlated with expansion potential of Vδ2 and Vδ1 T cells respectively, further characterization of donor characteristics is needed to delineate the cause of this variability.[42,43] Additionally, although this study focused on examining the effects of K562-mb-IL-21-based expansion in the absence of TCR stimulation, it is worth examining whether addition of Vδ1 TCR stimulation may increase the yield and purity of expanded Vδ1 T cells. Decreasing the length of the expansion process may be advantageous as long-term expansion could lead to γδ T cell exhaustion and potentially impair the expansion capacity of γδ T cells after adoptive transfer.

Phenotypic assessment showed that expansion enhanced γδ T cell expression of NK cell activation markers indicating improved anti-tumor function. Indeed, expanded γδ T cells exerted greater cytotoxicity against ovarian cancer cells than donor-matched freshly isolated γδ T cells. Expanded γδ T cells also highly expressed TIGIT and TIM3 exhaustion markers. The functional relevance of high TIGIT and TIM3 expression is not fully known as these markers are also upregulated on highly functional expanded NK cells and expanded Vδ1 T cells which have been characterized as having higher cytotoxicity than Vδ2 T cells.[16,42] Additionally, CD56 expression became upregulated with expansion, correlating with increased expression of NK cell activation receptors NKG2D, NKp30, and NKp44 but also NKG2A, CD158a, CD158b and co-inhibitory receptors, suggesting heighted activation status. Vδ1 T cells expressing CD158b possess cytotoxic activity against leukemic cell lines,[44] and while NKG2A expression in Vδ2 T cells has been associated with heightened cytotoxic effector functions, its role in mediating Vδ1 T cell anti-tumor activity remains poorly defined.[45] Future studies should investigate whether CD56-positive expanded γδ T cells display greater effector functions than their CD56-negative counterparts, as protocols that deplete CD56-expressing cells prior to expansion may remove γδ T cells with higher anti-tumor potential. To delineate the role of NK cell activation receptors and the γδ TCR in tumor cell recognition, we used blocking antibodies

against NKG2D, DNAM-1, and the γδ TCR on expanded γδ T cells prior to co-culture with tumor targets. Surprisingly, we saw no impact on γδ T cell cytotoxic function *in vitro* by blockade of these receptors alone or in combination.

While expanded γδ T cells displayed cytotoxicity against various solid tumor cell lines and significantly reduced tumor burden in an *in vivo* xenograft model, we surprisingly detected no to minimal CD107a and IFN-γ expression in response to tumor cells *in vitro*. However, stimulation with an IL-12, IL-15, and IL-18 cytokine cocktail revealed that expanded γδ T cells could express IFN-γ independently of tumor-mediated activation, albeit in a donor-dependent manner. Further, PMA/ionomycin stimulation also led to IFN-γ and TNF-α expression, demonstrating that TCR stimulation may lead to robust pro-inflammatory cytokine production. Other studies have similarly demonstrated that γδ T cells activated with zoledronate and IL-2 have low CD107a expression in response to stimulation with neuroblastoma cell targets despite showing high cytotoxicity against these cells.[31] Further, the serial killing ability of Vγ9 Vδ2 T cells is regulated by the co-localization of granzyme B and perforin and mainly occurs through a granzyme B-mediated mechanism.[46] Further investigation of the localization of lytic granules, perforin, and granzymes within expanded γδ T cells in response to tumor targets would enhance our understanding of their tumor killing mechanisms. Additionally, treatment of expanded γδ T cells with concanamycin A, a known inhibitor of perforin-mediated killing and degranulation, may be used to confirm whether their cell-mediated killing occurs through independently of lytic granule release.[30,47]

Maintaining cellular metabolic fitness within the nutrient scarce TME is needed for long-term tumor control against solid malignancies. To assess the ability of expanded γδ T cells to withstand the TME, we evaluated their metabolic functions under normal conditions and after prolonged exposure to patient-derived ovarian ascites fluid. To our knowledge this is the first time that the metabolism of K562-mb-IL-21 expanded γδ T cells has been investigated. We found that mb-IL-21-driven expansion enhances γδ T cell nutrient transporter expression from baseline. Further, expanded γδ T cells retained higher nutrient receptor expression in the presence of the malignant ascites TME, in contrast to freshly isolated γδ T cells and zoledronate activated Vδ2 cells which lost almost complete expression of CD71 transferrin receptor. Expanded γδ T cells also sustained their cytotoxicity, and glycolytic and oxidative phosphorylation capacity in the ascites TME, suggesting an ability to overcome metabolic immune suppression in the solid tumor. While expansion of Vδ1 T cells using a cytokine cocktail has shown to enhance their metabolic function, our protocol is advantageous as it does not necessitate the use of five different cytokines for activation.[48] Additionally, investigating the functional differences between mb-IL-21 expanded γδ T cells compared to expansion with mb-IL-15 is of interest. Studies with human NK cells have demonstrated that in addition to metabolic reprogramming, expansion with mb-IL-21 results in higher proliferation rates over long culture periods by inducing telomere lengthening.[22] Further, prolonged exposure to IL-15 has been shown to induce NK cell exhaustion characterized by lower *in vivo* anti-tumor efficacy and metabolic dysfunction.[39,49] Hence, mb-IL-21 may provide an alternative method of expansion to maintain sustained proliferation, metabolic fitness, and anti-tumor efficacy of γδ T cells.

Arming γδ T cells with CARs offers various advantages over conventional CAR-T cells including reduced antigen escape due to their innate and CAR-dependent killing mechanisms and a lower chance for GvHD without deletion of the TCR. To our knowledge, this study is the first to engineer expanded Vδ1 T cells with a HER2-targeted CAR containing a CD28 co-stimulatory domain and a CD3ξ intracellular domain using CRISPR/Cas9 and AAV. Using this method, CAR expression remained stable over several weeks of expansion, allowing efficient scale-up of the cells, which is crucial for a potential 'off-the-shelf' therapeutic product. Activation through the CAR induced expanded γδ T cell degranulation and greater *in vitro* killing against HER2+ breast cancer cells compared to the wild type controls. Similar results were seen *in vivo*, as HER2 CAR-Vδ1 T cells exerted the greatest tumor control in a HER2+ ovarian cancer xenograft model without eliciting toxicity. Previous studies have shown that αβ T cells expressing a CAR with the HER2 binding domain used in our study can cause fatal off-tumor toxicity due to cross-reactivity against healthy murine lung and heart tissues.[50,51] This toxicity was associated with high levels of production of pro-inflammatory cytokines involved in cytokine release syndrome, including IFN-γ and GM-CSF.[50,52] Further investigation is needed to compare the *in vivo* safety of CAR-Vδ1 T cells with CAR-αβ T cells, considering factors such as the route of administration, differences in CAR-mediated signaling, cytokine production profiles, and the potential for infiltration and expansion in healthy tissues. A limitation of this work is that

in vivo anti-tumor activity was assessed using xenograft models with tumor cell lines engrafted in immunocompromised mice which does not fully recapitulate the human tumor microenvironment and the pharmacokinetics of expanded γδ T cells was not assessed. This study opens avenues for future work to determine the optimal cell dosing regimen for anti-tumor efficacy, the *in vivo* persistence, and tumor and tissue trafficking of expanded γδ T cells.

Overall, our study demonstrates that K562 mb-IL-21 expanded Vδ1 T cells exhibit broad innate cytotoxicity which can be heightened in an antigen-specific manner with a CAR, while maintaining metabolic fitness in the immunosuppressive TME. These findings highlight their therapeutic potential as an 'off-the-shelf cell' therapy to target solid tumors.

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A.L.P and A.A.A conceived the project and designed the experiments. A.L.P and M.M performed the experiments. A.M, T.M.R, E.B, A.E.M, and M.S contributed to performing experiments. G.S and M.N.K generated the HER2 CAR Vδ1 T cells. D.A.L provided feeder cells used in the study. J.L.B provided the CAR construct used in the study. A.E.M, J. L.B, D.A.L, and M.N.K provided intellectual input. A.L.P and A.A.A wrote the manuscript. M.M, T.M.R, and E.B edited the manuscript. A.A.A secured the funding and supervised the project.

Author contributions

CRediT: **Ana L. Portillo:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft; **Misaal Mehboob:** Investigation, Validation, Writing – review & editing; **Genesis Snyder:** Investigation, Methodology; **Adnan Moinuddin:** Investigation, Writing – review & editing; **Tyrah M. Ritchie:** Investigation, Writing – review & editing; **Elizabeth Balint:** Investigation, Writing – review & editing; **Allyson E. Moore:** Investigation, Methodology; **Mohammadamin Sookhaklari:** Investigation; **Jonathan L. Bramson:** Methodology, Resources; **Dean A. Lee:** Methodology, Project administration, Resources; **Meisam Naeimi Kararoudi:** Methodology, Project administration, Resources, Supervision, Writing – review & editing; **Ali A. Ashkar:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Disclosure statement

MNK is a co-inventor of gamma delta T cell gene editing technology, he is also the co-founder and CSO of CARTx Therapeutics and owns stock in the company. All other authors declare no competing interests.

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Data and materials availability statement

All data and materials in the manuscript and supporting information can be made available upon reasonable request from the corresponding author.

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