A Simple and Robust Single-Step Method for CAR-Vδ1 γδT Cell Expansion and Transduction for Cancer Immunotherapy

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The γδT cell subset of peripheral lymphocytes exhibits potent cancer antigen recognition independent of classical peptide MHC complexes, making it an attractive candidate for allogeneic cancer adoptive immunotherapy. The Vδ1-T cell receptor (TCR)-expressing subset of peripheral γδT cells has remained enigmatic compared to its more prevalent Vγ9Vδ2-TCR and αβ-TCR-expressing counterparts. It took until 2021 before a first patient was dosed with an allogeneic adoptive Vδ1 cell product despite pre-clinical promise for oncology indications stretching back to the 1980s. A contributing factor to the paucity of clinical progress with Vδ1 cells is the lack of robust, consistent and GMP-compatible expansion protocols. Herein we describe a reproducible one-step, clinically translatable protocol for Vδ1-γδT cell expansion from peripheral blood mononuclear cells (PBMCs), that is further compatible with high-efficiency gene engineering for immunotherapy purposes. Briefly, γβTCR- and CD56-depleted PBMC stimulation with known-in-the-art T cell stimulators, anti-CD3 mAb (clone: OKT-3) and IL-15, leads to robust Vδ1 cell expansion of high purity and innate-like anti-tumor efficacy. These Vδ1 cells can be virally transduced to express chimeric antigen receptors (CARs) using standard techniques, and the CAR-Vδ1 exhibit antigen-specific persistence, cytotoxicity and produce IFN-γ. Practicable, GMP-compatible engineered Vδ1 cell expansion methods will be crucial to the wide-spread clinical testing of these cells for oncology indications.

Keywords: gamma delta (gammadelta) T cells, γδT cells, Vδ1 T cells, cancer immunotherapy, CAR-Vδ1 cells

INTRODUCTION

Characterized by expression of a T cell receptor (TCR) composed of gamma and delta chains (γδTCR), γδT cells are an innate-like subset of human T cells representing up to 15% of peripheral CD3-positive cells and up to 60% of intraepithelial lymphocytes in healthy donors. Whilst their physiological role in humans remains an area of active study and debate (1–3), γδT cells are a major
area of interest in adoptive cell therapy for oncology indications (4–7). Exome characterisation of >16,000 patient tumors identified infiltrating γδT cells as the immune cell species most positively associated with patient survival across all cancers (8). A recent examination of patient brain tumor samples further defined γδT cell infiltration as most predictive of patient survival, in unexpected contrast to αβT cells, which correlated negatively with survival (9).

Two subsets composed of MHC-unrestricted \( V_{\gamma V_{\delta}} \)-TCR and \( V_{\gamma V_{\delta 1}} \)-TCR-expressing cells (where \( x \) denotes one of 6 functional gamma chain genes) dominate the peripheral \( V_{\gamma V_{\delta}} \) cell compartment. In contrast to the oligoclonal and phosphoantigen-reactive \( V_{\gamma V_{\delta}} \)-TCR population, \( V_{\gamma V_{\delta 1}} \) cells (referred to hereafter as ‘\( V_{\delta 1} \) cells’) express a \( V_{\delta 1} \)-TCR chain paired with one of various \( \gamma \)-chains. The peripheral human \( V_{\delta 1} \) population has a polyclonal TCR repertoire that is reactive to a range of antigen types including peptides, lipids and various CD1 proteins of self and non-self origin (3, 10).

Adoptive transfer of the \( V_{\gamma V_{\delta}} \)-TCR cell subset has been clinically tested for anti-cancer efficacy for nearly 20 years (11). Several groups have also previously demonstrated methods to expand \( V_{\delta 1} \) cells (12–18). 2021 saw publication of data from two first-in-man \( V_{\delta 1} \) cell adoptive transfer clinical trials. GammaDelta Therapeutics Ltd are exploring unengineered, allogeneic ‘delta one’ T cell (‘DOT’; ‘GD012’ product) safety, tolerability, and preliminary antileukemic activity in patients with minimal residual disease-positive acute myeloid leukemia (trial ID: NCT05001451). Adicet Bio Inc are testing the safety and efficacy of ‘ADI-001’ product anti-CD20 CAR-engineered allogeneic \( V_{\delta 1} \) cells in adults with B cell malignancies, as a monotherapy or in combination with IL-2 (trial ID: NCT04735471). The discrepancy in the numbers of clinical investigations between \( V_{\delta 1} \) and \( V_{\gamma V_{\delta 2}} \) γδT cell subsets does not stem from a lack of pre-clinical promise of the \( V_{\delta 1} \) subset. Indeed, there is literature stretching back decades describing potent \( V_{\delta 1} \) cell responses against tumor targets in vitro and graft-versus-leukemic effects following bone marrow transplantation, hypothesized to be mediated by atypical T cell subsets (19–22).

The discovery that \( V_{\gamma V_{\delta 2}} \) cells can be expanded to high numbers and purity using phosphoantigens (e.g. IPP or BrHPP) or phosphoantigen-inducing aminobisphosphonates (e.g. zoledronic acid) enabled high-throughput \( V_{\gamma V_{\delta 2}} \) cell pre-clinical exploration, and consequently accelerated clinical translation. A promising two-step multi-cytokine clinical-grade protocol for \( V_{\delta 1} \) cell expansion was published and patented by Almeida and colleagues in 2016 (23) (referred to hereafter as the ‘DOT protocol’) and is set for clinical translation in trial NCT05001451. Herein we describe a one-step, single-cytokine gene-engineered \( V_{\delta 1} \) cell product manufacturing protocol that utilizes processes and reagents already employed to generate genetically modified αβT and \( V_{\gamma V_{\delta 2}} \) cell biotherapeutics. We show that \( V_{\delta 1} \) cells are readily expandable to high numbers and purity by stimulation of αβT-cell- and CD56-depleted PBMC with OKT-3 anti-CD3 mAb in the presence of IL-15-supplemented media. Thus-stimulated \( V_{\delta 1} \) cells are efficiently and stably transduced with a chimeric antigen receptor (CAR) using standard viral transduction protocols. The resulting \( V_{\delta 1} \)-CAR-T cells exhibit innate recognition of targets in addition to antigen-specific boosting of function, and do not exhibit alloreactivity to allogeneic PBMC.

**MATERIALS AND METHODS**

### Ethical Approval

Expansion of T cells from healthy donors was performed under the governance of the following UCL UK research ethics committee approvals: "Establishing cell cultures for pediatric cancers", IRAS project ID:154668. This ethical approval allows for expansion cell lines from tissue samples following written informed consent or from anonymized blood samples from healthy volunteers. For this study, only anonymized commercially available blood samples or anonymized small samples from healthy volunteers were used.

### γδT Cell Expansion

PBMC were isolated from purchased whole blood leucocyte cones via density gradient centrifugation using Lymphoprep (Stemcell) according to manufacturer’s instruction. PBMC were either cryopreserved in 90% FBS 10% DMSO or re-suspended in complete T cell culture media for further processing. Complete T cell culture media consisted of xenogen-free serum-free medium (Thermo Fisher) and 10% synthetic serum replacement (Thermo Fisher) and GlutaMAX (Thermo Fisher), all of which are available to research as well as GMP-grade from Thermo Fisher with the following product catalogue numbers: research-grade CTS-OpTmizer (A1048501) and GMP-compatible alternative GMP-grade OpTmizer-CTS (A3705003), synthetic immune cell serum replacement that is compatible with both manufacturing standards (A2596101) and GlutaMAX also compatible with both standards (35050061). If starting with cryopreserved material, PBMC were thawed and rested at 10x10^6 cells/mL in complete pre-warmed media overnight before further processing to avoid over-stressing the lymphocytes and to enhance depletion quality. PBMC at 2-4x10^6 cells/mL density were then either stimulated in standard cell culture plates right away or first depleted of αβT cells using the TCRγ/β Product Line (Miltenyi Biotec) according to manufacturer’s instructions concurrently with depletion of CD56-positive cells using CD56 MicroBeads (Miltenyi Biotec) according to manufacturer’s instructions. Briefly, cells were first labelled with anti-TCRγ/β-biotin, then a mix of anti-biotin microbeads and anti-CD56 beads, and then depleted using MACS Cell Separation LD Columns (Miltenyi Biotec). If cultured in G-Rex vessels (Wilson Wolf), depleted PBMC were initiated at 2-4x10^6 cells/cm^2. Thus-prepared PBMC were stimulated with either 1μg/mL OKT-3 (Miltenyi Biotec Cat# 130-093-387, RRID: AB_1036144) or 1μg/mL PHA (Merck) and various cytokine combinations: (i) 100 IU/mL IL-2 aldesleukin (Proleukin; Novartis), (ii) 70 ng/mL IL-15 (Peprotech), (iii) 20 ng/mL rhIL-7 (Peprotech), or the (iv) ‘DOT protocol’ cytokine
harvest, expanding cells were harvested, washed and labelled with
antibodies (Biorad) and further incubated with CD3 and CD28 anti-
mAbs (BioLegend Cat# 345023, RRID: AB_2564045) according to 
manufacturer’s instructions. All expansion and activation
samples were analyzed on a BD LSR II flow cytometer using
FACSDiva software (BD FACSDiva Software, RRID: SCR_001456), while CAR-V81 proliferation was analyzed on a
Beckman Coulter CytoFlex using CytExpert software (CytExpert
Software, RRID: SCR_017217). For setting of gates in analysis of
panels we employed fluorescence minus one (FMO) controls.
Post-acquisition data processing was carried out using FlowJo
software (FlowJo, RRID: SCR_008520). T-SNE analysis on flow
cytometry data was performed using FlowJo software and
concatenated using R language Statistical Computing (RRID:
SCR_001905).

**Vδ2 γδT Cell Depletion**

Vδ2 γδT cells were depleted from PBMC at one of three stages of
expansion: pre-initiation, at midway split or at harvest. All
depletions were done using anti-TCR/Vδ2 mAb clone B6
(BioLegend Cat# 331404, RRID: AB_1089228) at a concentration of
0.5µg/10⁶ PBMC. When depleting at initiation Vδ2 cell initiation
was incorporated into the ωTCR/CD56 depletion process. This
depletion was done as follows: PBMC were co-incubated with ωTCR-biotin
mAb and Vδ2 (clone: B6)-biotin mAb, washed, and then co-
incubated with anti-biotin and anti-CD56 microbeads according to
manufacturer’s protocol, then washed and depleted using
Miltenyi LD magnetic column separation, as above and according to
manufacturer’s protocol. If depleting at midway split or final
harvest, expanding cells were harvested, washed and labelled with
0.5µg clone B6/10⁶ PBMC, incubated for 20min, washed and
incubated and depleted using Miltenyi anti-biotin microbeads and
LD columns as above.

**Flow Cytometry**

The following fluorochrome-antibody conjugates and dyes were used
to detect different lymphocyte subpopulations in culture: Zombie Green Viability Dye (BioLegend), Zombie Yellow Viability Dye (BioLegend), LIVE/DEAD Fixable Near IR kit (Thermo Fisher), anti-CD3 PE/Dazzle594 (BioLegend Cat# 980006, RRID: AB_2715768), anti-ωTCR/CD56 APC (BioLegend Cat# 306717, RRID: AB_10612747), anti-TCRVδ1 APC-Vio770 (Miltenyi Biotec Cat# 130-120-440, RRID: AB_2752099), anti-TCRVδ2 VioBlue and PE (Miltenyi Biotec Cat# 130-101-152, RRID: AB_2660779), anti-CD69 FITC (BioLegend Cat# 310903, RRID: AB_314838), anti-NKG2D PerCP/Cy5.5 (BioLegend Cat# 320817, RRID: AB_2562791) anti-CD56 Alexa Fluor 488 (BioLegend Cat# 318311, RRID: AB_604094), anti-PD-1 APC/Fire750 (BioLegend Cat# 329953, RRID: AB_2616720) and BUV737 (BD Biosciences Cat# 612791, RRID: AB_2870118), anti-LAG-3 PE/Cy7 (BioLegend Cat# 369309, RRID: AB_2629752), anti-TIM-3 BV711 (BioLegend Cat# 345023, RRID: AB_2564045), anti-CD34 QBend10 Alexa Fluor700 (BioTechnie), anti-CD34 QBend10 Alexa Fluor488 (Novus Biologicals), anti-NKp44 PerCP/Cy5.5 (BioLegend Cat# 325114, RRID: AB_2616752), anti-NKp30 DyLight 650 (NovusBio, Cat# FAB1849W, clone 210845). When detecting intracellular and cell surface accumulation of
IFN-γ and CD107a, respectively, PBMC were challenged with
relevant targets overnight, and incubated at 37°C and 5% CO₂ with anti-CD107a FITC (BioLegend Cat# 328605, RRID: AB_1186058), then 1x monensin (Biolegend) was added followed by incubation for another 4h, stained for cell surface markers, and then permeabilized using Biolegend Intracellular Staining Permeabilization Wash Buffer and stained with anti-
IFN-γ Brilliant Violet 605 (BioLegend Cat# 502535, RRID:
AB_11125368), anti-IL17a PerCP/Cy5.5 (BioLegend Cat# 512313, RRID: AB_961397) or anti-Granzyme B Pacific Blue
(BioLegend Cat# 372217, RRID: AB_2728384) according to
manufacturer’s instructions. All expansion and activation
samples were analyzed on a BD LSR II flow cytometer using
FACSDiva software (BD FACSDiva Software, RRID: SCR_001456), while CAR-V81 proliferation was analyzed on a
Beckman Coulter CytoFlex using CytExpert software (CytExpert
Software, RRID: SCR_017217). For setting of gates in analysis of
panels we employed fluorescence minus one (FMO) controls.
Post-acquisition data processing was carried out using FlowJo
software (FlowJo, RRID: SCR_008520). T-SNE analysis on flow
cytometry data was performed using FlowJo software and
concatenated using R language Statistical Computing (RRID:
SCR_001905).

**Retroviral Production and T Cell Transduction**

293T cells (ATCC Cat# CRL-3216, RRID: CVCL_0063) were
plated at 1.5x10⁶ cells per 10cm² plate (Corning) in 10mL 10%
fetal bovine serum (FBS)-supplemented Gibco IMDM (Thermo
Fisher). At 70% confluence, 293T cells were transfected using
GeneJuice (Merck) according to manufacturer’s protocol. Triple
plasmid transient transfection was carried out using SFG-
gammaretroviral vectors (RRID: Addgene_22493). The anti
B7H3 CAR-T was synthesized within SFG and contains the
following components: IL-2 signal peptide, TE9 ScFv, CD8 hinge
and transmembrane, CD28 endomain, CD3zeta. The CAR was
co-expressed with the RQR8 sort suicide gene allowing
detection with anti-CD34 antibody.

The B7H3-CAR (second generation with CD28 and 325 CD3-
zeetas endomains synthesized in our laboratory), gag-pol 326
(RRID: Addgene_4499) and RD114 envelope (RRID: 327
Addgene_17576) plasmids were added at an equimolar ratio.
Retroviral supernatant was harvested at 48 and 72 hours
following transfection and used immediately for T cell
transduction. Briefly, non-tissue culture treated 24 well plates
(Costar) were coated with RetroNectin (Takara) in PBS
(final concentration of 1mg/mL) and incubated at 4°C for 24 hours.
The retronectin was removed and 1.5 mL of retroviral
supernatant was added to each retronectin coated well.
Following this, 3x10⁵ stimulated T cells in 500 µL was added
and plates were centrifuged at 1000 x g for 40 minutes, at room
temperature before incubation in complete T cell culture media
at 37°C, supplemented with IL-15 to a final concentration of
70ng/mL (~140 IU/mL). Transduced T cells were harvested after
three days, washed and re-suspended for expansion in specified
cytokine-supplemented complete T cell culture medium.
FIGURE 1 | Continued
Transduction efficiency was assessed by flow cytometric detection of the CD34 marker gene (26).

Cytotoxicity Assays
Cytotoxicity was determined either by staining for cell surface accumulation of CD107a as above where indicated, or by four-hour chromium ($^{51}$Cr)-release assay. Briefly, 1x10$^6$ target cells were labelled with 20 µl $^{51}$Cr amounting to 3.7 MBq (PerkinElmer) for 60 minutes at 37°C. Following this, target cells were co-cultured with effector CAR T cells at ratio of effector: target (E:T) ratios (10:1, 5:1, 2.5:1 and 1:2.5:1) for four hours at 37°C in 96 well U-bottom plates (Grenier). After incubation, the plates were centrifuged at 1500rpm for 5 minutes and 50 µl of the supernatant was transferred to 96 well OptiPlate-96 HB (PerkinElmer). 150 µl of scintillation fluid was added per well and the plates were sealed and incubated at room temperature overnight. $^{51}$Cr release from lysed target cells was counted on 1450 MicroBeta Trilux Scintillation Counter (PerkinElmer). The scintillation counts from wells with only target cells were counted as spontaneous release and target cells lysed with 1% Triton X-100 (ThermoFisher) were used as a maximum $^{51}$Cr release control.

Proliferation Assay
Proliferation of expanded and harvested V81 cells following repeated stimulation was evaluated to determine CAR-V81 persistence in the presence of an antigen-expressing target cell line. Briefly, CAR-V81 cells were labelled with CellTrace Violet proliferation dye (ThermoFisher) according to manufacturer’s instructions for 20 minutes at 37°C. Once labelled, CAR-V81 cells were plated at 5x10$^5$ per well of a 48-well plate (Corning) and co-cultured at a 1:1 E:T ratio with irradiated tumor targets, either B7H3-negative Jurkat wild type cells (Jurkat-WT) or isogenic Jurkat cells transduced to express high levels of B7H3 (Jurkat-B7H3). Plates were incubated at 37°C and 5% CO2 for 6 days, without exogenous cytokine supplementation. Freshly irradiated target cells were fed every two days following coculture and proliferation was evaluated by flow cytometry.

Cell Lines
Jurkat (ATCC Cat# TIB-152, RRID: CVCL_0367), HeLa (ATCC Cat# CCL-2.2, RRID: CVCL_0058), NOMO-1 (DSMZ Cat# ACC-542, RRID: CVCL_1609), K562 (ATCC Cat# CCL-243, RRID: CVCL_0004) and U87 (ATCC Cat# HTB-14, RRID: CVCL_0022) cell lines were all acquired from ATCC and cultured as recommended by the supplier. RRID: CVCL_0004) and U87 (ATCC Cat# HTB-14, RRID: CVCL_0022) cell lines were all acquired from ATCC and cultured as recommended by the supplier. Cell lines were screened monthly for mycoplasma contamination Briefly, Jurkat, K562 and NOMO-1 cells were grown in 10% FBS-supplemented RPMI1640 (Sigma Aldrich) suspension culture and kept at <1x10$^5$/mL density. LAN-1, HeLa and U87 cell lines were grown in 10% FBS-supplemented DMEM (Thermo Fisher) adherent culture and split regularly at around 80-90% confluence using trypsin (Thermo Fisher)-based disaggregation, to avoid overgrowth.
isoform of B7-H3 was purchased (Sinobiological) and cloned into a γ-retroviral expression cassette. Retroviral transduction was used to stably transduce Jurkat cells with 4Ig-B7-H3.

**Statistical Design**

Data was analyzed with GraphPad Prism (GraphPad Prism, RRID: SCR_002798). Data are displayed at mean ± SEM unless otherwise stated. For normally distributed numerical data, parametric tests were used to determine significance of difference between groups. Analysis of variance (ANOVA) was used, unless otherwise stated. Significance is represented by: *p<0.5, **P<0.01, ***p<0.001, ****p<0.0001.

**Calculation of Earth Mover’s Distance (EMD)**

EMD describes change in signal strength based on difference in probability distribution, with a higher EMD denoting a larger change. The use of EMD to describe changes in protein accumulation allows multiple biological replicates to be characterized with a high degree of consistency, without collapsing the data to mean or median values at the expense of interpretability (24). EMD was computed between bulk CAR-transduced T cell culture versus non-transduced culture. Samples were time- and donor-matched. EMD was calculated between T cell populations that had undergone the same processing. The Python (Python Programming Language, RRID: SCR_008394) module ‘wasserstein_distance’, which is a component of ‘scipy.stats’, was used to calculate EMD between samples.

**RESULTS**

**OKT-3 and IL-15 Stimulation of αβTCR- and CD56-Depleted PBMC Leads to Robust and Reproducible Vδ1 Cell Expansion**

Benchmarking our efforts to the two-step and multi-cytokine ‘DOT protocol’ [described in detail by Almeida and co-workers (23)], we compared its ability to expand Vδ1 cells with canonical ex vivo T cell expansion methodology, consisting of a single step PBMC stimulation with clone OKT-3 anti-CD3 mAb and IL-2 at 100 IU/mL. Briefly, the ‘DOT protocol’ entails a first 10 day culture in OKT-3 with IL-4, IFN-γ, IL-21 and IL-1β, followed by a second culture in OKT-3 with IL-15 and IFN-γ. Over 20 days of expansion, the ‘DOT’ cocktail of cytokines yielded a mean 100-times more Vδ1 cells than culture in IL-2 following activation of unsorted PBMC with a single dose of OKT-3 at initiation (Figure 1A). The inferiority of IL-2 monoculture to the ‘DOT’ cocktail of cytokines is consistent with what was reported in the original ‘DOT’ protocol publication (23). In pursuit of an allogeneically-applicable expansion protocol that generates a product without potentially alloreactive αβT cells, we then depleted the starting PBMC of αβT cells using a standard and GMP-compatible αβTCR-biotin and anti-biotin bead-based protocol from Miltenyi. Aside from removing contaminating αβT cells, αβTCR-depletion further enhanced the yield of Vδ1 cells following 20 day culture in the ‘DOT’ cocktail of cytokines with a single dose of OKT-3 at initiation (Supplementary Figures 1A, B). This may be at least in part be due to the removal of αβT cell competition for cytokines.

We then investigated, in the context of the DOT cocktail of cytokines, whether we could expand Vδ1 cells more efficiently by using a more specific γδTCR stimulus, such as an anti-γδTCR mAb (clone: B1) or specific anti-Vδ1-TCR mAb (clone: TS-1). A single stimulating 1 μg/mL mAb dose at initiation has been previously reported to be effective for TS-1/B1 mAb-driven Vδ1 cell expansion (25). Anti-CD3 OKT-3 stimulation led to an order of magnitude higher Vδ1 cell expansion from non-depleted PBMC than either γδT cell-specific clone (Figure 1B). Moreover, specific anti-γδTCR stimulation applied to non-depleted PBMC failed to prevent expansion of contaminating αβT cells in culture (Supplementary Figure 1C). Vδ1 cell numbers in DOT cytokine cocktail culture were evaluated and found equivalent between single stimulation with OKT-3 at initiation or repeated OKT-3 stimulation at 5 day intervals over the course of expansion, suggesting that a single OKT-3 administration is sufficient for optimal T cell expansion (Supplementary Figure 1D). As a result, αβTCR-depletion and a single stimulation with OKT-3 anti-CD3 mAb were progressed for further study.

We next evaluated Vδ1 cell expansion in this culture setup using either the two-step DOT cocktail of cytokines or continuous culture in 70ng/mL (corresponding to ~140 IU/μL) of IL-15 alone, it also being a component of the latter half of the DOT protocol regimen. Having discarded IL-2 alone as an optimal milieu, IL-15 was chosen as the second most commonly-employed GMP-compatible T cell manufacturing mitogen. Unexpectedly, IL-15 monoculture yielded at least equivalent or higher Vδ1 cell numbers to the DOT cocktail of cytokines (Figure 1C). We next examined whether IL-15-driven Vδ1 cell expansion could be improved by further depleting competition for cytokine from NK cells. We first confirmed that freshly-isolated PBMC Vδ1 cells do not express canonical NK cell-marker, CD56, while CD3-negative freshly-isolated PBMC and some Vδ2 cells do (Supplementary Figure 1E). We then combined the αβTCR-biotin and anti-biotin depletion step with GMP-compatible Miltenyi anti-CD56 magnetic beads according to manufacturer’s protocol. Three donor concatenated t-SNE analysis of culture initiation material demonstrates the difference between undepleted and αβTCR/CD56-depleted freshly-isolated PBMC (Figure 1D). The double-depleted material is predominantly CD3-negative, though with enriched Vδ1 and Vδ2 composition relative to undepleted PBMC.

Undepleted, αβTCR- and αβTCR/CD56-depleted PBMC starting material was then compared for its ability to expand Vδ1 cells when stimulated with IL-15 and OKT-3. Double-depleted PBMC yielded not only substantially greater Vδ1 cell numbers, but also purity (Figures 1E, F). Of note, no substantive differences in product composition from any of the starting materials could be found when comparing IL-15 monoculture with culture in the DOT cocktail of cytokines (Figure 1F). We, therefore, progressed a single-step OKT-3 + IL-15-based...
αβ TCR/CD56-depleted Vδ1 expansion protocol for further optimization. We note that in this setup, a majority of donor αβ TCR/CD56-depleted PBMC initially plated at 1x10^6 cells/cm^2 approached over-confluence by day 10 of culture (Supplementary Figure 1F). We, therefore, opted for a 1:4 culture split midway through the protocol (Figure 1G). While feasibly the cells can be cultured for shorter or longer periods as per desired product specification, we progressed a 20-day expansion period with a midway split for further analysis.

We benchmarked our OKT-3 and IL-15-based single step protocol against other published single step Vδ1 expansion methods that utilize phytohaemagglutinin (PHA) instead of anti-CD3 mAb (16–18). We stimulated αβ TCR- and CD56-depleted PBMC with OKT-3 and IL-15, or PHA with either IL-2 (16, 17) or IL-7 (18). OKT-3 with IL-15 outperformed both PHA-based protocols in terms of Vδ1 yield in all donors tested (Supplementary Figures 2A-D). The choice of anti-CD3 stimulation was further re-enforced by data indicating that, at harvest, OKT-3-stimulated Vδ1 cells expressed higher activation marker levels with concurrently lower exhaustion markers than PHA-stimulated Vδ1 cells, all the while expressing more NKG2D and CD56 receptors, indicative of favorable functional phenotype (Supplementary Figure 2E). Indeed, PHA-stimulated Vδ1 cells expressed higher apoptotic markers than CD3-stimulated Vδ1 cells at harvest (Supplementary Figure 2F).

Encouragingly for clinical practicality, OKT-3 with CD56 expanded not only freshly-isolated PBMC, but also Vδ1 cells from thawed cryopreserved PBMC that were αβ TCR-/CD56-depleted following an overnight rest upon resuscitation (Supplementary Figures 3A, B). We note that an overnight PBMC ‘rest and recovery’ step in complete media at standard culture conditions enabled retention of a pre-cryopreservation Vδ1/Vδ2 cell ratio (Supplementary Figure 3C), and substantially increased the quality of αβ TCR-/CD56-depletions as well as Vδ1 cell expansion. Resting was carried out at a high cell density (10^6 PBMC/mL) in complete expansion media, without cytokine supplementation.

To simulate a potential manufacturing process, we compiled expansion data of six arbitrarily chosen healthy donor cryopreserved leukapheresate-derived PBMC from two experimental runs, each of which consisted of PBMC thaw and cryopreserved leukapheresate-derived PBMC from two experimental runs, each of which consisted of PBMC thaw and overnight rest in complete media, followed by αβ TCR/CD56-depletion and OKT-3/IL-15 stimulation the following day, as described above, except that in this iteration Vδ1 cells were cultured in 6-well G-Rex (as opposed to standard cell culture) vessels. Expansions were split into new wells at a 1:4 culture surface area ratio on day 10 of expansion and harvested at day 20 for analysis. Out of six donors tested, three achieved >1,000-fold Vδ1 cell expansion, and all achieved >400-fold expansion (Figure 1H). While in every donor examined Vδ1 cell expansion rate was greater than that of Vδ2 cells, in five out of six donors the difference was minimal suggesting a relatively unbiased γδ T subset expansion by OKT-3/IL-15 (Figure 1H).

The total Vδ1 cell yield per harvested 6-well G-Rex well was >2x10^8 Vδ1 cells per 4x10^8 PBMC initiated in three out of six donors tested, delineating apparent ‘good’ and ‘poor’ expanders (Figure 1I). These donors further clustered by product composition. While all yielded 80-100% pure γδ T cells, γδ T cell composition varied greatly (Figure 1J). γδ T cell contamination was negligible, though some CD3-negative cells (mostly NK cells) persisted (Figure 1J). We investigated γδ T cell product composition further and found that in all G-Rex-expanded donor products, an apparently inverse relationship existed between high purity Vδ1 donors and Vδ2 donors (Figure 1K).

The relatively unbiased subset expansion by OKT-3/IL-15 we observed, we interrogated whether a high-purity (or ‘good’) Vδ1 donor could be predicted by examining the undepleted leukapheresates of donors entered for expansion.

The pre-depletion donor PBMC Vδ1:Vδ2 ratios were compared in six donors and correlated to Vδ1 cell purity and total Vδ1 cell count after 20 day stimulation of αβ TCR/CD56-depleted PBMC with OKT-3 and IL-15. In these donors, a pre-initiation Vδ1:Vδ2 ratio of greater than 0.4:1 was associated with at least 50% Vδ1 cell purity at harvest (R^2 = 0.74) (Figure 1L). The relationship between Vδ1:Vδ2 ratio and absolute Vδ1 cell yield was also investigated. We observed, however, that with excluding one donor from analysis for yield (indicated in red in Figures 1L, M), high Vδ1:Vδ2 ratio at initiation correlated with high Vδ1 cell yield at harvest in this small sampling of independent donors. A minimum pre-initiation Vδ1:Vδ2 ratio of 0.2:1 was associated with harvests of >2x10^8 Vδ1 cells per 4x10^8 PBMC initiated (R^2 = 0.79) (Figure 1M). We hypothesize, therefore, that a high Vδ1:Vδ2 ratio at initiation of expansion may serve as a biomarker for high ultimate Vδ1 cell yield and purity at harvest, though more donor material screening is required to substantiate this observation.

We next examined whether αβ TCR/CD56-depleted OKT-3/IL-15-stimulated product can be further enriched for Vδ1 cells by depleting contaminating Vδ2 cells. To this end, we identified three potential depletion points during the manufacture: at initiation concurrently with αβ TCR/CD56-depletion, midway at the split, or at harvest (Figure 1N). First examining the initiation depletion strategy, we compared the following depletions for Vδ1 cell purity among freshly-isolated PBMS, (i) αβ TCR,(ii) αβ TCR/CD56, and (iii) αβ TCR/CD56/Vδ2. The triple depletion was performed as follows: PBMC were co-incubated with αβ TCR-biotin mAb and Vδ2 (clone: B6)-biotin mAb, washed, and then co-incubated with anti-biotin and anti-CD56 microbeads according to manufacturer’s protocol. Each depletion step further increased Vδ1 cell purity among the initiation T cell compartment (Figure 1O). Not only was the clone B6 Vδ2-depletion highly effective at the outset, it also prevented re-growth of Vδ2 cells during expansion (Figure 1P). Though, it also encouraged non-Vδ1/Vδ2 γδ T cell expansion, resulting in a Vδ1: non-Vδ1/Vδ2 γδ T cell ratio of ~ 2:1. Product Vδ2 depletion midway was highly efficacious; at harvest it yielded a ~77% pure Vδ1 cell product, with a ~17% non-Vδ1/Vδ2 γδ T cell presence (Figure 1Q). Product depletion at harvest yielded a similar purity of ~72% Vδ1 cells and 20% non-Vδ1/Vδ2 γδ T cells (Figures 1R, S). Non-γδ T cell content was ~10% in all methods tested and was largely CD3-negative. A majority of these were NK cells (CD3-negative/CD56-positive PBMC).
(Supplementary Figure 4B), that we hypothesize either escaped initial depletion or upregulated CD56 during expansion. We note that, while initially negative, also 50-70% of Vδ1 cells upregulated CD56 upon expansion (Supplementary Figure 4C), negating the possibility of a CD56-based contaminant depletion at harvest.

Of the Vδ2 cell depletion strategies tested, we hesitate to recommend the best, nor indeed whether it is required at all - as optimal product specifications in terms of γδT cell subset purity for maximal therapeutic efficacy are yet to be determined. It is not necessarily the case that the purest Vδ1 cell product is the most efficacious against cancer, and it is feasible that other γδT cell subsets in the product will synergize rather than suppress Vδ1 cell anti-cancer functionality. Substantial further study in this area is required. Other factors that will impact the decision on Vδ2 cell depletion include post-harvest processing, such as...
FIGURE 3 | Continued
intention to cryopreserve, etc. The remainder of the functional data in this study is presented on Vδ1 cell products derived from double (αβTCR/CD56)-depleted PBMC.

We note that the above depletions could be reproduced to GMP-standard by replacing research-grade αβTCR and CD56 depletion reagents with GMP-grade alternatives from Miltenyi Biotec (CliniMACS TCRα/β Product Line cat nr: 200-070-407; CliniMACS CD56 Product Line cat nr: 170-076-713) and carried out on either CliniMACS Plus or CliniMACS Prodigy hardware. We note the lack of commercially-available GMP-compatible clone B6 Vδ2-biotin products, though anticipate that those could be obtained from suppliers through custom manufacture.

**OKT-3/IL-15-Expanded Vδ1 Cells Are Innately Cytotoxic Against a Range of Tumor Targets**

20-day-expanded OKT-3/IL-15 Vδ1 cells exhibited a similar memory and exhaustion profile to DOT cytokine cocktail counterparts. As indicated in concatenated t-SNE plots of various markers, Vδ1 cells were broadly positive for CD27, with a subpopulation brightly expressing CD45RA. While a proportion of CD27+/CD45RA− cells expressed PD-1, few Vδ1 cells bound anti-LAG-3 antibody above isotype control (Figures 2A, B). Nearly all Vδ1 cells were dimly but universally TIM-3-positive. OKT-3/IL-15-expanded Vδ1 cells further upregulated activation marker CD69 as well as cytotoxic differentiation marker NKG2D, but not Nkp44 (Figures 2C, D). A small subpopulation of expanded Vδ1 cells consistently upregulated Nkp30.

Functionally, OKT-3/IL-15-expanded Vδ1 cells exhibited highly consistent innate cytotoxicity against a range of hematological and solid tumor targets, including T cell leukemia Jurkat cells, cervical cancer HeLa cells, neuroblastoma LAN-1 cells, chronic myelogenous leukemia K562 cells and acute myeloid leukemia NOMO-1 cells (Figure 2E). This is of note, as donors were not specifically selected for only high Vδ1 cell purity, but rather represented a range of Vδ1-subset compositions (a range of harvested αβTCR/CD56-depleted PBMC-derived products is illustrated in Figure 2F). This suggests that maximal Vδ1 cell purity does not uniquely determine the cytotoxic potential of the OKT-3/IL-15-expanded product.

**OKT-3/IL-15-Expanded Vδ1 Cells Are Readily Transducible With Chimeric Antigen Receptors (CAR)**

To assess the suitability of this expansion protocol for generating genetically-modified immunotherapeutics, we evaluated Vδ1 cell retroviral transduction with an anti-B7H3 2nd generation 28ζ chimeric antigen receptor (CAR) (Figure 3A). A consistent ~50% transduction efficiency (ranging from 35.8% - 79.1%) was achieved transducing nine different donors in three experimental runs (Figure 3B).

We queried the impact of viral transduction with an ITAM-containing CAR on OKT-3/IL-15 Vδ1 cell product by comparing expression of a range of memory, exhaustion and functional markers within the transduced cell population. Cells were transduced on day 3 following initiation, and thereafter expanded for an additional 17 days until harvest at day 20. Anti-CD34 staining was used to detect expression of the RQR8 CAR marker gene (26) in the transduced cell product. Unexpectedly, none of the activation, memory or exhaustion markers we tested mapped neatly onto CAR CD34+ Vδ1 cells (Figure 3C). The closest matches were increased expression of NKG2D and a dim but consistent association of CD34 with PD-1 expression in CAR-Vδ1 compared to unmodified Vδ1 cells. Curiously, there was little association between CD69 and CD34 in any of the donors tested, suggesting that the Vδ1 cell product was highly activated regardless of CAR expression. The most notable difference between CAR-transduced Vδ1 cells as a whole compared to OKT-3/IL-15 Vδ1 cells that were never exposed to retrovirus (Figure 2A) was the downregulation of CD27 in virus-exposed compared to non-exposed cells. Most other queried markers were similar between both populations.

CAR-Vδ1 were then tested against a range of antigen-positive and negative hematological and solid tumor targets: B7H3-negative Jurkat cells, and B7H3-positive cell lines U87 (originating from glioblastoma) and LAN-1 cells (Figure 3D). Intracellular IFN-γ and cytotoxic degranulation cell surface
marker CD107a accumulation was compared in CAR-transduced versus unmodified VØ1 cells using flow cytometry (Figure 3E). The red line in Figure 3E indicates marker median fluorescence intensity (MFI) of unmodified target-free VØ1 cells, while the blue line indicates the MFI of target-free CAR-VØ1 cells. These measures are included to account for the innate, B7H3-independent reactivity of VØ1 cells, as well as potential baseline activation mediated by CAR-transduction. Differences between histograms were quantified using the statistical analysis tool Earth Mover’s Distance (EMD), which quantifies the dissimilarity between two dimensional distributions whilst respecting the single-cell nature of the dataset; a greater value of EMD indicates greater difference (see methods). EMD scores were generated measuring the difference between bulk VØ1 cell IFN-γ or CD107a accumulation when either unmodified or transduced with a B7H3-28ζ-CAR and challenged with different tumor targets. An EMD score of 0 indicates no relative change between transduced and non-transduced VØ1 cells. Interestingly, while CAR-VØ1 CD107a-mediated cytotoxic degranulation was significantly higher upon challenge with antigen-positive U87 and LAN-1 targets than without challenge or challenge with antigen-negative Jurkat cells, IFN-γ production was less consistently impacted by the presence of target antigen and highly variable on a donor-donor basis (Figure 3F). This inconsistency was caused not by the inability of CAR engagement to mediate IFN-γ production, but rather high innate and non-CAR-dependent IFN-γ production in some of the donors. All donor VØ1 cells demonstrated intracellular IFN-γ with and without CAR transduction. A significant, antigen-dependent upregulation of intracellular IFN-γ could be observed when gating on specifically CAR-positive VØ1 cells, rather than bulk VØ1 cells in culture (Figure 3G). IFN-γ production correlated positively with VØ1 cell CAR marker gene, CD34, expression when challenged with antigen-positive but not negative targets (Supplementary Figure 5). Consistent with a high and sustained cytotoxic potential, granzyme B levels were at least as high or higher in matched unmodified VØ1 cells compared to CAR-VØ1 cells before and after challenge with targets (Figure 3H).

To test proliferative and persistence capacity, CAR-VØ1 were harvested post-expansion, plated with no exogenous cytokine and challenged twice at a 1:1 E:T ratio at three day intervals with irradiated B7H3-negative Jurkat wild type cells (Jurkat-WT) or isogenic Jurkat cells transduced to express B7H3 (Jurkat-B7H3) (Figures 3I, J). Expansion was monitored via dilution of CellTrace Violet proliferation dye, as well as cell counts performed using Precision Count beads and flow cytometry. While all CAR-VØ1 were highly activated and continued lower-grade proliferation after re-plating, more proliferation was seen upon challenge with Jurkat-B7H3 compared to no targets or Jurkat-WT (Figure 3K). The black line in Figure 3K indicates the CellTrace Violet MFI of VØ1 cells at plating, the red line of effectors only after 6 days in culture, and the blue line – of effectors co-cultured with antigen-positive targets. Normalized to effectors only, VØ1 cells expanded more when expressing a CAR but only in response to antigen-positive Jurkat cells (Figure 3L).

Unmodified VØ1 cells expanded ~2-fold over target-free matched effectors, likewise CAR-VØ1 in response to Jurkat-WT. In response to Jurkat-B7H3, meanwhile CAR-VØ1 cells expanded 4-fold.

**DISCUSSION**

We set out to develop a single-step, GMP-compatible CAR-VØ1 cell expansion and transduction protocol that utilizes standard T cell therapy expansion reagents already employed in the CAR-T field. To that end, we focused on pan-T cell stimulating anti-CD3 mAb, clone OKT-3, and the classic T cell cytokine expansion milieu of IL-2 and IL-15. While OKT-3 with IL-2 failed to support sufficient VØ1 cell expansion, OKT-3 with IL-15 led to substantial VØ1 cell expansion that was further boosted by depletion of CD56-positive cells. The additive effect of CD56-positive cell depletion was likely at least in part mediated by decreasing competition for IL-15 from CD56-expressing PBMC, such as NK cells. Given the pan-T cell stimulatory nature of both OKT-3 and IL-15, stringent αβT cell depletion prior to initiation was obligate for achieving VØ1 cell yield and purity. αβTCR/CD56-depleted OKT-3/IL-15-stimulated VØ1 cells were highly tumor-reactive in their own right and amenable to transduction to high efficiency with a second generation B7H3-28ζ CAR using standard retroviral protocols. CAR-VØ1 cells retained innate tumor responsiveness while also engaging in CAR-directed reactivity. Upon challenge with targets, B7H3-28ζ-VØ1 exhibited antigen-specific persistence, cytotoxicity and IFN-γ production. Taken together, we have described a fully GMP-compatible CAR-VØ1 manufacturing protocol that utilizes reagents and processes well practiced in the CAR-T field.

We further examined the additional purification of VØ1 cell product with V62 cell depletion. V62 cells were effectively removable using anti-V62TCR mAb clone B6 conjugated to biotin, magnetically removed with anti-biotin microbeads. These depletions could be successfully carried out at initiation of culture with a triple αβTCR/CD56/V62 depletion, midway through depletion at culture split or at harvest. We reserve judgement as to the best approach in this instance, or whether V62 cell depletion is required at all. We hypothesize that an ultra-pure VØ1 cell product may not exhibit improved efficacy over a product that contains other γδT cell populations. Though, this warrants substantial further investigation with a range of donors. Indeed, it will be difficult to assess optimal product composition until such products are tested clinically. As the debate for “which γδT cell subset is best?” pervades the immunotherapy field, we expect that only clinical testing and conscientious and scientific clinical trial design will shed light on these questions. Ultimately, it may be that no single subset is superior, but rather that a correct balance of the different subsets is optimal for anti-cancer targeting.

In developing the optimized protocol described herein we used the “DOT protocol” cytokine cocktail described by Almeida and colleagues (23) as a comparator, investigating whether we can design a simplified process. VØ1 cell yield and phenotype
were broadly similar between cells expanded with either OKT3/IL-15 or the “DOT protocol” cocktail of cytokines. There are four main modifications in our process compared to the published “DOT protocol”: (1) a simultaneous αβTCR- and CD56-bead depletions step replaces the αβTCR-depletion only, (2) the “DOT protocol” employs a second OKT-3-based CD3 positive selection step while our protocol adds OKT-3 to the depleted product without the need for a second selection step, 3) the multi-cytokine cocktail of the “DOT protocol” is replaced by IL-15 alone, 4) a second OKT-3 stimulation in the “DOT protocol” midway through expansion in omitted in our protocol. Together these changes represent a considerable simplification of the Vδ1 cell expansion process, and a reduction in cost. It was beyond the scope of the current study to perform a detailed side-by-side comparison in terms of in vitro and in vivo effector function. Further studies are warranted to compare the long-term effector function between these approaches.

We anticipate an increase of pre-clinical and clinical gene-engineered Vδ1 cell investigations for oncology indications in what is a rapidly evolving immunotherapeutic landscape. With the clinical success of canonical autologous CAR-αβT for a range of B cell malignancies, a role may be carved out for allogeneic non-cannonical cell therapies. This includes γδT cells of Vδ1 and Vγ9Vδ2 subsets, as well as NK cells, for the targeting of solid tumor indications and CAR-αβT refractory hematological cancers. Allogeneic approaches of this type may further play an important role in democratizing access to a new generation of gene-engineered cell therapy drugs that can be manufactured in bulk from healthy donor material, with accompanying reductions in price and supply chain complexity, as well as possible improvement in product clinical efficacy.

An important area of ongoing research remains the identification of ‘optimal’ donors for allogeneic cell therapy products. It remains unclear whether high product yield during manufacture is a sure indicator of maximum therapeutic performance, or as recent data from the CAR-αβT field suggests (5) – that cell ‘quality’, including memory and exhaustion status, is a more predictive metric than quantity. The elucidation of the factors that govern γδT cell product ‘quality’ will be crucial to sustained clinical success. Vδ1 cells expanded with this one-step IL-15/OKT-3 process expressed high CD27 and CD45RA, in a pattern that is consistent with naïve and central memory in γδT cells and was diminished upon transduction with B7H3-28Z-CAR. It is unclear whether this marker expression profile correlates with γδT-like memory phenotypes in Vδ1 cells. Indeed, relatively little is known of γδT cell memory, and less still how such cell surface marker phenotypes correlate with anti-tumor functionality. Expanded and CAR-transduced Vδ1 cells weakly upregulated PD-1 and strongly upregulated TIM-3 ‘exhaustion’ markers, the significance of which on γδT cells is little understood. It is unclear whether their presence is indicative of true T cell exhaustion, activation or something other still.

These properties may further vary between the types of indications targeted and gene engineering applied. Intelligent clinical trial design and study of adoptively transferred γδT cells pre- and post-infusion into patients will be crucial in elucidating the specific qualities of cells that confer the greatest therapeutic benefit.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical approval was granted by the UCL UK research ethics committee under IRAS project ID-154668.

AUTHOR CONTRIBUTIONS

MB and JA designed the experiments and wrote the manuscript. GF, CA, MF, and SD performed the data-generating experiments for this paper. JF provided data analysis. KC co-supervised CA. All authors contributed to the article and approved the submitted version.

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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.2022.863155/full#supplementary-material
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Conflict of Interest: MF and SD are employed by TC BioPharm Ltd. JA and JF are both inventors on a patent pertaining to CCRs in γδT cells, which was licensed to TC Biopharm (WO/2016/174661). JF has undertaken paid consultancy work for TC BioPharm Ltd. MB was previously employed by TC BioPharm Ltd. JA holds founder stock in Autolus Ltd and share options in TC BioPharm Ltd and holds patents in CAR-T technology. JA received consultancy payments from TC-Biopharm between 2018-2021.

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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