

Cytometric analysis of T cell phenotype using cytokine profiling for improved manufacturing of an EBV-specific T cell therapy.

Rachel Cooper¹, Aleksandra Kowalczyk¹, Gwen Wilkie¹, Mark Vickers², John Campbell¹, Marc Turner¹, and Alasdair Fraser¹

¹Scottish National Blood Transfusion Service

²University of Aberdeen

January 7, 2021

Abstract

Adoptive immunotherapy using Epstein-Barr Virus (EBV)-specific T cells is a potentially curative treatment for patients with EBV-related malignancies where other clinical options have proved ineffective. We describe improved GMP-compliant culture and analysis processes for conventional lymphoblastoid cell line (LCL)-driven EBV-specific T cell manufacture, and describe an improved phenotyping approach for analyzing T cell products. We optimized the current LCL-mediated clinical manufacture of EBV-specific T cells to establish an improved process using xenoprotein-free GMP-compliant reagents throughout, and compared resulting products with our previous banked T cell clinical therapy. We assessed effects of changes to LCL: T cell ratio in T cell expansion, and developed a robust flow cytometric marker panel covering T cell memory, activation, differentiation and intracellular cytokine release to characterize T cells more effectively. These data were analyzed using t-Stochastic Neighbour Embedding (t-SNE) algorithm. The optimized GMP-compliant process resulted in reduced cell processing time and improved retention and expansion of central memory T cells. Multi-parameter flow cytometry determined the optimal protocol for LCL stimulation and expansion of T cells and demonstrated that cytokine profiling using IL-2, TNF- α and IFN- γ was able to determine the differentiation status of T cells throughout culture and in the final product. We show that fully GMP-compliant closed-process culture of LCL-mediated EBV-specific T cells is feasible and profiling of T cells through cytokine expression gives improved characterization of start material, in-process culture conditions and final product. Visualization of the complex multi-parameter flow cytometric data can be simplified using t-SNE analysis.

Title:

Cytometric analysis of T cell phenotype using cytokine profiling for improved manufacturing of an EBV-specific T cell therapy.

Short Title:

T cell cytokine analysis for T cell therapy manufacture.

Authors:

Rachel S Cooper¹, Aleksandra Kowalczyk², Gwen Wilkie², Mark A.Vickers², Marc L. Turner¹, John D.M. Campbell¹ and Alasdair R Fraser¹

Affiliation:

1. Tissues, Cells and Advanced Therapeutics, Scottish National Blood Transfusion Service, Jack Copland Centre, Edinburgh EH14 4BE 2. Blood Transfusion Centre, Scottish National Blood Transfusion Service, Foresterhill Road, Aberdeen AB25 2ZW

Corresponding Author:

Dr Alasdair Fraser, Research Manager, Tissues, Cells and Advanced Therapeutics, Scottish National Blood Transfusion Service, Jack Copland Centre, 52 Research Avenue North, Heriot-Watt Research Park, Edinburgh EH14 4BE.

Phone: 0131 314 5687 / email: alasdair.fraser@nhs.scot

Keywords:

T cell, Epstein-Barr, Phenotyping, Cell Therapy

Summary:

Adoptive immunotherapy using Epstein-Barr Virus (EBV)-specific T cells is a potentially curative treatment for patients with EBV-related malignancies where other clinical options have proved ineffective. We describe improved GMP-compliant culture and analysis processes for conventional lymphoblastoid cell line (LCL)-driven EBV-specific T cell manufacture, and describe an improved phenotyping approach for analyzing T cell products.

We optimized the current LCL-mediated clinical manufacture of EBV-specific T cells to establish an improved process using xenoprotein-free GMP-compliant reagents throughout, and compared resulting products with our previous banked T cell clinical therapy. We assessed effects of changes to LCL: T cell ratio in T cell expansion, and developed a robust flow cytometric marker panel covering T cell memory, activation, differentiation and intracellular cytokine release to characterize T cells more effectively. These data were analyzed using t-Stochastic Neighbour Embedding (t-SNE) algorithm.

The optimized GMP-compliant process resulted in reduced cell processing time and improved retention and expansion of central memory T cells. Multi-parameter flow cytometry determined the optimal protocol for LCL stimulation and expansion of T cells and demonstrated that cytokine profiling using IL-2, TNF- α and IFN- γ was able to determine the differentiation status of T cells throughout culture and in the final product.

We show that fully GMP-compliant closed-process culture of LCL-mediated EBV-specific T cells is feasible and profiling of T cells through cytokine expression gives improved characterization of start material, in-process culture conditions and final product. Visualization of the complex multi-parameter flow cytometric data can be simplified using t-SNE analysis.

Introduction

Epstein Barr virus (EBV) is a human herpes virus with a prevalence of over 90% in adults [1]. Acute infection is generally asymptomatic, but can present clinically as infectious mononucleosis (glandular fever) and infection persists latently throughout life in B lymphocytes. Intermittent viral reactivation drives proliferation of infected cells, which can transform to malignant lymphoblasts in the absence of immune surveillance. In immunocompetent individuals, EBV-specific T cells control these reactivations [2]. However, iatrogenic immunosuppression of patients following haematopoietic stem cell transplant (HSCT) or solid organ transplant (SOT) can result in potentially fatal EBV-driven post-transplant lymphoproliferative disorder (PTLD), where EBV-transformed B cells develop into an aggressive B cell lymphoma [3].

Adoptive immunotherapy using Epstein-Barr Virus (EBV)-specific T cell lines has proven to be an effective clinical treatment for patients with rituximab-resistant or refractory PTLD [4]. The Scottish National Blood Transfusion Service (SNBTS) EBV-specific T cell bank has delivered third-party partially HLA-matched EBV-specific T cells for PTLD patients for over 15 years, allowing a rapid therapeutic intervention following diagnosis, with an initial 52% complete response rate in a phase 2 multicentre trial [5]. As of December 2020, over 100 SOT and HSCT patients have been treated with the more recent second-generation SNBTS T cell bank, issued under a Medicines and Healthcare Regulatory Agency (MHRA) Manufacturing Specials licence for therapeutic use, and a recent follow-up study of 64 patients with refractory disease treated between 2011 and 2017 indicates that overall survival at 3 years post treatment was more than 40%, and higher (62%) in

SOT patients [6]. This corresponds well with other clinical studies using adoptive therapy with EBV-specific T cells, where overall response rates were 63-68% [7-9].

Clinical manufacture of allogeneic EBV-specific T cells for therapy requires a generation protocol that conforms to current good Manufacturing Process (cGMP) regulations. Manufacture of the current SNBTS second-generation SNBTS T cell bank was undertaken using a conventional process for generating EBV-specific T cells, using mononuclear cells (MNC) co-cultured over 6-8 weeks with autologous EBV-transformed lymphoblastoid cell lines (LCL). The LCL act as antigen-presenting cells (APC) to induce proliferation of T cells recognizing EBV viral proteins such as BZLF1 and BMLF1 involved in early lytic cycle transactivation, and viral latency proteins such as EBNA1, EBNA2, EBNA3, LMP1 and LMP2 [10,11]. Multiple rounds of stimulation with irradiated autologous LCL are required to induce EBV-specific T cell expansion to a suitable therapeutic dose [12]. The introduction of rapid expansion protocols in development of new T cell therapies [13] indicates a need for both robust, rapid methods for EBV-specific T cell manufacture, and improved analytic methods to assess the quality of the material throughout.

Replacement of standard flask culture with gas-permeable rapid expansion devices (G-Rex flasks, Wilson Wolf) is now established for T cell culture, and T cell lines are generally initiated in culture with a ratio of 40 MNC : 1 LCL for the first stimulation round. Subsequent stimulation rounds are generally at a 1 T cell : 5 LCL ratio in rapid expansion protocols [14], though variations in this approach are evident between studies [15-18]. As part of this study, we determined the optimal number of stimulation rounds and optimized our previous manufacture protocol by transfer to EU GMP-compliant culture reagents (medium and cytokine) and G-Rex flask culture throughout. We confirmed appropriate T cell: LCL stimulation ratio parameters for culture and in each case utilised an extensive multi-parameter flow cytometric analysis approach to dissect the composition of the cultures.

The critical quality attributes of the SNBTS therapeutic EBV-specific T cells products are stringent and include viability, T cell lineage, and absence of other lymphocytes; for this process optimization study we introduced a broader flow cytometric analysis panel which allowed characterization of the T cell compartment to a much higher level of discrimination. The new panels were able to determine T cell development, differentiation and activation status, and provide markers of efficacy through intracellular cytokine analysis. We utilized these panels to determine the efficacy of the modified protocol in comparison with the current process, and assess quality from starting mononuclear cells through to final T cell product. Visualizing this data is complex, and we applied t-Stochastic Neighbour Embedding (t-SNE) algorithms to incorporate multiple parameters into a single image, allowing visual comparison of the T cell phenotype. This approach provides clear evidence for an improved process for manufacture of virus-specific T cells to a standard suitable for clinical use.

Materials and Methods

Donor material

Stocks of donor leukapheresis mononuclear cells, derived EBV-specific T cells and corresponding LCL from the SNBTS second-generation bank were supplied from frozen stored stocks. For this bank material, initial leukapheresis donations were collected by Spectra Optia apheresis system (Terumo BCT) and all donations were fully consented for research use and were either from New Zealand (NZ code, free of variant CJD and suitable for therapy); or were from local Scottish donors, for research only. Autologous EBV-transformed lymphoblastoid cell lines (LCL) for the second-generation bank were generated from NZ donor PBMC by infection with concentrated EBV-positive supernatant, for use as autologous antigen presenting cells as previously described [12]. Briefly, supernatant from a live EBV-expressing cell line was added to donor PBMC and cultured for several rounds until stable LCL lines were established. The LCL were frozen in 1ml aliquots in human serum albumin (HSA) + 10% DMSO until use. Initial work on phenotypic development was run using peripheral blood mononuclear cells (PBMC) derived from buffy coats from normal blood donors, supplied through SNBTS under Sample Governance 14~11.

Generation of EBV-specific T cells in standard culture is improved in G-Rex flasks

Standard cultures were generated by re-deriving new products from frozen stored leukapheresis samples from 6 donors. These new cultures were grown in standard medium (RPMI + 10% FCS and glutamine) in T75cm² flasks by mixing thawed apheresis MNC with autologous LCL (irradiated at 40Gy) at an initial ratio of 40 MNC : 1 LCL, and then re-stimulated with fresh autologous LCL for a total of 6-8 stimulation rounds. Final T cell product (1.0x10⁷ cells/ml) were frozen in cryovials from each stimulation round and stored for later analysis by flow cytometry.

A comparison study set up to assess improvement of culture of EBV-specific T cells using closable G-Rex flasks (Wilson-Wolf) was carried out using thawed donor leukapheresis material and compared against cultures in conventional open culture flasks. Cells in suspension from each donor were split and cultured in either T75cm² culture flasks (1.0x10⁸ MNC + 2.5x10⁶ LCL, final density = 1.33x10⁶ MNC per cm²) or G-Rex100cm² flasks (1.0x10⁸ MNC + 2.5x10⁶ LCL, final density = 1.0x10⁶ MNC per cm²). At day 10, cells in T75cm² culture were counted, and a second stimulation of autologous irradiated LCLs were added at a ratio of [4 T cell : 1 LCL] and split to new T75cm² flasks to give a final density of 0.5-1.0x10⁶ T cells per cm². For G-Rex cultures at day 10, cells were counted and irradiated LCL added at a ratio of [1 T cell : 5 LCL] and split to new G-Rex100cm² flasks to give a final density of 0.1x10⁶ T cells per cm² as indicated in the rapid expansion protocol [14]. At day 14, cultures were re-fed with interleukin-2 (IL-2) at a final concentration of [20IU/mL]. Thereafter, cultures were counted every 3-4 days and fed/split to new flasks as necessary. All cell counts were given as viable cells via trypan blue exclusion of dead cells.

Comparison of GMP-compliant reagents and stimulation intensity

Small scale studies of EBV T cells cultured in G-Rex flasks comparing reagents and stimulation round densities were performed using frozen vials of matched MNC and LCL supplied from the second-generation SNBTS T cell bank. As before, on day of initiation LCL were irradiated at 40Gy and mixed with autologous MNC at an initial stimulation ratio of 40 MNC : 1 LCL. Cells from each donor were divided and cultured in 40mL culture medium: either RPMI with glutamine + FBS [10%] + research-grade IL-2 [50IU/mL] or xeno-free GMP-grade TexMACS (Miltenyi Biotec) + GMP-grade IL-2 [50IU/mL] (both Miltenyi Biotec) and transferred to G-Rex10cm² flasks (1x10⁷ MNC + 2.5x10⁵ LCL, final density = 1.0x10⁶ MNC per cm²). Cell counts were taken every 3-4 days and cultures re-fed at these time-points by removing 90% volume of media and replacing with fresh media plus IL-2. At day 10, cells were harvested from flasks and re-cultured at 0.5-1x10⁷ per G-Rex10 for re-stimulation with irradiated LCL at 1 T cell : 5 LCL as per standard G-Rex protocol. Cultures in fully GMP reagents were further subdivided into secondary stimulation ratios of 1 T cell : 5 LCL (standard protocol) or 1 T cell : 1 LCL (reduced-intensity modified protocol). A final round of stimulation was performed at day 20, with either 1 T cell : 5 LCL or 1 T cell : 1 LCL for the standard or modified protocol respectively. At day 30, cells were harvested from the G-Rex and assessed for yield and viability by trypan blue exclusion and tested in the flow cytometric assays below.

Buffly Coat PBMC Isolation

Donor PBMC were isolated from healthy donor buffy coats by ficoll-paque (GE Healthcare) density centrifugation (450g for 40 minutes) to set up flow cytometry panels (surface phenotype and intracellular cytokine assay). Donor PBMC from n=6 buffy coats were also characterised for cytokine expression in relation to T cell memory markers (see intracellular cytokine assay).

Surface Phenotyping

Flow cytometric characterisation of T cells was carried out with surface marker panel: CD45RA-Vioblu, CD8-Viogreen, CD62L-FITC, CD3-PE or CD57-PE, CD45RO-PE-Vio700, CD4-PE-Vio770, CD28-APC (all Miltenyi Biotec) and dead cell marker DRAQ7 (BioLegend, UK). Following stimulation by LCL, samples were taken weekly to monitor phenotypic changes using a modified panel, where CD57-PE (Miltenyi) was used to replace CD3 to monitor terminal differentiation / senescence.

Briefly, 2x10⁶ cells were washed with PBS plus 2.5mM EDTA (Invitrogen) and 0.5% human serum albumin (Alburex) (PEA buffer), blocked with 5µl human Fc Receptor block (Miltenyi), labelled with antibody

cocktail at 4°C for 20 minutes, washed and DRAQ7 added prior to analysis. Approximately 100,000 live events were acquired using a MACSQuant10 (Miltenyi) or BD Fortessa LSR (Becton Dickinson) flow cytometer with data analysis using FlowJo V10 software (Tree Star, Inc). All flow cytometric analyses were based on a manual gating strategy as described in Figure 1A, with lymphocyte population identified by scatter, then doublets and dead cells excluded (singlet gated / DRAQ7- cells). Data were collected as percentages of gated populations, as mean fluorescence intensity (MFI), or as a corrected MFI (target MFI – isotype MFI).

Intracellular Cytokine Assay

Intracellular staining to characterize T cell populations based on cytokine profile was developed. Cells were stimulated with 2µl/ml Cell Activation Cocktail (PMA/ionomycin, BioLegend, UK), for 2 hours, then brefeldin A (5µg/ml, BioLegend, UK) was added for a further 3 hours. Non-activated cells treated with brefeldin were used as negative controls. Cells were harvested, washed twice in PEA and surface markers labelled with CD8-Vio700, CCR7-FITC, CD57-PE and CD45RO-PE-Vio700 (Miltenyi) as before, washed in PBS plus 2.5mM EDTA (PE buffer) and stained with 1µl/ml Fixable Viability Dye eFluor 780 (eBioscience, UK) for 30 minutes at 4°C. Cells were washed again in PE, then fixed and permeabilized as per manufacturer's instructions (BD Biosciences, UK) and labelled with anti-IFN-γ-eFluor450 (eBioscience, Thermo UK), anti-TNF-α-PE-Vio770 and anti-IL-2-APC (both Miltenyi) for 30 minutes. Cells were washed again and resuspended in PEA and up to 100,000 live events recorded on MACSQuant10 / BD Fortessa LSR.

Flow Cytometry Gating Strategy and t-SNE analysis

Phenotypes were defined using a combination of expression of surface markers with intracellular cytokine expression after stimulation with PMA/ionomycin (versus relevant negative and isotype controls). Representative flow cytometric data from second generation bank EBV-specific T cell final product with initial gating of lymphocytes/ singlets/ live cells is outlined in Supplementary Figure 1. Lineage was identified through CD4 versus CD8 labelling, and differentiation status through surface expression of CD45RO/ CD45RA and CD62L or CCR7 (Supplementary Fig 1B). Analysis of cytokine expression (Supplementary Fig 1C) was based on gating CD8+/CD45RO+ viable lymphocytes, dividing into IL-2^{low} and IL-2^{high} populations, and then quadrant gating each IL-2 subpopulation for IFN-γ and TNF-α co-expression. A contrasting profile of T cell cytokine expression of a heterogeneous pan T cell compartment in PBMC freshly isolated from buffy coat donors is shown in Supplementary Figure 2.

Stochastic Neighbour Embedding (t-SNE) analysis was applied to multi-parameter flow cytometric data. Briefly, analyses were gated on lymphocyte/singlet/live as above using FlowJo, and reduced to a representative 10,000 events using Downsample. The t-SNE maps were generated using the FlowJo plugin for 800 iterations at perplexity 20. Events from each Downsample population were spatially correlated in terms of likeness for all fluorescent parameters outlined previously. Manual gating overlays were used to subdivide CD8+ and CD4+ cells into T cell memory populations based on surface marker expression: naive (CCR7+/CD45RO-), central memory TCM (CCR7+/CD45RO+), effector memory TEM (CCR7-/CD45RO+) and terminally-differentiated effectors expressing CD45RA TEMRA (CCR7-/CD45RO-).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7.01 software (GraphPad Software, California, USA). Comparisons between groups were made on the basis of the difference in mean percentage of cells positive for surface marker expression for phenotypic analyses, the difference in cMFI of anti-cytokine antibodies for intracellular cytokine production analyses, and the difference in mean cell number/yield for cell expansion analyses. Significance was calculated using unpaired 2-tail Student t-tests with Holm-Sidak correction for multiple tests, where a p value of <0.05 was considered significant.

Results

Determining optimal stimulation round for effective EBV-specific T cell manufacture

Third-party EBV-specific T cell banks based on LCL stimulation involve leukapheresis-derived T cells co-

cultured with irradiated autologous LCL through several rounds to generate a relatively homogenous virus-specific T cell product. We hypothesized that multiple rounds of LCL-mediated antigen presentation could potentially lead to exhaustion of virus-specific T cells. To determine this, we re-derived exemplars from our second-generation EBV-specific T cell bank leukapheresis and LCL stocks and analyzed cells by flow cytometry at 7-9 days following each successive stimulation round to characterize phenotypic development. Changes in CD4/CD8 ratio were seen through each round of stimulation (Figure 1A), indicating that the CD4 population forms less than 1% of the total T cells from stimulation round (SR) four onwards. The corresponding IFN- γ /TNF- α expression in the CD8 central memory (TCM), effector memory (TEM) and differentiated effector (TEMRA) compartment shows the majority of cells (86%) co-express IFN- γ and TNF- α in response to PMA/ionomycin stimulation (Figure 1A, lower panel). From SR2 onwards this increases to over 97% IFN- γ + /TNF- α +, indicating the CD8 compartment rapidly progresses to functional cytotoxic T cells. However, percentage cytokine expression stabilizes after SR3, indicating no significant benefit to functional capacity with multiple rounds of LCL stimulation. This was quantified in six different EBV-CTL lines (data is represented as mean \pm SEM), and confirmed that outgrowth of the CD8 population was consistent (Figure 1B). The mean percentage of CD8+/CD45RO+ cells peaks at SR3 (Figure 1C) then starts to decrease, indicating downregulation of CD45RO with continued stimulations. Expression of the activation / senescence marker CD57 also increases throughout each stimulation round. While the mean percentage of TNF- α /IFN- γ -expressing CD8+ T cells cytokine does not vary significantly throughout, it is clear that the mean fluorescence intensity corrected to negative control (cMFI) of TNF- α and IFN- γ peaks at SR3 or 4 and then starts to decrease (Figure 1D), indicating that extending stimulation beyond four rounds potentially compromises functional cytokine levels. Further cytometric analysis of exhaustion markers (PD-1 / LAG-3 / Tim-3) demonstrated no significant co-expression of these indicators of loss of function (data not shown). The results from Figure 1 suggest that EBV-specific T cell cultures reach peak product quality by SR3/4, after which functional parameters decline.

GMP-translatable culture vessels improve manufacturing outcomes

The SNBTS second-generation EBV-specific T cell bank was manufactured in standard culture flasks, and we determined whether transfer to more GMP-compliant G-Rex culture flasks would affect T cell phenotype or functional markers. Sero-positive apheresis material from healthy EBV-positive donors (n=6) were used to establish fresh EBV-specific T cell cultures, and each donor culture was split to directly compare culture in closable G-Rex flasks (G-Rex100) versus standard tissue culture flasks (Corning) through an optimized three-round stimulation process. The G-Rex flasks used had a standard research cap, but are identical to GMP-compliant flasks which have a closed-process cap, so can be considered GMP-translatable. At day 30 (9 days after the third LCL stimulation), cells were harvested from both culture vessels and analyzed by surface and intracellular cytokine phenotyping. The mean percentage of CD8+ T cells was significantly higher (p=0.012) in G-Rex flask than in standard flask (90.2 \pm 3.49% versus 78.4 \pm 2.11% respectively, Figure 2A). However, T cell surface marker phenotype did not differ significantly between culture flasks (Figure 2B). Intracellular cytokine analysis after PMA/ionomycin stimulation demonstrated that there was no significant difference in either quantity of cytokine expression (measured by cMFI, Figure 2C) or cytokine co-expression subpopulations in CD8+ T cells between flasks (Figure 2D). These results demonstrate using manufacturing-scale G-Rex culture flasks not only allows increased cell expansion (data not shown), but also develops a more consistent T cell phenotype in a shorter period of time than culture in conventional Corning flasks. This process is then readily transferable to G-Rex closed-system flasks to allow a fully closed manufacturing process via use of the Gatherex cell harvester (Wilson Wolf Ltd).

Comparability of EBV-specific T cells generated using GMP-compliant reagents.

The SNBTS second generation EBV-specific T cell bank utilized some non-GMP standard reagents, including fetal bovine serum for LCL culture. We assessed whether EBV-specific T cell products cultured in GMP-compliant reagents including serum-free culture medium throughout would generate comparable T cell products. Culture was performed in G-Rex100 flasks using the optimized three-round stimulation protocol and assessment of T cell characteristics was carried out as before. In final product EBV-specific T cells

grown in GMP compliant reagents throughout, surface phenotype (Figure 3A-3B) was substantially similar to those grown in research-grade reagents. A key improvement was a significantly higher percentage of TCM (CD62L+ / CD45RO+) cells in the GMP-compliant conditions (n=4).

There were no significant differences between the groups for cytokine co-expressing populations (Figure 3D), though a trend towards increased cytokine levels was observed in GMP-compliant cultures (Figure 3C). These data demonstrate that GMP compliant reagents used with G-Rex culture flasks results in EBV-specific T cell cultures that are comparable to current bank material, and have improved retention of the TCM compartment.

Optimization of T cell: LCL ratio for EBV antigen-driven stimulation

Conventionally, *ex vivo* expansion of EBV-specific cytotoxic T cell lines (CTLs) has been performed using a ratio of 40 PBMC : 1 LCL for the initial stimulation, and subsequent stimulation rounds at a ratio of 1 T cell : 5 LCL in G-Rex rapid expansion protocol which could potentially drive anergy, senescence or activation-induced cell death (AICD). We determined whether modulation of the stimulation ratio conferred benefits in terms of functional subpopulation profile.

T cell cultures from four EBV-positive donors were initiated using an initial concentration of 40 PBMC : 1 LCL as per standard protocol, then at SR2 or SR3 cultures were split and co-cultured at standard (1 T cell : 5 LCL) or at lower intensity (1 T cell : 1 LCL). At day 30 (the end of SR3), a direct comparison of each stimulation ratio was assessed. Mean harvest cell counts indicated a 28.1 ± 13.63 -fold expansion for 1 T cell : 5 LCL cultures, and 21.68 ± 9.24 -fold expansion for 1 T cell : 1 LCL cultures (Figure 4A). Samples taken at day 30 were comparable for surface marker analysis, with ~90% CD8+ cells and equivalent levels of each surface marker subset (Figure 4B). While the percentage of T cells with co-expression of IFN- γ / TNF- α was equivalent between the two groups (Figure 4D), the cMFI of IFN- γ (Figure 4C) was significantly higher (p=0.00218) in 1 T cell : 1 LCL cultures (mean = 162.5 ± 16.03) than in 1 T cell : 5 LCL cultures (mean = 77.65 ± 25.50). Similarly, TNF- α cMFI was significantly increased (p=0.0202) in 1 T cell : 1 LCL cultures (mean = 112.18 ± 18.56) than in 1 T cell : 5 LCL cultures (mean = 53.69 ± 12.2). The increased cytokine cMFI indicates co-culture with a lower intensity LCL stimulation result in EBV-specific T cells with an enhanced capacity for functional cytokine secretion. These data clearly demonstrate the robustness of the current LCL-based method for EBV-specific T cell stimulation and expansion, as most parameters are unaffected by the modification of LCL ratios. However, the finding that reducing LCL dose corresponds with increased secretion of IFN- γ and TNF- α which may enhance effector functions of these cells *in vivo*, suggests that the EBV-specific T cell culture product can be optimized. We have therefore demonstrated that our optimised culture approach provides a significant improvement in retention of TCM cells with increased cytokine expression. The expanded phenotyping approach allows for a better characterisation of start material and final products, and we assessed how to analyse the data from this multi-parameter flow cytometric approach more effectively.

Comparing T cell memory status using surface marker and cytokine profiles

The flow cytometric characterization of EBV-specific T cells in the current second-generation SNBTS bank was restricted to a small panel of essential lineage markers: CD8, CD4, CD19 and CD56 plus 7-AAD for viability. Manufacturing release criteria were confined to the presence of <2% CD19 B cells (as a marker of LCL contamination) and >10% specific lysis against autologous LCLs by cytotoxicity assay. In this study we extended this analysis to characterize T cell products on the basis of lineage, memory and differentiation status, and correlate this with cytokine profile to improve the assessment of quality and functionality of T cell material used for clinical therapy. This was used to assess the outcomes from optimisation of the LCL-based manufacturing method.

Optimised phenotyping and t-SNE analysis for T cell products

Conventionally, T cell memory subtypes are identified through co-expression of homing receptors such as CD62L and CCR7 with leukocyte common antigen (CD45) isoforms RO and RA, but these memory types

can also be classified by cytokine co-expression (Supplementary Figure 3). We characterised T cells from start material through to final product for memory / differentiation status using a combined surface marker/ intracellular cytokine panel, and used t-SNE analysis to simplify multi-parameter phenotyping.

The t-SNE dimensionality reduction identified a clear sequential spatial correlation in both the CD4 and CD8 differentiation from naïve (CCR7+/CD45RO-) to TCM (CCR7+/CD45RO+) to TEM (CCR7-/CD45RO+) to TEMRA (CCR7-/CD45RO-) stages. Furthermore, fluorescent intensity heat maps applied over this same t-SNE plot (Figure 5B) indicated highest intensity of IFN- γ in the CD8+ TEM region; highest intensity of TNF- α in the CD8+ TEM and CD4+ TEM regions; and highest intensity of IL-2 in the CD8+ TCM and CD4+ TCM/TEM compartments, confirming the correlation of differentiation status with cytokine profile.

As CD8+ T cells form the principal component of the final cell therapy product, the CD8+ naïve, TCM, TEM and TEMRA populations were then sequentially analysed by expression of cytokines IFN- γ , TNF- α and IL-2. The mean percentage of each cytokine subpopulation from six buffy coat-derived PBMC donors stimulated with PMA/Ionomycin is expressed in pie chart format in Figure 5C. The majority of CD8+ naïve T cells are cytokine-null, with the remaining 26.2% mostly comprising IL-2 or TNF-expressing cells. The CD8+ TCM cells predominantly express all cytokines (IFN- γ +/TNF- α +/IL2+), though there is also a distinct TNF- α +/IL2+ population (27%) within this subset which represents early central memory development. Within the CD8 TEM compartment, there is developmental transition from central memory (retention of IFN- γ +/TNF- α +/IL2+) into more effector-like IFN- γ /TNF- α co-expressing cells which form almost 50% of the TEM subpopulation. The CD8 TEMRA population shows further transition, with IFN- γ +/TNF- α + or IFN- γ + only T cells forming the principal population, with a small residual population retaining some TCM (IFN- γ +/TNF- α +/IL2+) characteristics. There is also an increased presence (17%) of cytokine-null T cells suggesting that some cells may have become anergic, with no functional secretory response. Importantly, this data demonstrates the limitations of trying to discern T cell memory phenotype purely through surface marker expression alone. For example, even within the conventionally defined naïve cells (CCR7+ CD45RO+) which are typically described as cytokine null, there are evident small subpopulations within that secrete cytokines.

The use of t-SNE dimensionality reduction generates a single-image analysis of complex multi-parameter flow cytometric data which can be utilised to gain a clear representation of the quality and composition of the final T cell product in comparison with the starting mixed leukocyte material (Figure 5D).

Discussion

The SNBTS currently provides allogeneic third-party EBV-specific T cells for patients with relapsed/refractory post-transplant lymphoproliferative disease (PTLD). As of November 2020 more than 100 patients with relapsed or refractory PTLT have been treated from the current bank under a Specials license, with a mean overall survival rate of over 40% at three years post treatment. Patients in this cohort with PTLT arising after solid organ transplant had better outcomes, with survival of over 60% at three years post-treatment, and with minimal adoptive cell therapy-related side-effects [5].

The current bank of EBV-specific T cells was manufactured from 2007-2014, and changes to GMP standards since this period have driven a requirement to optimize and refine the current manufacturing processes [20]. More recent methods for generation utilise cytokine release assays to capture virus-specific T cells, though this requires a different expansion process. LCL-based stimulation and expansion protocols are still in regular use for development of anti-cancer therapies [34-36], and therefore there is a need to identify optimal methods for production and analysis.

In this study we demonstrated that optimization of the standard autologous LCL-based method of EBV T cell manufacturing to a fully GMP-compliant closed-process process is feasible without compromise in quality of final cell product. The modifications to protocol, reagents and culture process were assessed principally using flow cytometry, which provides a rapid and quantitative method for analysis. Robust, validated flow cytometric assays are a cornerstone of effective reproducible cell therapy manufacture [21]. The use of flow cytometric analysis and functional profiling of EBV-specific T cells through cytokine expression in this study

resulted in improved characterization of both start material and final product, and effective assessment of in-process culture optima, which has been used for analysis of other T cell therapeutics including a SARS-CoV-2 T cell product for COVID-19 treatment [37].

Intracellular cytokine staining for IL-2, TNF- α and IFN- γ provides a reliable method for discriminating the differentiation state of T cells [19, 22]. The combination of multi-parameter cytokine secretion-based phenotyping with t-SNE analysis forms a powerful tool for dissecting functional subpopulations within the CD8+ cytotoxic T cell compartment, and was used as the basis for analysis of improvements and refinements in manufacturing of the current SNBTS EBV-specific T cell therapy used for treatment of PTLD [20].

Using the combined surface marker and intracellular cytokine flow cytometric phenotyping approach we were able to identify that multiple rounds of LCL stimulation were unnecessary, and that extending stimulation may increase the level of anergy or loss of function in the T cells, as identified by a loss of absolute IFN- γ secretion and increased expression of CD57, a marker of terminal effector differentiation [23]. However, the reduction in stimulation round to maximize functional responses needs to be balanced with the requirement for high yields of cells for treatment of multiple patients from a single manufacturing run.

Adoptive T cell therapy relies on large-scale expansion of functional T cells to manufacture clinically relevant numbers for patient infusion, conventionally through use of standard culture flasks or gas-permeable bags. The introduction of large volume, high gas exchange culture vessels (G-Rex flask, Wilson Wolf) has significantly improved the rate and extent of T cell expansion capacity [24]. The G-Rex flasks are GMP-compliant and are scalable up to 1L flasks which are qualified as an FDA Class 1 medical device allowing full closed process manufacture. This closed process manufacture involves suitable sealed flasks, transfer bags, heat sealed tubing and the GatheRex cell harvester pump (Wilson Wolf) to ensure sterility in the clinical product. We identified that cell yields could also be improved by using G-Rex flasks for culture with no significant changes in phenotype. A minor change in T cell composition was identified in the G-Rex cultures, with increase in the percentage of CD8 cells. This consistency of final product phenotype was also retained when all reagents were converted to fully GMP-compliant standards. GMP-compliant medium and cytokines with no exogenous xenoproteins ensured that the modified process complied with current regulatory requirements. T cells generated with GMP compliant reagents and flasks suitable for closed process culture had a significant increase in retention of the TCM compartment. This has advantages for persistence of the cell therapy once administered to a patient [25,26].

A principal concern with the current LCL-based stimulation process is that high LCL (and therefore viral antigen load) ratio to T cells combined with multiple rounds could drive T cell exhaustion [27,28] and the reduced T cell: LCL ratio process outlined here quantified whether this resulted in functional differences. The LCL process appears robust, as reduced intensity stimulation over three rounds did not significantly affect the phenotype of the T cells at end-point, although the reduced ratio exhibited a significantly enhanced CD8+ cell secretion of IFN- γ and TNF- α . The only modulation of culture processes that was not undertaken was to replace or supplement IL-2 with other gamma-chain specific T cell growth factors. However, other studies have concluded that changing the cytokine-mediated expansion method from IL-2 to other cytokines such as IL-7, IL-15, or IL-21 has no significant effect on the overall phenotype or function of T cells for therapy [29]. The increased production of IFN- γ and TNF- α in response to stimulation in the reduced intensity LCL stimulation may suggest products made using this protocol could have increased effector functions against viral-infected cells following patient engraftment.

A key feature of this work was to identify a robust panel of surface and intracellular markers which could effectively classify the T cell differentiation status and development from initial material through to final product. Our approach supplies clear data for this, and demonstrates the utility of this approach for T cell therapies [37]. In addition, the use of t-SNE dimensionality reduction was very effective at condensing multiple parameters into a single image which could be used to identify the status of the material at any stage of manufacture. These images are both illustrative and quantitative and could therefore be used as part of a standardised product release process. This cytometric phenotype and analysis approach is sufficiently adaptable and inclusive that it would be suitable for phenotypic and functional assay of other cell therapies

including virus-specific and genetically-modified T cell therapies [30-33,37].

Acknowledgements

We gratefully acknowledge the voluntary donations of peripheral blood and leukapheresis samples used in this study. This study was supported by the staff of the Scottish National Blood Transfusion Service (SNBTS) Clinical Apheresis Unit, Aberdeen and medical colleagues at the New Zealand Blood Transfusion Service for collection of the donor leukapheresis material for the second iteration of the EBV-T cell bank. This work was supported by a Wellcome Trust Translational Award. The ongoing operation of the UK EBV-specific T cell bank is supported by cost-recovery fees and supported by SNBTS.

Contributions

RSC and AK were responsible for the acquisition and analysis of data, and RSC wrote the principal manuscript; GW and MV were involved in supply of material and interpretation of data; MLT, JDMC and ARF were responsible for study conception and design, data analysis and interpretation, and revision of the final manuscript.

Declaration of interests:

The authors have no conflicts of interests.

This work has not been published previously (except in the form of a poster abstract and MSc thesis), is not under consideration for publication elsewhere, and its publication is approved by all authors. If accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

References

1. Ball RJ, Avenell A, Aucott L, Hanlon P and Vickers MA (2015) Systematic review and meta-analysis of the sero-epidemiological association between Epstein-Barr virus and rheumatoid arthritis. *Arthritis Res Therap* 17: 274
2. Hislop AD, Taylor GS, Suace D, Rickinson AB. (2007) Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Ann Rev Immunol* 25: 587-617.
3. Crombie JL, LaCasce AS. (2019) Epstein Barr Virus Associated B-Cell Lymphomas and Iatrogenic Lymphoproliferative Disorders. *Front Oncol* . 9:109.
4. Bollard CM, Gottschalk S, Torrano V, Diouf O, Ku S, Hazrat Y, Carrum G, Ramos C, et al (2014) Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. *J Clin Oncol* . 32:798-808. doi: 10.1200/JCO.2013.51.5304
5. Haque T, Wilkie GM, Jones MM, Higgins CD, Urquhart G, Wingate P, Burns D, McAulay K, et al (2007) Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood* 110: 1123-1131
6. Kazi S, Mathur A, Wilkie GM, Cheal K, Battle R, McGowan N, Fraser N, Hargreaves E., et al (2019) Long-term follow up after third party viral-specific cytotoxic lymphocytes for immunosuppression- and Epstein-Barr virus-associated lymphoproliferative disease. *Haematologica* 104:e356-e359. doi: 10.3324
7. Doubrovina E, Oflaz-Sozmen B, Prockop SE, Kernan NA, Abramson S, Teruya-Feldstein J, Hedvat C, Chou JF, et al (2012) Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy proven EBV+ lymphoma after allogeneic hematopoietic cell transplantation. *Blood* 119: 2644-55.
8. Leen AM, Bollard CM, Mendizabal AM, Shpall EJ, Szabolcs P, Antin JH, Kapoor N, Pai SY, et al (2013) Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. *Blood* 121:5113-5123

9. Prockop SE, Doubrovina E, Feng A, Koehne G, Dahi P, et al (2018) Long-term outcome of Tabelecleucel (Third-party allogeneic EBV-targeted cytotoxic T-lymphocytes) for Rituximab-refractory post-transplant EBV + lymphomas: a single centre experience. Poster PF401. Presented on June 15, 2018 at 23rd Congress of the European Hematology Association (EHA)
10. Pallesen G, Hamilton-Dutoit SJ, & Zhou X (1993) The association of Epstein-Barr virus (EBV) with T cell lymphoproliferations and Hodgkin's disease: two new developments in the EBV field. *Advances Cancer Res* 62, 179-239
11. Bollard CM, Aguilar L, Straathof KC, Gahn B, Huls MH, Roussea A, Sixbey J, Gresik MV, et al (2004) Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *J Exp Med* 200: 1623-1633
12. Wilkie GM, Taylor C, Jones MM, Burns DM, Turner M, Kilpatrick D, Almot PL, Crawford DH, et al (2004) Establishment and characterization of a bank of cytotoxic T lymphocytes for immunotherapy of Epstein-Barr virus-associated diseases. *J Immunother* 27:309-16.
13. Hao QF, Yang YX, Wang Y, Chen LM, Sheng GY & Luan Z (2014) Rapid generation of Epstein-Barr virus-specific T cells for cellular therapy. *Transplant Proc* 46:21-5
14. Vera JF, Brenner LJ, Gerdemann U, Ngo MC, Sili U, Liu H, Wilson J, Dotti G, et al (2010) Accelerated production of antigen-specific T cells for preclinical and clinical applications using gas-permeable rapid expansion cultureware (G-Rex). *J Immunotherapy* 33 (3), 305-315
15. Bollard CM, Gottschalk S, Huls H, Leen AM, Gee AP & Rooney CM (2011) Manufacture of GMP-grade cytotoxic T lymphocytes specific for LMP1 and LMP2 for patients with EBV-associated lymphoma. *Cytotherapy* 13: 518-522
16. Lapteva N & Vera JF (2011) Optimization manufacture of virus- and tumor-specific T cells. *Stem Cells International* 434392. doi: 10.4061/2011/434392434392
17. Sili U, Leen AM, Vera JF, Gee AP, Huls H, Heslop HE, Bollard CM & Rooney CM (2012) Production of good manufacturing practice-grade cytotoxic T lymphocytes specific for Epstein-Barr virus, cytomegalovirus and adenovirus to prevent or treat viral infections post-allogeneic hematopoietic stem cell transplant. *Cytotherapy* 14: 7-11
18. McLaughlin LP, Gottschalk S, Rooney CM & Bollard CM (2017) EBV-directed T cell Therapeutics for EBV-associated lymphomas. *Methods Mol Biol* 1532: 255-265
19. Cox MA, Kahan SM & Zajac AJ (2013) Anti-viral CD8 T cells and the cytokines that they love. *Virology* 435, 157-169
20. Vickers MA, Wilkie GM, Robinson N, Rivera N, Haque T, Crawford DH, Barry J, Fraser N, et al (2014) Establishment and operation of a Good Manufacturing Practice-compliant allogeneic Epstein-Barr virus (EBV)-specific cytotoxic cell bank for the treatment of EBV-associated lymphoproliferative disease. *Br J Haematol* 167, 402-410
21. Campbell JDM & Fraser AR (2018) Flow Cytometric Assays for Identity, Safety and Potency of Cellular Therapies. *Cytometry Part B (Clin Cytom)* 94B:569-579
22. Pennock ND, White JT, Cross EW, Cheney EE, Tamburini BA & Kedl RM (2013) T cell responses: naive to memory and everything in between. *Adv Physiol Education* 37 (4), 273-283
23. Kared H, Martelli S, Ng TP, Pender SL & Larbi A (2016) CD57 in human natural killer cells and T-lymphocytes. *Cancer Immunol Immunother* 65:441-52
24. Bajgain P, Mucharla R, Wilson J, Welch D, Anurathapan U, Liang B, Lu X, Ripple K, et al (2014) Optimizing the production of suspension cells using the G-Rex "M" series. *Molecular Therapy – Methods & Clinical Development* 1, 14015

25. Busch DH, Fräßle SP, Sommermeyer D, Buchholz VR & Riddell SR (2016) Role of memory T cell subsets for adoptive immunotherapy. *Semin Immunol* 28:28-34.
26. Klebanoff CA, Gattinoni L & Restifo NP (2012) Sorting through subsets: which T-cell populations mediate highly effective adoptive immunotherapy? *J Immunother* 35:651-60.
27. Shourian M, Beltra JC, Bourdin B & Decaluwe H (2019) Common gamma chain cytokines and CD8 T cells in cancer. *Semin Immunol* . 42:101307.
28. Blank CU, Haining WN, Held W, Hogan PG, Kallies A, Lugli E, Lynn RC, Philip M, et al (2019) Defining 'T cell exhaustion'. *Nat Rev Immunol* . 2019 Sep 30. doi: 10.1038/s41577-019-0221-9. [Epub ahead of print]
29. Merlo A, Turrini R, Trento C, Zanovello P, Dolcetti R & Rosato A (2010) Impact of γ -chain cytokines on EBV-specific T cell cultures *J Transl Med* 8: 121 doi: 10.1186/1479-5876-8-121
30. Icheva V, Kayser S, Wolff D, Tuve S, Kyzirakos C, Bethge W, Greil J, Albert MH, et al. (2013) Adoptive transfer of epstein-barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. *J Clin Oncol* 31:39-48.
31. Horlock C, Skulte A, Mitra A, Stansfield A, Bhandari S, Ip W, Qasim W, Lowdell MW, et al (2016) Manufacture of GMP-compliant functional adenovirus specific T-cell therapy for treatment of post-transplant infectious complications. *Cytotherapy* 18: 1209-1218
32. Gerdemann U, Katari UL, Papadopoulou A, Keirnan JM, Craddock JA, Liu H, Martinez CA, Kennedy-Nasser A, et al (2013) Safety and clinical efficacy of rapidly-generated trivirus-directed T cells as treatment for adenovirus, EBV, and CMV infections after allogeneic hematopoietic stem cell transplant. *Mol Therapy* 21: 2113-2121
33. Tzannou I, Papadopoulou A, Naik S, Leung K, Martinez CA, Ramos CA, Carrum G, Sasa G, et al (2017) Off-the-Shelf Virus-Specific T Cells to Treat BK Virus, Human Herpesvirus 6, Cytomegalovirus, Epstein-Barr Virus, and Adenovirus Infections After Allogeneic Hematopoietic Stem-Cell Transplantation. *J Clin Oncol* 35:3547-3557.
34. Khalaf WS, Garg M, Mohamed YS, Stover CM, Browning MJ. (2019) In vitro Generation of Cytotoxic T Cells With Potential for Adoptive Tumor Immunotherapy of Multiple Myeloma. *Front Immunol*. 10:1792. doi: 10.3389/fimmu.2019.01792.
35. Chicaybam L, Abdo L, Carneiro M, Peixoto B, Viegas M, de Sousa P, Fornazin MC, Spago MC et al. (2019) CAR T Cells Generated Using Sleeping Beauty Transposon Vectors and Expanded with an EBV-Transformed Lymphoblastoid Cell Line Display Antitumor Activity In Vitro and In Vivo. *Hum Gene Ther*. 30:511-522. doi: 10.1089/hum.2018.218.
36. Sánchez-Martínez D, Azaceta G, Muntasell A, Aguiló N, Núñez D, Gálvez EM, Naval J, Anel A, et al (2014) Human NK cells activated by EBV(+) lymphoblastoid cells overcome anti-apoptotic mechanisms of drug resistance in haematological cancer cells. *Oncoimmunology* .4:e991613. doi: 10.4161/2162402X.2014.991613.
37. Cooper RS, Fraser AR, Smith L, Burgoyne P, Imlach SN, Jarvis LM, Zahra S, Turner ML, Campbell JDM (2020) Rapid GMP-compliant expansion of SARS-CoV-2-specific T cells from convalescent donors for use as an allogeneic cell therapy for COVID-19. *BioRxiv*. doi: <https://doi.org/10.1101/2020.08.05.23786>.

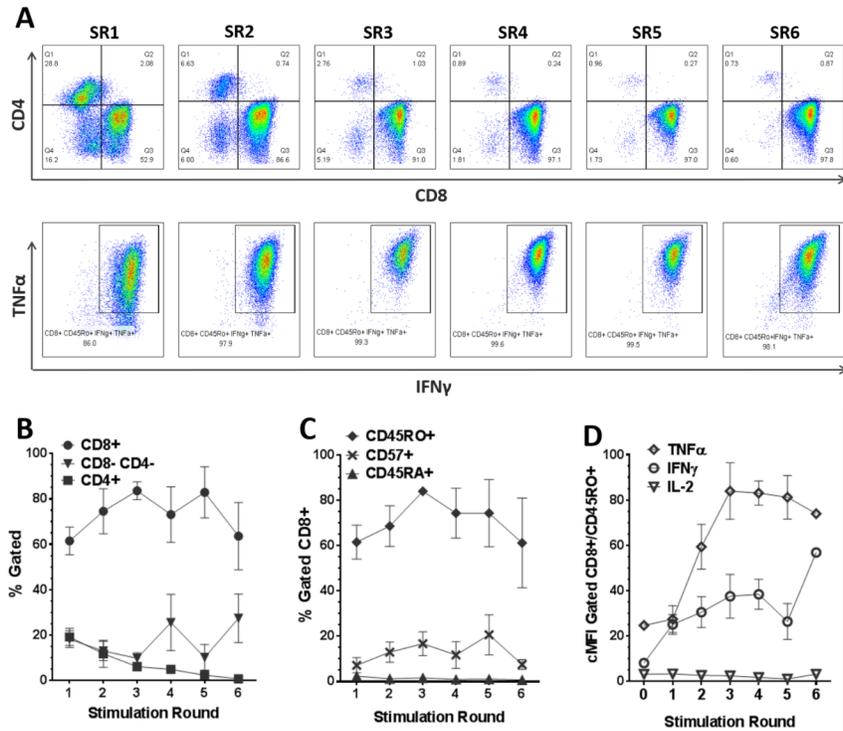


Figure 1. Sequential LCL stimulation affects EBV-specific T cell development . Samples were taken 7-9 days after each stimulation round (SR) from six different donor CTL lines. (A) The T cell ratio shows rapid transformation to a highly-enriched CD8+ T cell product by stimulation round 3-4, with most gated CD8+/CD45RO+ cells co-expressing IFN- γ and TNF- α by stimulation round 2, peaking at SR3. Flow plots are from representative CTL line NZ873. The mean percentage of (B) T cell subpopulations (CD8+, CD4+, CD8-/CD4-) and (C) CD45RO+, CD45RA+ and CD57+ in CD8 cells at each SR (n=6) is shown, demonstrating consistent development of CD8+ enriched products and consistent differentiation status by SR3. (D) The mean cMFI of IFN- γ , TNF- α and IL-2 expression in CD8+/CD45RO+ cells reaches a plateau by SR3 then decreases, indicating that peak functionality occurs within 3 stimulation rounds. Data are represented as means \pm SEM.

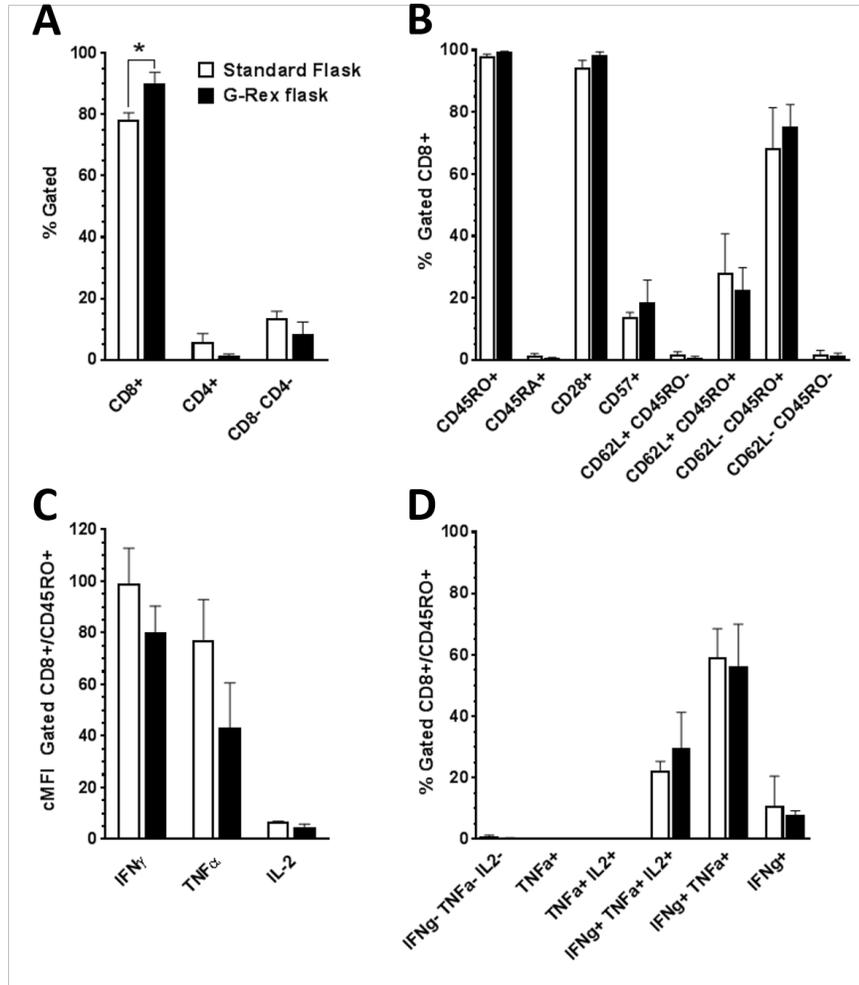


Figure 2. Comparison of culture vessel for T cell products . Co-cultures of apheresis MNC and autologous LCLs were set up (n=6 donors) to compare culture in Corning flasks versus G-Rex100 flasks through an optimized three stimulation round protocol. Harvested samples were compared for phenotypic and functional profiles as before. (A) Cultures from G-Rex flasks had a significantly higher percentage of CD8+ cells than standard flask grown lines (p<0.05). (B) CD8+ cell phenotype was consistent between both culture methods for all markers analysed. Intracellular analysis of the gated CD8+/CD45RO+ cells showed no difference between standard flask and G-Rex cultured T cells for (C) expression of cytokines by cMFI or (D) relative frequencies of differentiated subpopulations by cytokine expression. Data are represented as means \pm SEM.

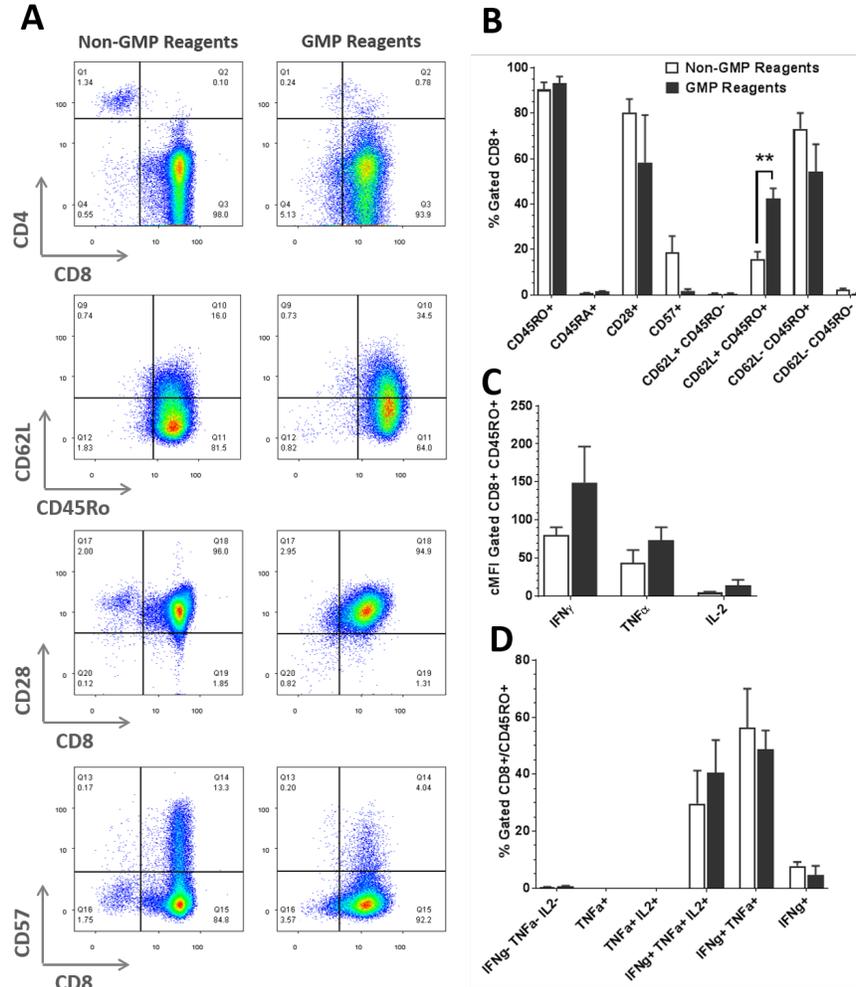


Figure 3 . GMP-compliant reagents improve T cell phenotype and function . Samples taken at day 30 harvest from G-Rex10 flasks (stimulation round 3 + 9 days) analysed as before. (A) Direct comparison of representative line LAD1 cultured using GMP-compliant reagents and standard culture reagents demonstrated both T cell cultures had comparable expression of most markers, but reduced CD57 expression in GMP-compliant process. (B) Analysis of mean surface marker expression showed a significantly higher percentage of CD62L+/CD45RO+ Central Memory T cells in GMP-compliant culture (n=4) compared to standard (n=4, p=0.002). Intracellular staining showed no significant difference in (C) cytokine expression between the two groups, or (D) relative frequency of differentiated T cell subpopulations by cytokine expression. Data are represented as means \pm SEM.

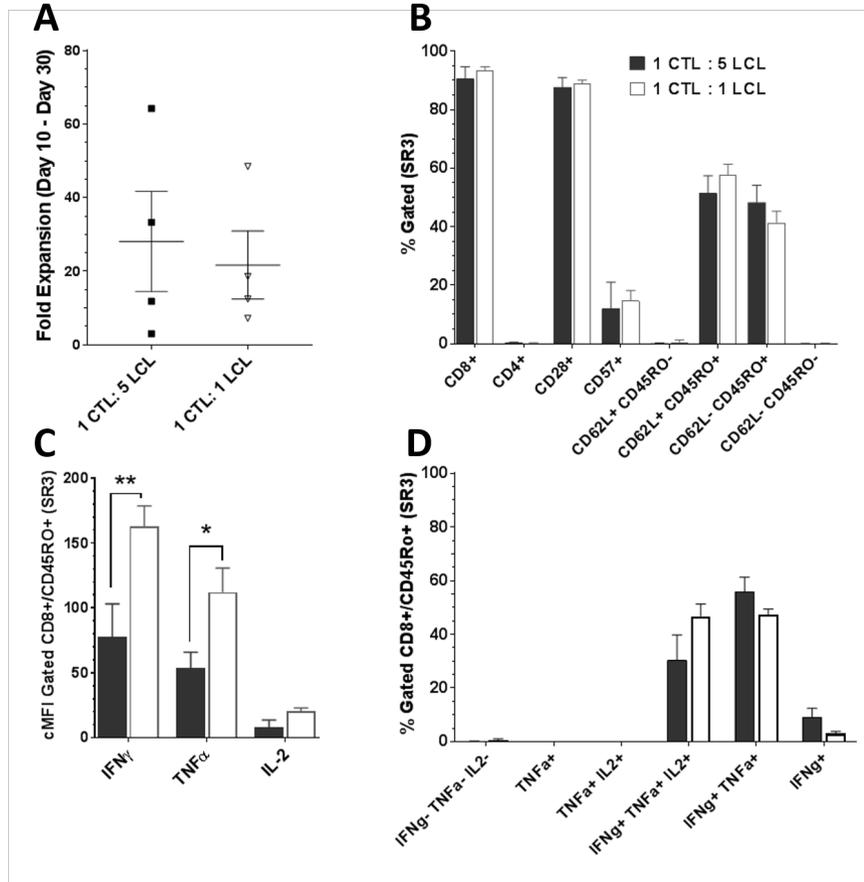


Figure 4 . Modifying CTL: LCL ratio improves cytokine expression . T cell cultures from n=4 donors were split to directly compare stimulation round (SR) 2 and 3 of co-culture at standard (1 T cell : 5 LCL), or reduced intensity (1 T cell : 1 LCL). (**A**) Fold expansion of viable lymphocytes between SR2 initiation (day 10) and end of SR3 (day 30) was calculated from viable cell count. (**B**) Surface phenotype of final products remain comparable between the two groups for all markers analyzed. (**C**) Reduced intensity culture had a significantly higher cMFI of IFN- γ (p=0.00218) and TNF- α (p=0.0202) in response to stimulation (corrected to negative controls) compared to the standard ratio cultures. (**D**) In CD8+/CD45RO+ T cells, there was an even distribution of Central Memory and Effector Memory compartments with reduced intensity conditioning, and a skew towards Effector Memory in standard process. Data is represented as mean \pm SEM.

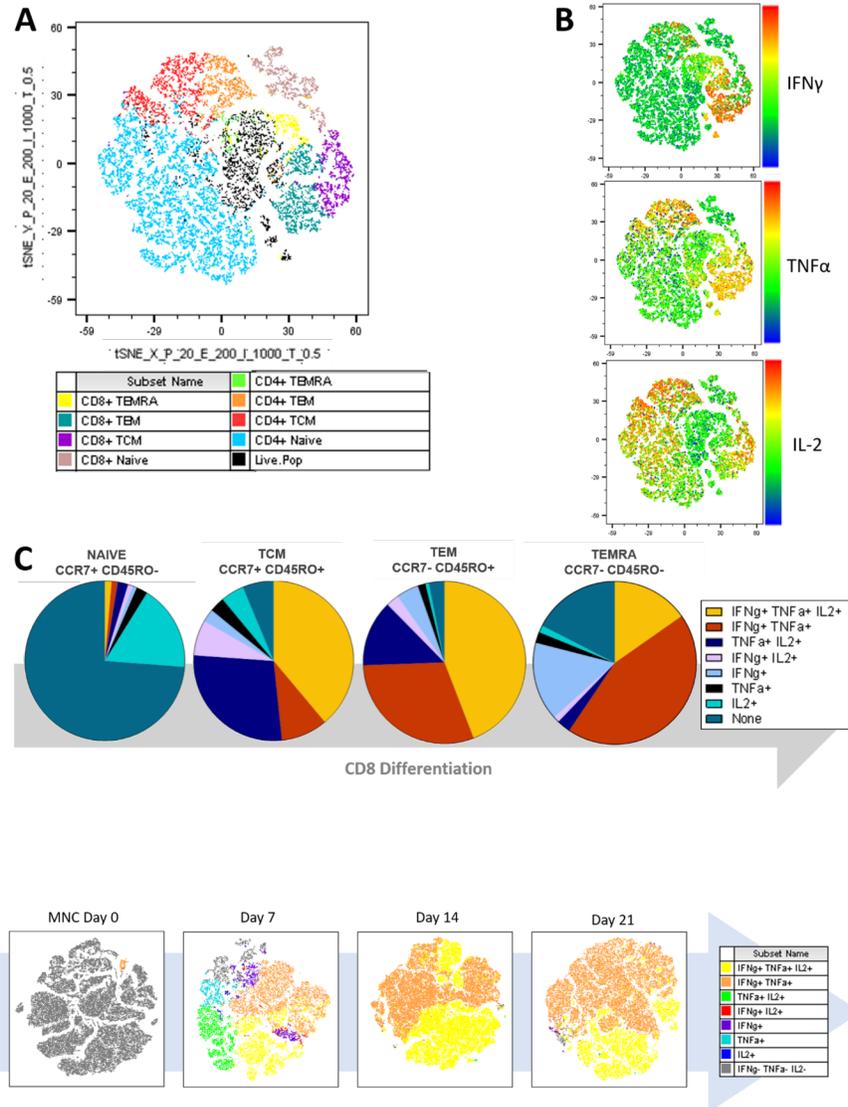


Figure 5. Analytic phenotyping of T cell memory subpopulations. Differentiation profiles of the T cell compartment were identified by combination of surface marker and intracellular cytokine detection. Donor PBMCs freshly isolated from buffy coats were stimulated to induce cytokine expression, and CD8+ and CD4+ populations were gated into T cell memory subsets dependent on surface marker expression: naive (CCR7+/CD45RO-), T_{CM} (CCR7+/CD45RO+), T_{EM} (CCR7-/CD45RO+) and T_{EMRA} (CCR7-/CD45RO-). Stochastic Neighbour Embedding (t-SNE) analysis was used to cluster cellular sub-populations. **(A)** Manual gating overlays of an exemplar demonstrate the distribution of CD8 and CD4 memory subtypes in the total lymphocyte population. **(B)** The data were also analyzed for individual cytokines by fluorescence intensity. Each subtype was then quantified for expression of single or multiple cytokines as outlined in the gating strategy. **(C)** The relative frequency of cytokine subpopulations from representative PBMC demonstrates changes in cytokine expression throughout T cell differentiation (mean of n=6). **(D)** Exemplar of t-SNE analysis of a T cell product from initial PMBC fraction through to final product indicating the accumulation of TCM / TEM CD8 T cells over time.