The stimulation and expansion of antigen-specific T cells in a single closed environment using the Xuri™ Cell Expansion System

The Xuri Cell Expansion System is used as an alternative to static cell culture methods for the stimulation and expansion of antigen-specific T cells (ASTs). The automated process includes a semi-static phase (low rocking/angle) for antigen stimulation, a rocking phase for cell expansion, and a feeding regimen with perfusion to maintain optimal culture conditions in a single closed environment. Phenotypic analysis of expanded ASTs shows the Xuri Cell Expansion System provides a viable alternative to traditional static culture techniques while also removing the time-consuming sample manipulations and minimizing the risk of contamination commonly associated with open systems.

Introduction

Administration of ASTs, in particular for viral infections in transplantation, is a key focus in immunotherapy. Solid organ and hematopoietic stem cell transplantations have been performed over decades. However, viral infections, most commonly with Adenovirus (Adv), Cytomegalovirus (CMV), or Epstein-Barr virus (EBV) can cause major complications after transplantation due to the severely compromised immune system of the patient. Adoptive transfer of virus-specific T cells has been shown to prevent and treat viral complications (1–4), offering an alternative treatment to conventional viral drugs, which are often ineffective and toxic.

The generation of ASTs is usually achieved by *in vitro* stimulation with the antigen presenting cells (APCs) that express the antigen or peptide of interest in the presence of one or more cytokines. This process results in an increased frequency of T cells specific for the antigen and a decrease of T cells with adverse specificities.

Standard culturing protocols for ASTs require an initial stage of cell-to-cell contact for T cell stimulation. Minimal movement of the cell suspension is necessary to enable these critical cell interactions, and thus static culture using flasks or static culture bags are typically used. Static culture methods support extended physical cell contact that can promote T cell stimulation against the antigen. However, as the cells proliferate, additional culture vessels are required, therefore the number of vessels and culture volume increase with time. Due to the complexity of the workflow and the need to manipulate multiple vessels, experienced staff are required to perform time-consuming and cumbersome procedures to reduce the risk of human error and contamination.

For the production of a regulatory compliant cell therapy, adaptation of these conventional protocols to a more automated and contained process is required. Various bioreactor systems, including rocking platforms, have been used by several clinical research groups for the production of genetically modified T cells and tumor infiltrating lymphocytes (TILs) (5). The rocking bioreactors have been considered beneficial as they support the standardization and scale-up of cell therapy production in a manufacturing setting. Use of a closed bioreactor system for all phases of the culturing process is a significant advantage because the cells are contained in a single vessel thereby minimizing the risk of contamination due to adventitious agents and operator error.

The process described here demonstrates use of the Xuri Cell Expansion System from the initiation to the generation of virus-specific T cells. The data show that Xuri Cell Expansion System provides a functionally closed environment suitable for the expansion of ASTs.

Materials and methods

Initiation of culture

Fresh peripheral blood mononuclear cells (PBMCs) from CMV seropositive healthy donors were isolated using Ficoll-Paque™ PLUS (GE Healthcare). A portion of the PBMCs were kept for initiation of culture (~ 300 × 10^6 cells) and the remaining cells were cryopreserved in 90% heat-inactivated human serum (TCS Biosciences) + 10% DMSO (Sigma) for later use as autologous feeders.

A starting cell number of 300 to 350 × 10^6 cells were re-suspended in 10 mL complete medium: X-VIVO™ 10 medium (Lonza) supplemented with 5% heat-inactivated human serum, 2 mM GlutaMAX™ L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies), and 300 units/mL IL-2 (GE Healthcare). The cells were pulsed with HCMVA PepMix™ PM-IE1 (JPT) and HCMVA PepMix PM-pp65 (JPT) at 10 ng/15 × 10^6 cells in a 50 mL Falcon™ tube for 2 h at 37°C.

A 2 L Cellbag™ bioreactor (GE Healthcare) was placed on either a Xuri Cell Expansion System W5 or W25 (GE Healthcare), filled with 5% CO_2 and a reservoir containing complete media and a waste bag was connected. An initial volume of between 200 and 250 mL complete media was added to the bioreactor and allowed to equilibrate to 37°C for 2 h.

After 2 h, 300 × 10^6 of the peptide-pulsed cells were inoculated into the bioreactor and additional medium added, giving a total of 300 mL with 1 × 10^6 cells/mL per bioreactor. The cells were cultured in semi-static conditions of 2 rocks per minute (rpm) at a 2° angle and gas flow rate of 0.05 Lpm for 3 to 5 days. To minimize evaporation of the culture media during the semi-static phase the Cellbag bioreactors were either wrapped in aluminium foil (for the Xuri Cell Expansion System W5) or the lid of the instrument was used (for the Xuri Cell Expansion System W25).

Once initiated, the bioreactors were left undisturbed (i.e., no sampling) for 3 days to allow cell to cell interactions to occur. From day 3, fresh medium was added to the bioreactor, reaching between 400 and 500 mL by day 7. Daily sampling was performed for cell number and biochemical analysis (lactate, ammonium, glucose, and pH). From day 5 or 6, the rocking rate was increased to 4 rpm and the angle to 4° until day 7. 24 h before the addition of feeders for re-stimulation, 200 mL of media was perfused to control lactate, ammonium, and glucose levels.

Re-stimulation and expansion of cells

On day 7, the cultures were re-stimulated with autologous peptide-pulsed feeders. Cryopreserved autologous PBMCs were thawed in complete medium, irradiated at 30 Gy and pulsed for 2 h with the PepMixes pp65 and IE-1 (10 ng/15 × 10^6 cells). The cells were added to Cellbag bioreactors at a ratio ranging between 1:10 and 1:1 feeders to T cells. The cultures were incubated for 3 days without interference at 2 rpm and a 2° angle. From day 10, Cellbag bioreactors were fed-batched to maintain at least 1 × 10^6 cells/mL until the maximum volume of 1 L was obtained. Thereafter, a perfusion rate of 200 to 700 mL/24 h was adjusted according to the metabolite readings to control glucose and the waste metabolites lactate and ammonium. The gas flow was increased to 0.1 to 0.2 Lpm, when the working volume reached 500 mL. Daily monitoring, cell counts and biochemical analysis were carried out. From day 11 the rocking rate and angle was increased gradually every day to 4 rpm/4°, 8 rpm/6°, 10 rpm/6° (2 to 20 × 10^6 cells/mL) and 12 rpm/6° if the cells were above 20 × 10^6/mL.

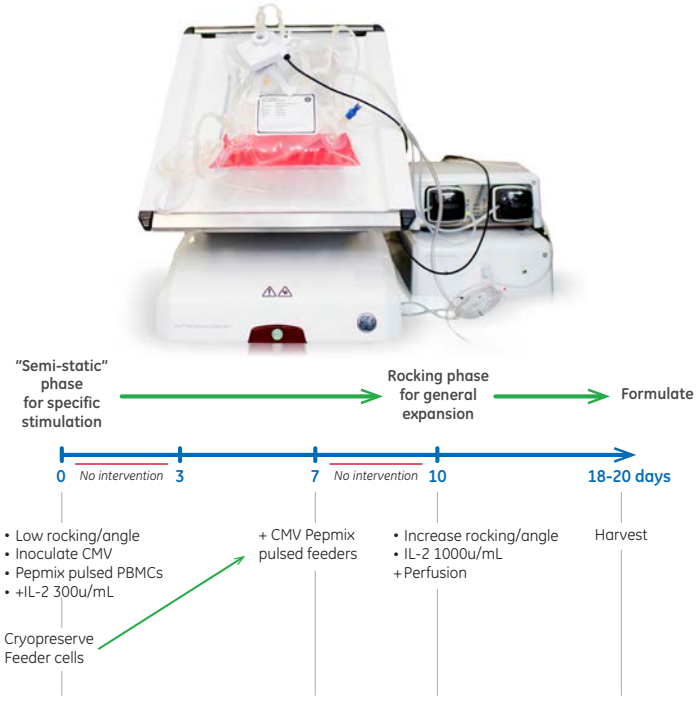

Fig 1. General workflow for the generation of ASTs directly in Xuri Cell Expansion System W25.

Phenotypic and functional analysis

Samples for flow cytometry analysis were taken and cryopreserved on days 0, 7, 15, and 18. Cells were thawed and stained with antibody panels detecting immunophenotypic markers for lymphocyte populations, T cell memory, activation, and exhaustion. 1 × 10^6 cells were stained with CD3-PerCP-Cy™5.5 (BD Biosciences), CD4-V500 (BD Biosciences), or –PE (BD Biosciences), CD8-Alexa Fluor™ 488 (BD Biosciences) together with CD56-PE (BD Biosciences) and CD19-APC (BD Biosciences), or CCR7-PE (R&D Systems) and CD45RO-PECy7 (BD Biosciences). For analysis of cytokine expression the cells were divided into negative control (cells+PBMC), along with testing for specificity (cells+PBMC+PepMix peptide pools). These were incubated overnight at 37°C. A cytokine secretion assay was carried out to test for antigen specificity using the MACs™ IFN Secretion Assay - Detection Kit, according to manufacturing instructions (Miltenyi Biotec). The stained cells were analyzed on a FACS™ Fortessa flow cytometer using FACSDiva™ software, according to the manufacturer's instructions (BD Biosciences).

Results

Cell growth and culturing conditions in Xuri Cell Expansion Systems

A process has been developed for the expansion of ASTs in Xuri Cell Expansion Systems. This process was evaluated using PBMC from CMV-seropositive donors in the presence of CMV peptide pools (IE-1 and pp65). Four PBMC cultures were initiated with a concentration of approximately 1.0 × 10^6/mL in 300 mL using Cellbag bioreactors on the Xuri Cell Expansion System W25 (Donors 1 and 3) and W5 (Donors 2 and 4) with low rocking rate and angle. Two modes of operation were applied to the cultures: semi-static phase (2 rpm/2°) during the steps requiring cell-to-cell interactions (days 0 to 3 and 7 to 10) and a rocking phase with increasing rocking rates and angles depending on the cell number and volume (4 to 12 rpm/4° to 6°) for a general expansion (from day 10). To avoid disturbing the cell-to-cell contact, the Cellbag bioreactors were left undisturbed (i.e., no sampling) for 3 or 4 days after inoculation and re-stimulation steps. Both Xuri Cell Expansion Systems (W5 and W25) supported the growth of cells, with final cell numbers ranging from 2 × 10^9 to 2.3 × 10^{10}. High viability was maintained throughout the culture. Diverse growth kinetics was observed between donors, which reflect donor variation. Nevertheless, an overall increase in cell number occurred for all donors after re-stimulation with APCs as they entered their expansion phase (Fig 2).

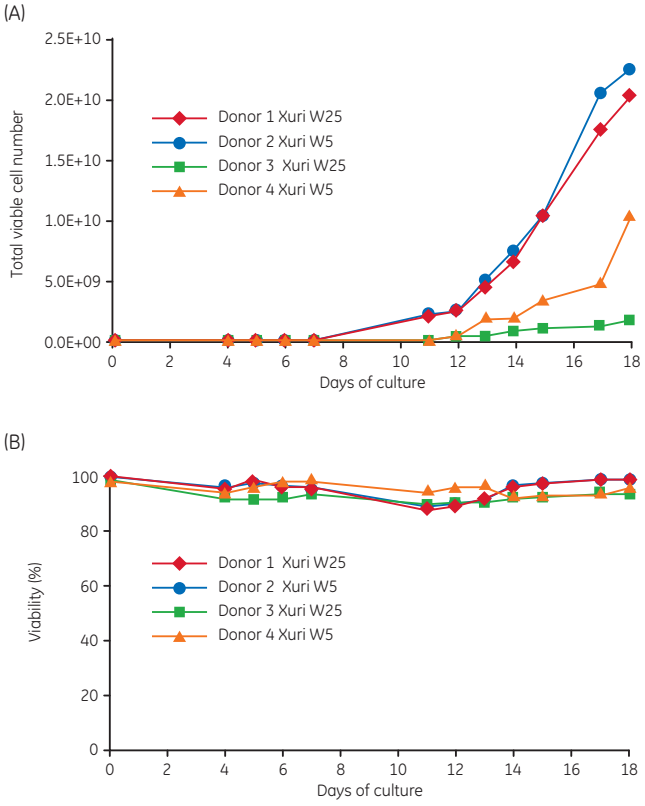

Fig 2. (A) Total cell numbers, and (B) percentage viability of cells grown in Cellbag bioreactors. Cells from Donors 1 and 3 were cultured in Xuri W25 systems and Donors 2 and 4 in Xuri W5 systems.

A medium addition and perfusion regimen were performed at defined periods during culture, driven according to cell number and to maintain an optimal culture environment. Medium was added during culture days 3 to 7 increasing the volume from the initial 300 mL to reach 400 to 500 mL to circumvent evaporation and control osmolality and waste metabolites. Small volumes (e.g., 50 to 100 mL) were added at any one time, keeping the temperature ≥36°C. The volume of medium added per day was dependent on the cell concentration. Besides media addition, perfusion should also be considered during the early stages if high osmolality and waste metabolites accumulate in the culture. The accumulation of waste metabolites is detrimental to the culture and affects T cell proliferation (6). A perfusion rate of 200 to 250 mL/day should be set for 24 to 48 h before re-stimulation to ensure optimal culturing conditions during this semi-static phase. From day 11, perfusion was then continuously set to control the high levels of waste metabolites, which coincided with an increased proliferation rate of the cells after re-stimulation (Fig 3).

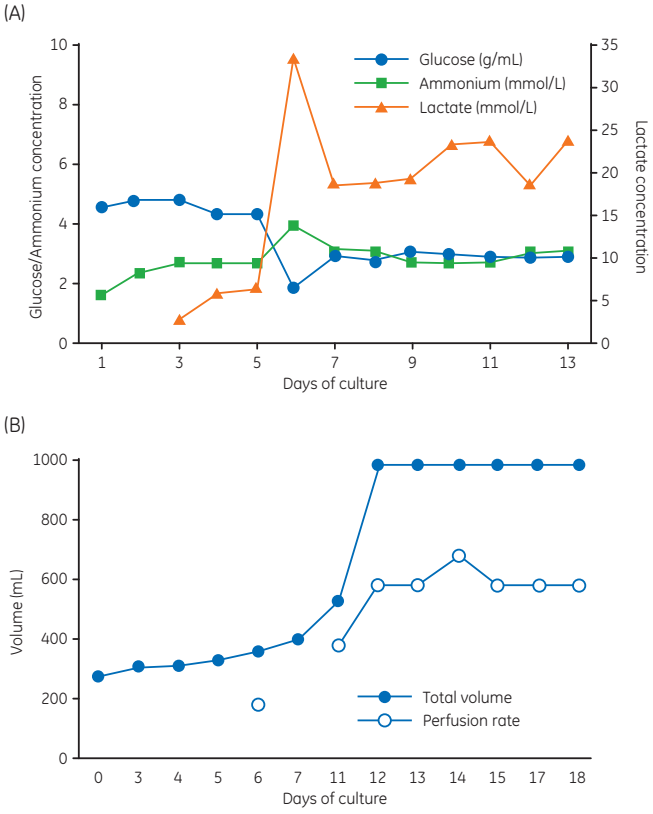

Fig 3. (A) Concentration of metabolites glucose (g/L), lactate (mmol/L), and ammonium (mmol/L), and (B) Total volume and perfusion rates throughout culture. Representative data are shown for culture from Donor 1 in the Xuri W25 system.

Immunophenotyping and functionality of cells grown in the Xuri Cell Expansion System

The expression pattern of several cell surface molecules was assessed by flow cytometry to determine the phenotypic composition throughout the culturing period in the Cellbag bioreactors. The percentages of the lymphocyte populations of NK cells (CD56+CD3-), B cells (CD19+), and T cells (CD3+) were evaluated throughout the culturing period. CD3+ T cells were expanded in all bioreactors, with a corresponding decrease of B cells and NK cells (Fig 4).

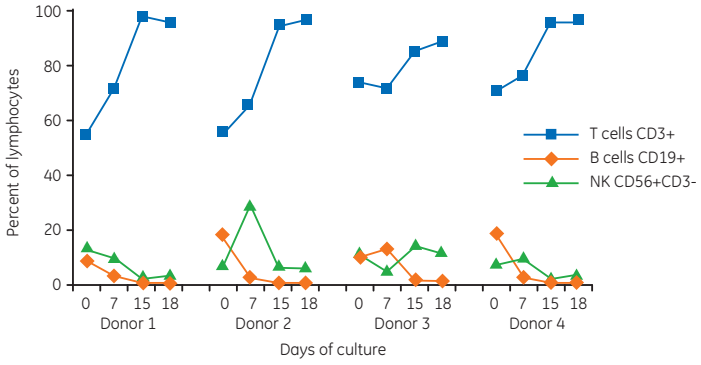

Fig 4. Frequency of the peripheral blood lymphocyte populations throughout the culture period in the Xuri W5 and W25 systems. The number of positive cells was determined by flow cytometry (FACS Fortessa). Lymphocytes were gated based on their forward and side scatter profile. Percentage B cells (CD19+), CD3+ T cells, NK (CD56+CD3-), and NKT-like cells (CD3+CD56+) are shown within the lymphocyte population.

The proportion of CD4 and CD8 T cell populations were also evaluated in the cultures and found to be diverse and donor-dependent. These results indicate that the Xuri Cell Expansion System has the ability to promote growth of CD4 and CD8 T cell populations using these culture conditions. At the end of the expansion, for Donors 2 and 3 there was an increase in CD8 population whilst for Donor 1 the proportion of CD4+ and CD8+ T cells was maintained and for Donor 4 an increase of CD4 population was seen (Fig 5).

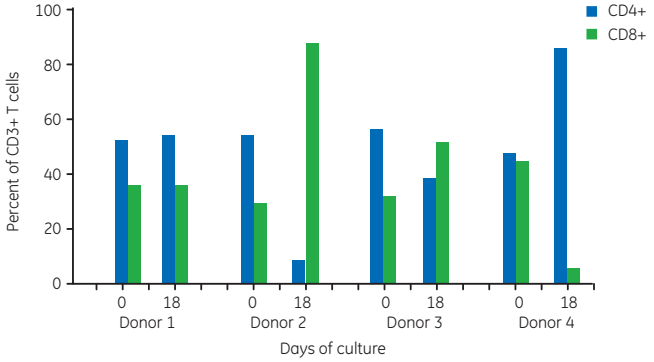

Fig 5. Frequency of CD4+ and CD8+ T cells throughout the culture period in the Xuri W5 and W25 systems. Percentages of these populations are shown within the combined lymphocyte/CD3 populations.

The T cell memory subsets were defined by dividing both CD4 and CD8 compartments on the basis CD45RO and CCR7 into naive (CCR7+CD45RO-), central memory (CCR7+CD45RO+), effector memory (CCR7-CD45RO+), and terminally differentiated effector cells (CCR7-CD45RO-). The subsets were evaluated throughout the culture period. Flow cytometry analysis revealed that although there was a donor-dependent heterogeneity at the beginning of the culture, most of the CD4 and CD8 T cells became effector memory (CCR7-CD45RO+) at the end of the culture for all the donors and no accumulation of terminally differentiated effectors cells was seen (Fig 6).

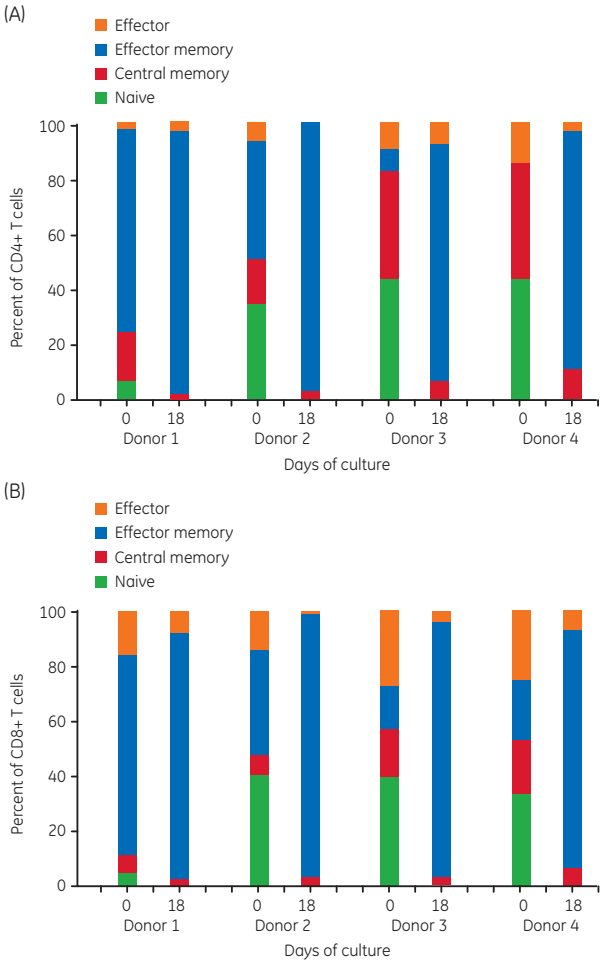

Fig 6. (A) CD4+, and (B) and CD8+ T cell memory subsets throughout the culture period in Xuri W5 and W25 systems. The number of positive cells was determined by flow cytometry (FACS Fortessa). Lymphocytes were gated based on their forward and side scatter profile. T cells were gated on (A) CD3+ CD4+, or (B) CD3+CD8+. T cells subsets were defined based on the expression of CCR7 and CD45RO. Naïve/ central memory "stem like" (CCR7+CD45RO-), Central Memory (CM) (CCR7+CD45RO+), Effector Memory (EM) (CCR7-CD45RO+), and Effector (EMRA) (CCR7-CD45RO-).

The ability of T cells to produce cytokine IFNγ was investigated by cytokine secretion assay using flow cytometry. The frequency of non-specific IFNγ producing T cells (T cells+autologous PBMC) and CMV-specific IFNγ secreting T cells [(T cells+PBMC+CMV peptide) – (T cells+PBMC)] were examined (Figs 7 and 8). An increase in CMV-specific T cells from a very low starting frequency of positive cells (<0.6% and <0.8% for CD4+ T cells and CD8+ T cells, respectively) was observed in all the cultures (Fig 8). The Xuri Cell Expansion Systems supported expansion of the CMV-specific T cell populations for all the donors, with >10 fold-increase of specificity, ranging from a 12.4 to 462.4 fold increase for CD4+ T cells and 49.9 to 183.9 fold increase for CD8+ T cells.

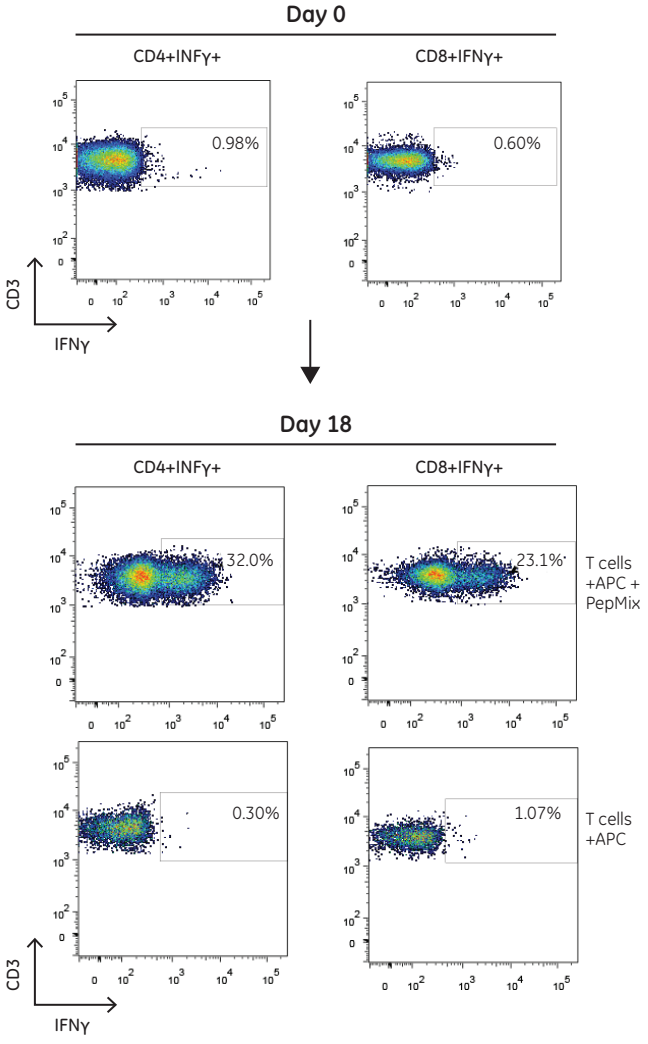

Fig 7. Representative flow cytometric plots for IFNγ secretion of CD8+ T cells and CD4+ T cells on day 0 and day 18 for Donor 1. T cells were either stimulated with PBMC (APC) alone (non-specific IFNγ secreting T cells, negative control) or with APC+CMV PepMixes (test for specificity).

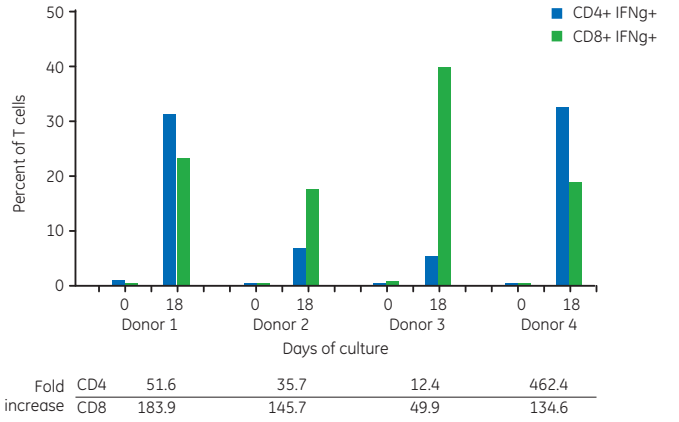

Fig 8. IFNγ secretion of CD8+ T cells and CD4+ T cells throughout the culture in Xuri W5 and W25 systems. Percentage of CD4+ or CD8+ T cells is shown on the graph and fold increase in specificity is shown for all the donors below graph. T cells were either stimulated with PBMC alone (non-specific IFNγ secreting T cells) or with PBMC+CMV PepMixes (test for specificity). CMV-specific T cells = (T cells+PBMC+CMV peptide) – (T cells+PBMC).

Conclusion

The Xuri Cell Expansion System has been used for all stages of AST culture to create an optimized procedure for the generation of ASTs in a closed culturing system. The process described here is scalable and enables the translation of T cell therapies beyond the academic level. The Xuri platform does not require additional open vessels and allows a cell therapy manufacturing process to scale-up in an automated, controlled, and optimized environment, without compromising the cell culture product.

The flexibility of the Xuri rocking platform allowed the stimulation of cells at low rocking rate and angle (semi-static phase) and the increased rocking rate and angle (rocking phase) supported the expansion of high cell numbers suitable for cell therapy doses. Although there was heterogeneity within the initial donor population, all the cultures were able to achieve a high number of cells of interest with a memory T cell phenotype. The cultures have shown an increased frequency of antigen-specific T cells at the end of the culturing period. This study demonstrates that the initial culturing phase can be achieved using a semi-static setting and the entire culture can be performed in a single functionally closed platform. Using the semi-static approach, the Xuri Cell Expansion System supports not only the generation of ASTs but has the potential to be adapted to other cell culture procedures that require extended static steps during the culture period.

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