Leukoreduction system chambers are a reliable cellular source for the manufacturing of T-cell therapeutics

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BACKGROUND: Following solid organ or hematopoietic cell transplantation, refractory opportunistic viral reactivations are a significant cause of morbidity and mortality but can effectively be controlled by virus-specific T-cell transfer. Among effective and safe strategies is the use of "third-party" (neither from the transplant donor nor recipient) virus-specific T cells that can be manufactured from healthy donors and used as "off-the-shelf" therapies. Leukoreduction system chambers (LRSCs), recovered after routine plateletpheresis, were evaluated as a potential source of peripheral blood mononuclear cells (PBMCs) for the manufacturing of clinical-scale virus-specific T cell.

STUDY DESIGN AND METHODS: PBMCs from the same donors obtained either from LRSCs or peripheral blood were compared, focusing on T-cell function and phenotype as well as the potential to generate cytomegalovirus (CMV)-specific T-cell lines from both CMV seropositive and seronegative donors.

RESULTS: PBMCs from both sources were comparable except for a transient downregulation of CD62L expression on freshly extracted PBMCs from LRSCs. Both nonspecific stimulation using anti-CD3/CD28 antibodies and CMV peptides revealed that LRSCs or blood T cells were equivalent in terms of expansion, differentiation, and function. Moreover, PBMCs from LRSCs can be used to generate autologous monocyte-derived dendritic cells to prime and expand CMV-specific T cells from seronegative donors.

CONCLUSION: LRSCs are a reliable source of PBMCs for the generation of virus-specific T cells for immunotherapy. These findings have implications for the development of third-party therapeutic T-cell products from well-characterized blood product donors.

Blood banks apply universal leukoreduction to all blood products to reduce adverse events associated with the transfusion of blood products.[1–4] Héma-Québec's leukoreduced platelet concentrates are produced with apheresis instruments. During the past year, more than 36,000 leukoreduced platelet concentrates were produced, generating as many leukoreduction system chambers (LRSCs), which were discarded at the end of the process. LRSCs contain high numbers of peripheral blood mononuclear cells (PBMCs) and are a reliable source of human cells for research. As such, LRSCs are an alternative to peripheral blood collections or apheresis for the study of B cells, monocytes, dendritic cells (DCs), stem cells,

ABBREVIATIONS: CMV = cytomegalovirus; DCs = dendritic cells; IFN-γ = interferon-γ; IL = interleukin; LRSCs = leukoreduction system chambers; moDCs = monocyte-derived dendritic cells; PBMCs = peripheral blood mononuclear cells; PHA = phytohemagglutinin; TNF-α = tumor necrosis factor-α.

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and T cells.[2,5–10] Regular platelet apheresis donors undergo extensive testing for transmissible diseases, are often well characterized in terms of human leukocyte antigen (HLA) typing as well as serostatus for common latent pathogens. As such, PBMCs harvested from LRSCs following collection of platelets by apheresis may be exploitable as a safe source of PBMCs for therapeutic applications.

Over the past 2 decades, several immunotherapeutic approaches involving the ex vivo manipulation and expansion of immune cells, particularly T cells, have been developed. While some of these adoptive immunotherapy strategies aim primarily at expanding naturally occurring T cells,[11,12] others rely on gene engineering to impart new properties to T cells.[13,14] Both autologous and allogeneic cellular sources have been used to generate potent immunotherapeutic products. The possibility of using allogeneic T-cell products offers many new opportunities such as the manufacturing of large and well-characterized lots, as well as the development of cell banks enabling the rapid delivery of T-cell immunotherapeutics.[15–18] The PBMCs contained in LRSCs used to filter blood products obtained from regular and well-characterized platelet donors may be an ideal source for the manufacturing of such therapies.

Solid organ transplantation and allogeneic hematopoietic cell transplantation are, respectively, the best therapeutic option for end-stage organ dysfunction and several refractory hematopoietic cancers, bone marrow failure syndromes, and primary immunologic diseases.[19–21] The mandatory use of immunosuppression to prevent graft rejection or graft-versus-host disease predisposes patients to several infections. Opportunistic viral reactivations from established latent viruses (or transferred through the graft) are especially challenging in the context of compromised T-cell immunity. Among others, the reactivation of herpes family viruses such as cytomegalovirus (CMV), Epstein–Barr virus and human herpesvirus 6, as well as polyomaviruses such as BK virus, are a significant burden in transplantation, impacting both morbidity and mortality rates.[22–25] While reducing immunosuppression and administering antiviral drugs remain the cornerstone of current treatments for viral reactivations after transplantation, adoptively transferred virus-specific T cells have yielded remarkable results for the prevention and control of virus-related complications, even in patients refractory to conventional treatments.[25] The use of ex vivo stimulated and expanded virus-specific T cells from either the patients themselves (or allogeneic hematopoietic cell transplantation donor) or so-called allogeneic third-party donors (neither the graft recipient nor donor) are associated with a high rate of durable responses with little toxicity.[16–18,26–31] However, virus-specific T cells are not widely available, limiting the use of these promising therapies.

We evaluated the feasibility of generating virus-specific T-cell lines from PBMCs harvested from LRSCs. The PBMCs obtained from LRSCs and paired peripheral blood samples were comparable at reliably generating clinical-scale CMV-specific T-cell lines from seropositive donors. Such T-cell lines displayed T-cell memory phenotypes and no evidence of T-cell exhaustion, predicting good performance after adoptive transfer.[32,33] Finally, using LRSCs from CMV-seronegative donors, we further show that monocyte-derived dendritic cells (moDCs) can be prepared from LRSC-extracted PBMCs and used to prime and expand CMV-reactive CD4+ and CD8+ T cells from CMV-naïve donors. Overall, our work shows that LRSCs are a reliable source of PBMCs for the manufacturing of clinical scale T-cell therapies from memory and naïve T-cell repertoires. As such, the use of LRSCs in research and clinical applications may contribute to maximize the impact of every apheresis procedure.

MATERIALS AND METHODS

LRSC and whole-blood procurement and handling

This study was approved by Héma-Québec's and Hôpital Maisonneuve-Rosemont Research Ethics Committees (CÉR 13030, HQ 2017–004). Regular platelet donors who agreed to participate in this study all signed an informed consent. LRSCs were collected after plateletpheresis procedures on an automated blood collection system (Trima Accel, Version 6, Terumo BCT). Immediately at the end of collection, the tubing linking the LRSC to the collection set was clamped, sealed, and cut. Peripheral blood from five LRSC platelet donors was also recovered from the diversion pouch in citrated tubes (Vacutainer, BD Labware) at the time of plateletpheresis.[34] The LRSC and blood sample were stored at room temperature and processed within 24 hours. The isolation of PBMCs from the LRSC was performed as previously described.[6,35] Briefly, individual LRSCs were back-flushed using 40 mL of Hank's balanced salt solution (Gilco) supplemented with 10% anticoagulant citrate dextrose solution (USP formula A, Fenwal). The recovered PBMCs were isolated by centrifugation on a sterile medium for isolation of lymphocytes as recommended by the manufacturer (Ficoll-Paque Plus, GE Healthcare). The cells were then used fresh or were cryopreserved in RPMI-1640 media supplemented with 30% fetal bovine serum and 10% dimethyl sulfoxide for future use.

T-cell polyclonal stimulation

PBMCs (1×10^5 cells) were cultured in 96-well round-bottom plates precoated with anti-CD3 antibodies (5 μg/mL; BD Pharmingen), in 0.2 mL T-cell media supplemented with 1 μg/mL anti-human CD28 (BD Pharmingen). On Days 3 and 5, half-media was removed and replenished with fresh T-cell media (advanced RPMI-1640 media, 2 mmol/L L-glutamine and 10% human serum).

Pathogen-specific T-cell line generation from CMV-seropositive donors

The rapid expansion of virus-specific memory T cells was performed based on previous studies.[36,37] Briefly, 15×10^6

PBMCs were pelleted and pulsed with 100 ng of CMV 65 kDa phosphoprotein (pp65) overlapping peptide library (JPT Peptides) and incubated 30 minutes at 37°C. Cells were then mixed with 30 mL of T-cell media supplemented with 400 U/mL interleukin (IL)-4 (Stem Cell Technologies) and 10 ng/mL IL-7 (Stem Cell Technologies). Cells suspensions were incubated at 37°C and 5% CO_2 in culture vessels (G-Rex, Wilson Wolf Manufacturing) for 14 days. On Days 5, 9, and 12, half of the media was removed and cultures were replenished with fresh T-cell media containing IL-4 and IL-7. Cells were counted using trypan blue exclusion and an automated cell counter (Countness, Invitrogen). If cell concentration exceeded 1.5×10^6/mL, cultures were divided 1:2 and replenished with fresh medium.

Generation of human moDCs

Based on our methods,[38,39] monocytes from PBMCs were isolated by plastic adherence and cultured in DC medium (X-vivo 15 - Lonza, 5% human AB serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-Glutamine, and 1 mM sodium pyruvate) supplemented with 800 IU/mL granulocyte-macrophage colony-stimulating factor and 1000 IU/mL IL-4; both cytokines from Feldan). After 7 days, moDCs were matured for 48 hours with granulocyte-macrophage colony-stimulating factor (800 IU/mL), IL-4 (1000 IU/mL), 10 ng/mL tumor necrosis factor-α (TNF-α), 10 ng/mL IL-1β, 100 ng/mL IL-6 (Feldan) and 1 μg/mL prostaglandin E2 (Sigma-Aldrich). 1000 U/mL (interferon-γ) IFN-γ was added during the last 24 hours of the maturation.

Pathogen-specific T-cell line generation from CMV-seronegative donors

Based on previous work,[38] pathogen-specific T cell lines were generated using 15×10^6 PBMCs cocultured with autologous, peptide-loaded (1 μg/mL of pp65 peptide library for 2 h at 37°C) mature moDC at a 1:10 ratio (moDC: PBMC). PBMCs and peptide-pulsed mature moDCs were cocultured for 7 days in T-cell medium supplemented with 30 ng/mL IL-21 and 10 ng/mL IL-12 (Feldan) in G-Rex culture vessels. On Day 7, T cells were washed and restimulated with peptide-pulsed moDC and incubated in T-cell medium supplemented with 30 ng/mL IL-21, 100 IU/mL IL-2, 10 ng/mL IL-7, and 5 ng/mL IL-15 (Feldan). After 14 days, IFN-γ–secreting cells were immunomagnetically enriched (IFNγ Secretion Assay-Detection Kit, Miltenyi Biotec) according to the manufacturer's instructions. Briefly, T cells were stimulated for 4 hours with the pp65 overlapping peptide library, labeled with an IFN-γ catch reagent and an IFN-γ detection antibody conjugated to phycoerythrin (PE) and magnetically separated using anti-PE MicroBeads and a magnetic-activated cell sorting separator (Miltenyi Biotec). Selected IFN–γ-secreting T cells were expanded using a rapid expansion protocol. For the rapid expansion protocol, 8.5×10^4 T cells were resuspended in 25 mL of T-cell medium containing 25×10^6 irradiated (40 Gy) autologous PBMCs, 30 ng/mL soluble anti-CD3 (clone OKT3) and 50 IU/mL IL-2 and transferred to a T25 tissue culture flask (maintained in the vertical position) for 12 days. After 4 days, cultures were harvested and resuspended in 25 mL of fresh T-cell medium with IL-2. Half medium changes were performed every 4 days.

Flow cytometry

Cells were surface stained with monoclonal antibodies to CD3 (BUV395 or PE-Cy7), CD4 (PE-Cy5), CD8 (APC-CY7), CD45RO (fluorescein isothiocyanate), CCR7 (Alexa fluor 700), CD11c (APC or PerCP-Cy5.5), CD56 (Pacific blue), CD14 (PE or APC-Cy7) (all from BD Biosciences), CD62L (Pacific blue), PD-1 (Bv605), CD57(efluor 605), KLRG1 (fluorescein isothiocyanate) (all from BioLegend) or CD8 (BV605, eBiosciences), washed and fixed in phosphate-buffered saline 2% fetal bovine serum 1% paraformaldehyde. Intracellular cytokine detection was performed after cell stimulation for 4 hours with dimethyl sulfoxide, pp65 peptide library, a nontargeted irrelevant peptide library (Epstein–Barr virus protein, late-membrane protein 2 -LMP2, JPT Peptides) or 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich). All conditions included intracellular transport blockade (7.5 mg/mL Brefeldin A, Sigma-Aldrich). Cells were then permeabilized and fixed (Foxp3 Transcription Factor Stainin buffer set, eBioscience). The cells were stained with anti–IFN-γ, anti–IL-2, anti–TNF-α (BD Biosciences) and resuspended in phosphate-buffered saline 2% fetal bovine serum 1% paraformaldehyde before acquisition. For degranulation assessment, protein transport inhibitor (BD GolgiStop solution), anti-CD107a, and anti-granzyme B (BD Biosciences) were used. T cells treated with 50 ng/mL PMA and 500 ng/mL ionomycin were used as positive controls. Data were acquired on a flow cytometer (LSRII or Fortessa, BD Biosciences) and analyzed with computer software (Flow LogiC, Inivai Technologies) or FlowJo (LLC).

IFN-γ enzyme-linked immunospot (ELISpot) and cytotoxicity assays

IFN-γ ELISpot assays were performed with 5×10^4 cells per well, according to manufacturer instructions (Mabtech). The assessment of cytotoxicity was performed by flow cytometry. Autologous or allogeneic phytohemagglutinin (PHA) blasts (3×10^6 PBMCs/mL were incubated in T-cell media with 20 μg/mL PHA for 3 days at 37°C and 5% CO_2) were pulsed (or not) with the pp65 peptide library and labeled using violet or yellow cell proliferation kits (CellTrace, Invitrogen) for 20 minutes at 37°C and 5% CO_2. The PHA blasts were then exposed to responder T cells at various ratios for 4 hours. Following this coculture, a stain kit was used according to the manufacturer's instructions (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Life Technologies). Determination of live PHA

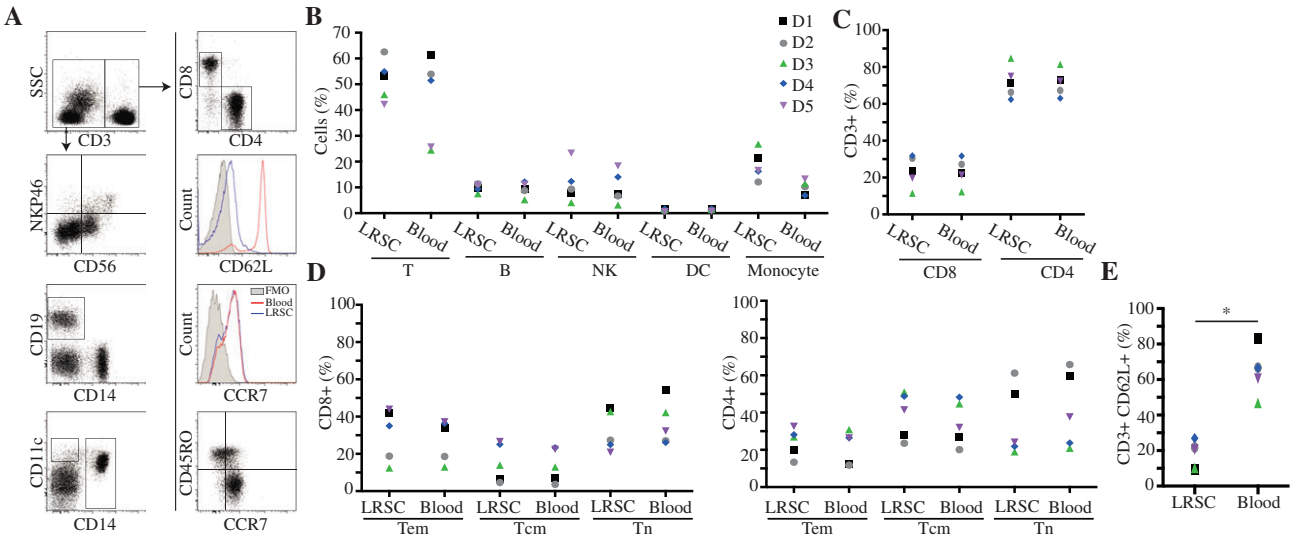

Fig. 1. Similar PBMCs profiles from paired peripheral blood and LRSC samples. (A) Gating strategies for the identification of NK cells (NKP46, CD56), B cells (CD19), monocytes lineage (CD14), DC (CD11c), T cells (CD3) as well as the CD4+ and CD8+ T-cell subsets along with representative staining of the T-cell differentiation markers CD45RO, CCR7 and CD62L. FMO refers to fluorescence minus one. (B) Proportions of T-cell, B-cell, NK cell, DC, and monocyte lineage from LRSCs and blood of five donors (D1 to D5, identified by different symbols). (C) Proportions of CD4+ and CD8+ T cells among CD3+ cells. (D) Distribution of CD8+ (left) and CD4+ (right) T-cell subpopulations of effector memory (Tem) (CCR7−CD45RO+), central memory (Tcm) (CCR7+CD45RO+) and naïve (Tn) (CCR7+CD45RO−) T cells among the CD3+ population. (E) Percentage of CD62L-expressing CD3+. All comparisons between LRSC and blood-derived PBMCs were non-statistically significant, except for the expression of CD62L (*p < 0.05).

counts was done by flow cytometry (LRSII, BD Bioscences; and Flow LogiC software). Specific cytotoxicity was calculated using the difference between the absolute number of live target cells (PHA blasts) in the control condition where no effectors (responder T cells) were added, and the absolute number of live target cells in conditions where effectors were added at different effector-to-target ratios. The percentage of live cell number relative to the control was then subtracted from 100 and reported as percent cytotoxicity. Absolute cell counts were determined using absolute counting beads (CountBright, Invitrogen) according to the manufacturer's instructions.

Statistic analysis

Statistical analyses were performed with the use of R software, Version 3.2.4. Two-tailed paired Wilcoxon or Kruskal-Wallis (nonparametric analysis of variance) tests were used, and p values of 0.05 or less were considered significant.

RESULTS

LRSCs contains representative PBMCs subsets relative to peripheral blood

Following plateletpheresis at the collection center, LRSCs were shipped as previously described[40] to the laboratory where they were processed the following day. On average, 7 mL of PBMC-enriched blood was extracted from the LRSCs (n = 8), yielding 1.3 ± 0.6 billion PBMCs after density gradient isolation. We performed phenotypic characterization of the PBMCs from five LRSCs for which paired peripheral blood (sampled during the plateletpheresis) was available. The percentage of T cells, natural killer cells, B cells, DCs, and monocytes were comparable among the PBMCs obtained from LRSCs or blood (Figs. 1A and 1B). Further characterization of the T-cell compartment revealed similar proportions of CD4+ and CD8+ T cells and distribution across the subpopulations of naïve (Tn –CD45RO–, CCR7+), central memory (Tcm – CD45RO+, CCR7+) and effector memory (Tem – CD45RO+, CCR7–) T cells (Figs. 1C and 1D).[32] Importantly, the cell surface molecule CD62L, which is often used as a marker of Tn and Tcm, was expressed at much lower levels on LRSC T cells (Fig. 1E). It has previously been observed that CD62L can be cleaved from the cell surface,[41] and we surmise that the LRSCs are conducive to CD62L cleavage. We nonetheless conclude that PBMCs and T cells extracted from either LRSCs or blood are similar in proportion and phenotypes.

T cells harvested from LRSCs and blood are similarly functional

A prerequisite to the use of LRSC-extracted T cells in adoptive immunotherapy is their capacity to expand and acquire effector functions after stimulation. We first assessed T-cell responses after nonspecific T-cell activation using agonistic anti-CD3/CD28 antibody stimulation. After 7 days in culture, the total cell count increased 6.6 ± 1.8-fold for the LRSC and 5.9 ± 1.6-fold for the peripheral blood with T

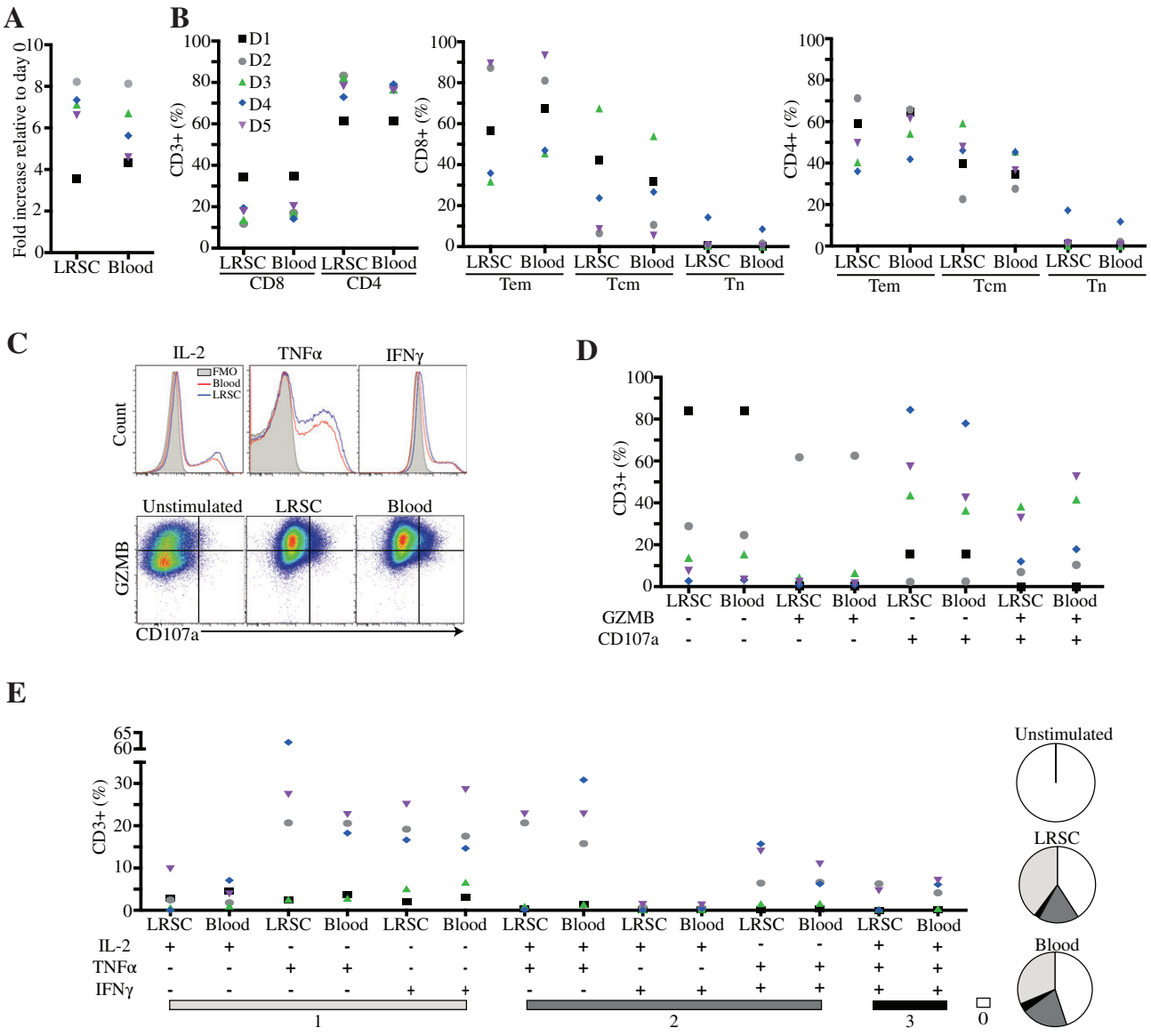

Fig. 2. Donor-matched peripheral blood and LRSC-extracted T cells show similar expansion and activation potential. (A) T-cell expansion following anti-CD3/CD28 stimulation of 1×10^5 PBMCs. (B) Proportions of CD4+/CD8+ T cells and their differentiation profile (Tem, Tcm and Tn) at the end of the culture based on the expression of CD45RO and CD62L. (C) Representative flow cytometry profiles (gated on CD3+) showing cytokine production (IL-2, TNF-α, IFN-γ) and degranulation marker expression (CD107a) relative to granzyme B (GZMB) expression following stimulation (right) or not (left) with PMA/ionomycin. (D) Frequencies of T cells expressing GZMB and the degranulation markers CD107a. (E) Cytokine expression in T cells from all donors (left) and average proportion of T cells expressing none, one of several cytokines (pie charts, right). All comparisons between LRSCs and blood-derived T cells were not statistically significant.

cells in equivalent CD4+/CD8+ proportions representing the majority of cells (LRSC 92% and blood 83%) in the culture (Fig. 2A). Moreover, the proportions of Tn, Tcm, and Tem generated using the cells from LRSCs were similar to those from the paired blood samples (Fig. 2B). As expected, Tem tended to predominate after activation, irrespective of the origin of the T cells. The proportion of Tn and Tcm based on the expression of CD45RO and CD62L or CCR7 were similar (data not shown), confirming that the low expression of CD62L on freshly LRSC-extracted T cells is transient and does not impact T-cell differentiation phenotypes in culture. To evaluate whether expanded T cells from LRSCs or blood had comparable effector function and cytokine secretion potential, we stimulated the T cells at the end of the culture using PMA/ionomycin.[37] Intracellular staining for IFN-γ, TNF-α and IL-2 revealed similar patterns of cytokine secretion between the two groups (Figs. 2C–2E). Likewise, PMA/ionomycin stimulation led to an equivalent proportion

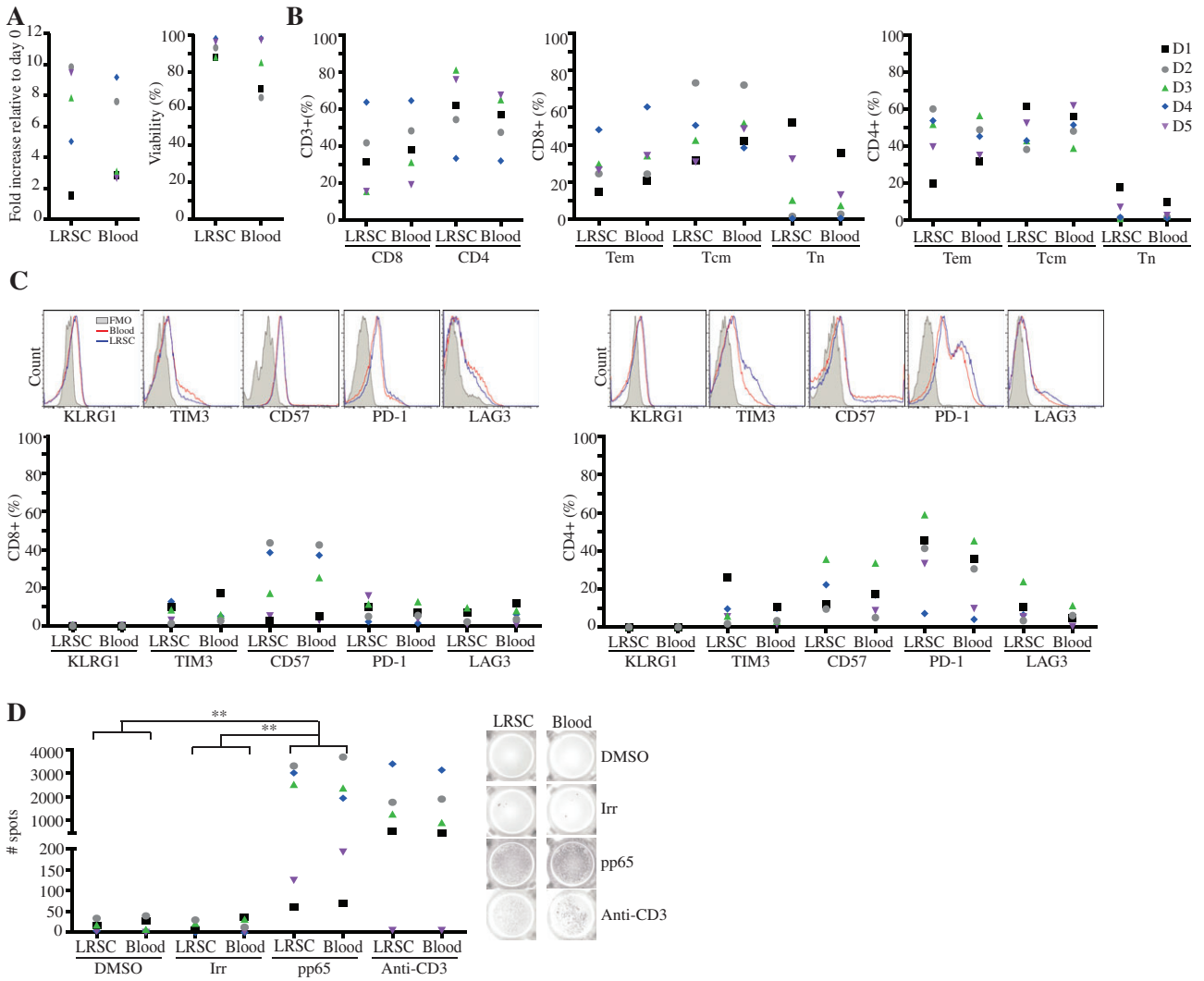

Fig. 3. LRSC-extracted PBMCs can be used to generate pathogen-specific T-cell lines. (A) T cells fold expansion based on cell counts at the beginning and end of the culture (left) and viability at the end of the culture (right). (B) Proportion of Tem, Tcm and Tn CD4+ and CD8+ T cells at the end of the culture as defined by CD45RO and CD62L. (C) Representative staining for terminal differentiation and exhaustion cell surface markers on CD4+ and CD8+ T cells (top) and data from five donors (bottom). (D) Compiled (left) and representative (right) ELISpot results showing IFN-γ–secreting T cells among 5×10^4 T cells at the end of the culture following no stimulation (cells exposed to the peptide diluent only, dimethyl sulfoxide [DMSO]) or exposure to a nontargeted irrelevant peptide library (Irr), the targeted pp65 peptide library (pp65) or agonistic anti-CD3 (positive control). Statistically significant comparisons relative to the pp65 condition are indicated (p < 0.01). All comparisons between LRSCs and blood-derived T cells were not statistically significant.**

of T cells showing evidence of degranulation as assessed by intracellular granzyme B and cell surface expression of CD107a (Figs. 2C and 2D). Taken together, these data support the notion that peripheral blood T cells obtained through LRSCs and fresh blood have a similar potential to expand and acquire effector functions.

PBMCs from LRSCs can be used to rapidly generate pathogen-specific T-cell lines

In transplantation settings, CMV is one of the most common opportunistic viral reactivations.[42,43] Based on the premise that T cells from LRSCs are equivalent to T cells harvested in the peripheral blood, we next tested whether PBMCs from LRSCs could be used to generate CMV-specific T cells. To this end, we used a variation of a protocol that can rapidly generate virus-specific T-cell lines in 9 to 14 days by expanding virus-specific memory T cells from PBMCs, and that has previously been used in adoptive immunotherapy trials.[18,36,37,44] This process involves exposing whole PBMCs to an overlapping peptide library that provides extensive coverage of the entire sequence of the CMV antigenic pp65 protein followed by expansion in IL-4 and IL-7

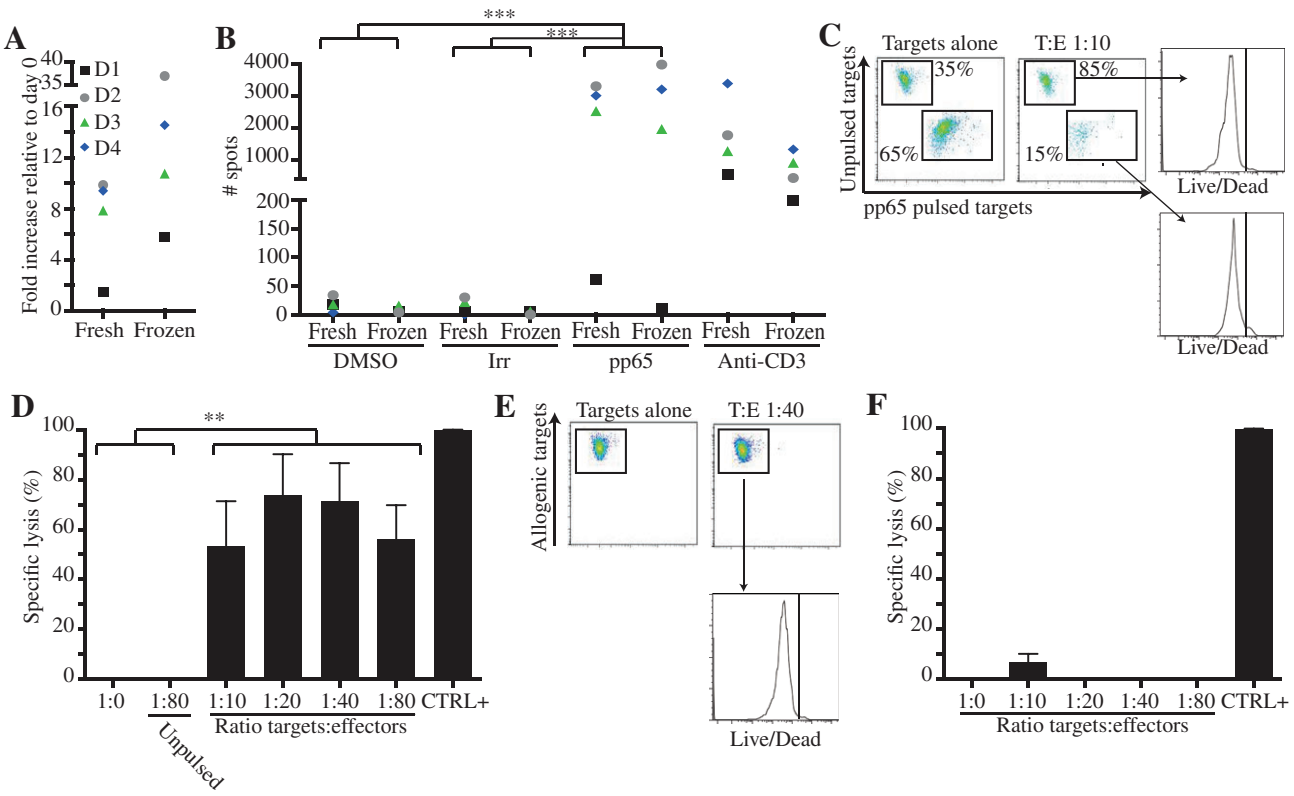

Fig. 4. Fresh and cryopreserved LRSC-derived PBMCs are comparable at generating pathogen-specific T-cell lines. (A) T-cell line expansion using fresh or thawed PBMCs from the same LRSC (four donors assessed). (B) ELISpot results at the end of the culture in four conditions: no stimulation (peptide diluent only, dimethyl sulfoxide [DMSO]) or stimulation with a non-targeted irrelevant peptide library (Irr), the targeted pp65 peptide library (pp65) or agonistic anti-CD3 (positive control). (C) Representative and (D) compiled (n = 5) cytotoxicity data showing specific killing of unpulsed, or pp65 peptide-pulsed autologous PHA targets at the indicated target-to-effector ratios using cryopreserved PBMCs for T-cell line generation and PHA blast preparation. (E) Representative and (F) compiled cytotoxicity data obtained using unpulsed allogeneic PHA blasts as targets (n = 5). The CTRL+ (positive control) condition represents target cells incubated in 70% ethanol and 1% bleach. Statistically significant comparisons relative to the pp65 condition are indicated (p < 0.01, ***p < 0.001). Comparisons between fresh and frozen PBMCs from LRSC were not statistically significant. Error bars represent standard error of the mean.**

supplemented media. Again, paired LRSC and blood PBMCs from the same donors collected at the same time were used. From 15×10^6 PBMCs at the start of the culture, cell counts had increased by 6.7 ± 3.5 (LRSC) and 5.1 ± 3.1 (blood) fold, with T cells representing the vast majority of cells at the end of the culture (Figs. 3A and 3B). The generated T-cell lines had balanced CD4+/CD8+ T-cell and Tcm/Tem ratios (Fig. 3B). We extended the phenotypic assessment to determine whether the generated cells expressed markers associated with exhaustion and terminal differentiation. In all cases but one, the proportions of CD4+ or CD8+ T cells expressing the terminal differentiation markers KLRG1 and CD57 or exhaustion markers PD-1, TIM3 and LAG3 were always less than 50% after 14 days in culture (Fig. 3C). Hence, virus-specific T-cell lines generated from LRSC and blood PBMCs displayed memory differentiation without prominent signs of terminal differentiation or exhaustion. Finally, LRSC and blood-derived PBMCs generated equivalent proportion of pp65-reactive T cells as evaluated by the IFN-γ ELISpot assay (Fig. 3D). We conclude that the PBMCs harvested from LRSCs and peripheral blood are equivalent to generate pathogen-specific T-cell lines from memory repertoires.

Cryopreservation does not affect rapid CMV T-cell line generation from LRSC-extracted PBMCs

Few applications would require the use of all the PBMCs harvested from a single LRSC. Considering that LRSCs yield on average more than a billion PBMCs, capable of supporting the manufacturing of several T-cell products, we next assessed whether LRSC-extracted PBMCs could be used after cryopreservation.[7,45] To this end, fresh and thawed PBMCs from the same LRSCs were used to generate CMV-specific T-cell lines. Cryopreserved PBMCs tended to expand slightly more than fresh PBMCs, but this did not

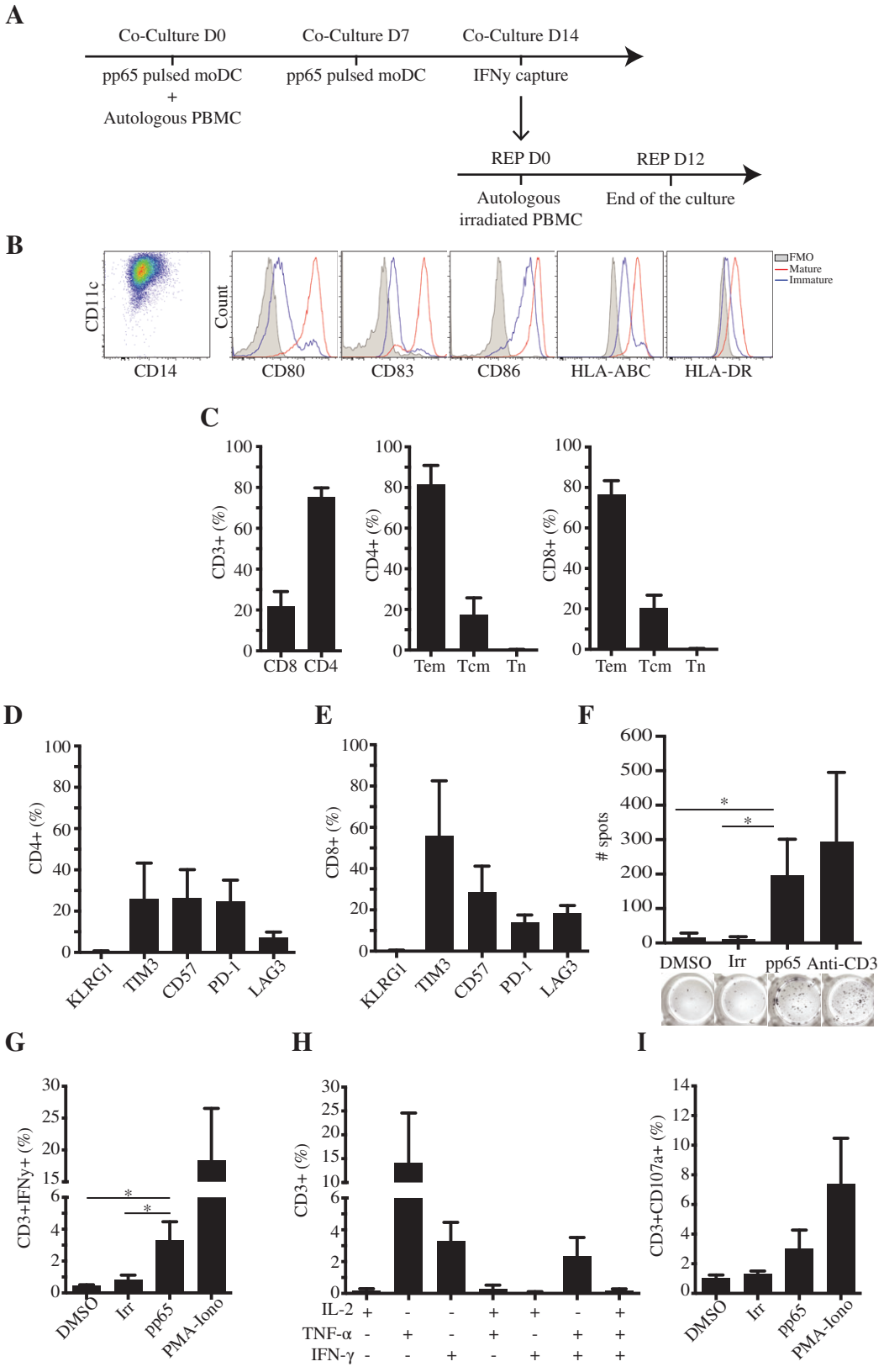

Fig. 5. Legend on next page.

reach statistical significance (Fig. 4A). Moreover, the antigenic specificity of the T-cell lines generated from fresh or thawed LRSC PBMCs was comparable as assessed by the ELISpot assay (Fig. 4B). To further characterize the T-cell lines generated from thawed PBMCs, we performed cytotoxicity assays using PHA blasts also generated from cryopreserved PBMCs as targets. As predicted by previous studies,[36,37] the pp65-specific T-cell lines killed autologous PHA blasts pulsed with the pp65 peptide library but not their unpulsed counterparts (Figs. 4C and 4D). To assess for the risk of unwanted off-target reactivity against allogeneic cells, as this is relevant in a transplantation context, we also performed cytotoxicity assays using allogeneic PHA blasts (Figs. 4E and 4F). Our data confirmed that CMV-reactive T-cell lines do not kill allogeneic T cells. Hence, our results suggest that fresh and cryopreserved LRSC-extracted PBMCs can be used to generate pathogen-specific T-cell products.

Stimulation of a CMV naïve T-cell repertoire using LRSCs-derived DCs

The priming and expansion of naïve T cells in vitro require several signals that are dispensable when expanding a memory repertoire. The use of professional antigen-presenting cells such as DC and stimulatory cytokines can successfully support a primary anti-viral response in vitro.[46–49] Based on a protocol to prime and expand naïve T cells[38] and previous evidence that LRSC-extracted PBMCs can be used to generate moDCs,[8] we devised a strategy to prime and expand pp65-specific T cells from CMV-seronegative LRSC donors. This process hinges on two stimulations of 15×10^6 PBMCs with peptide pulsed moDC, followed by an enrichment procedure (cytokine capture) and a reexpansion (Fig. 5A). As anticipated, CD11c+CD14− moDC were generated at high purity and expressed HLA and costimulation molecules at high levels after maturation (Fig. 5B). At the end of the full process, an average of 57 ± 8 million T cells were harvested. The T-cell lines had a CD4+ and Tem predominance (Fig. 5C). However, less than 30% of CD4+ and CD8+ T cells expressed the late differentiation markers KLRG1 and CD57, or exhaustion markers PD-1, LAG3 and TIM3 (with the exception of TIM3-expressing CD8+ T cells) (Figs. 5D and 5E). Specific reactivity to pp65 peptides was confirmed by intracellular cytokine staining and ELISpot (Figs. 5F–5H). Moreover, degranulation assays using CD107a staining suggested preferential reactivity towards pp65 peptides but this did not reach statistical significance (Fig. 5I). Taken together, LRSC-derived PBMCs can be used to generate mature moDC capable of priming virus-specific T-cell lines from virus-naïve (seronegative) donors.

DISCUSSION

The study of human immune cells and the development of human T-cell therapeutic products require access to a steady supply of PBMCs. However, volunteer donor recruitment can be tedious, and the use of commercial providers is expensive. Blood banks are leukoreducing their blood products, generating thousands of LRSCs that are discarded annually. Blood banks, such as the Canadian Blood Services, are increasingly proposing such by-products, or unusable products, to researchers to support their work on transfusion medicine.[50] The content of LRCS represents for sure an opportunity for researchers and clinicians and could maximize the potential human benefits of every leukoreduction procedure. Based on previous work suggesting that PBMCs from LRSCs can be used to study a variety of human leukocytes,[5,6] we undertook to assess whether this source of PBMCs was reliable to generate clinical-scale CMV-specific T-cell lines. We chose CMV for several reasons: 1) CMV reactivation is a significant medical problem, especially in the context of transplantation;[24,43] 2) the CMV serostatus of several regular platelet donors was known, enabling experiments to be done with naïve and memory T-cell repertoires; and 3) antiviral (including CMV) adoptive immunotherapy using third-party donors has a promising track record[18,26] and as such, blood agencies may want to invest into third-party T-cell banks through the participation of well-characterized regular apheresis donors.

In this study we confirm and extend previous studies suggesting that PBMCs obtained from LRSCs are an

FIGURE 5 Pathogen-specific T-cell expansion from naïve repertoires using LRSC-derived PBMCs. (A) Schematic representation of the naïve T-cell priming and expansion protocol. (B) Representative moDC (CD11c+CD14−) staining after monocyte isolation and culture in IL-4 and granulocyte-macrophage colony-stimulating factor followed by maturation to evaluate the expression level of maturation markers (CD80, CD83, CD86, HLA-ABC, HLA-DR). (C) Histograms representing the mean proportions of Tem, Tcm and Tn $CD4^+$ and $CD8^+$ T cells at the end of the culture based on CD62L and CD45RO expression (3 donors). (D) Histograms showing mean proportions of CD4+ and (E) CD8+ T cells expressing terminal differentiation and exhaustion markers. (F) Mean IFN-γ ELISpot results at the end of the culture from three donors using four conditions: no stimulation (peptide diluent only, dimethyl sulfoxide [DMSO]) or stimulation with a non-targeted irrelevant peptide library (Irr), the targeted pp65 peptide library (pp65) or agonistic anti-CD3 (positive control). (G) Histograms representing the percentage of CD3+ cells producing IFN-γ from three donors as evaluated by intracellular flow cytometry following either no stimulation (DMSO), irrelevant (Irr), pp65 peptide exposure, or PMA-ionomycin (PMA-Iono, positive control). (H) Histograms representing the percentage of CD3+ cells expressing IL-2 and/or TNF-α and/or IFN-γ (3 donors). (I) Mean percentage of degranulating T cells following peptide stimulation. Statistically significant comparisons relative to the pp65 condition are indicated (*$p < 0.05$).

adequate surrogate for the study of human leukocytes. Our data using paired PBMCs from LRSC and blood collected during the same procedure, show that there is high concordance in the relative counts of all major mononuclear subsets.[2,6] This was also the case in the conventional T-cell compartment, where CD4+ and CD8+ T cells at different stage of memory differentiation were found in similar percentages. However, the determination of T-cell subsets could not be based on CD62L expression, which was considerably reduced on T cells from LRSCs. Although unclear at this time, the activation of proteases from cells in the LRSC may be responsible for this finding, which is often transient.[41] In any event, this had no impact on T-cell function, expansion, and Tcm phenotype (assessed by CD62L expression in all other figures) following stimulation and culture. In fact, PBMCs from LRSCs and blood were remarkably similar after stimulation with anti-CD3/CD28 antibodies or viral peptides.

An objective of this study was also to assess the feasibility of using LRSC-derived PBMCs to generate antiviral T cells at a clinical scale. Based on our previous experience and commonly used virus-specific T-cell doses used in adoptive immunotherapy studies, our T-cell expansions reached clinically relevant levels, displayed pathogen-restricted reactivity and included memory cells with only a minority expressing terminal differentiation or exhaustion markers. Considering that only a fraction of the PBMCs extracted from a single LRSC (as low as 1.5×10^7 of 1.3×10^9 cells) yields therapeutic scale numbers of virus-reactive T cells, LRSCs represent an interesting source of PBMCs for the manufacturing of virus-specific products. Indeed, recent studies have used T-cell doses of 20×10^6/meter square of body surface area, equating to approximately 40 million T cells for an average adult.[18,44] Our data comparing cryopreserved versus fresh PBMCs as a source of T cells and moDCs indicate that the large number of PBMCs extracted from a single LRSC may be used either immediately after collection or later for the manufacturing of different products. This is particularly relevant in the context where third-party donors are increasingly used in antiviral adoptive immunotherapy and where a single LRSC or serial LRSCs from regular, well-characterized (HLA type, serotyped for several viruses, tested for communicable diseases, etc.) donors may be used for several indications. However, not all T-cell lines generated for this report displayed strong effector functions after culture. This variability between donors remains incompletely understood but is in line with what we and others have observed previously.[36–38] However, blood and LRSC T cells from the same donors behaved similarly, consistent with the notion that LRSCs are equivalent to blood as a source of T cells. Our results nonetheless suggest that in clinical settings, LRSC donors generating robust responses may be preferentially selected for the manufacturing of specific therapeutic T-cell products.

In conclusion, our work supports the use of LRSCs as a source of PBMCs for the development of adoptive T-cell therapy protocols from both a memory and a naïve repertoire and infers that this largely unused source of PBMCs may be used to manufacture clinical-grade T-cell products for a variety of indications.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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