

Manufacturing of Human T-lymphoid Progenitors from Two Different Hematopoietic Stem Cell Sources and Perspective for New Immunotherapies

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Abstract

The adaptive immune system depends on the efficient response of lymphocytes, protecting the organism from infection or malignant diseases. T-cell immunodeficiency, innate or acquired, put the patient at risk for developing opportunistic infections and cancers. We previously described a novel approach to overcome the major limitations of T-cell immunotherapy and hematopoietic stem cell transplant (HSCT) by transplanting human T-lymphoid progenitors (HTLP), with the aim to achieve shortened immune reconstitution and fully functional naïve T-cell repertoire in immunodeficient or immunocompromised patients. By complementing our DL4-based cell culture with TNF α we developed and scaled-up clinical strategies involving hematopoietic stem and progenitor cells (HSPC) differentiated into T-lymphoid progenitors. We discuss here recent advances made in the characterization of different cell sources used as starting materials for T-lymphoid progenitors manufacturing, as well as gene modification of these cells, highlighting new perspectives for the development of therapeutic strategies.

Keywords: Clinical immunology, Immune reconstitution, Immunodeficiency disorders, Immunotherapy, Stem cell, T-cell progenitors, Thymus, Transplantation immunology

Introduction

Adaptive immunity relies on an efficient lymphocyte response, enabling an organism to defend itself against infections or malignancies [1]. Lymphocyte repertoire may be impacted by various factors, including conditions such as primary immune deficiencies or Acquired Immunodeficiency Syndrome (AIDS), or by treatments such as chemotherapy [2]. The development of therapeutic strategies to restore and

boost adaptive immunity is imperative for patients suffering from immune deficiencies.

As described by Moirangthem and colleagues [3], immunotherapeutic human T-lymphoid progenitors (HTLP) have demonstrated efficient thymus engraftment in NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice and could play an important role in conferring a shortened immune recovery following adoptive transfer into immunodeficient patients. Only a rare

subset of blood circulating T-lymphoid progenitors is capable of seeding the thymus, hampering their use as a cell therapy product source. Thus, their *ex vivo* generation at a large scale is of great interest. A 7-day feeder-cell free culture process allows their GMP-grade generation from hematopoietic stem and progenitor cells (HSPCs) [3].

First described for its key role in the inflammatory and antiviral responses, Tumor Necrosis Factor α (TNF α), has been linked with a significant effect on HSPCs when cultured in a Notch receptor-pressuring environment, both in terms of cell expansion and T-lymphoid differentiation [4]. By taking advantage of this behavior, we developed and scaled-up clinical strategies involving HSPCs differentiated into T-lymphoid progenitors.

Such cell-based therapy could not only considerably improve allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT) prognosis [5], but also constitute an interesting platform of engineered cell products for cancer immunotherapy. Here, we propose a commentary on the use of different cell sources as starting materials for T-lymphoid progenitors manufacturing, namely CD34⁺ cells isolated from mobilized peripheral blood (mPB) or from Cord Blood (CB) units.

We lead a thorough analysis of cell therapy products obtained from both types of cell sources using a complementary approach relying on single-cell transcriptomic and on mass cytometry. Thus, we are able to highlight the similarity of T lymphoid progenitor cells' phenotypes and underline some source-related specificities.

Based on the proof of concept realized by Moirangthem and colleagues to confirm the compatibility of T-lymphoid progenitors manufacturing and gene modification, we demonstrate here the possibility to express a CAR construct through lentiviral transduction, allowing the efficient production of high CAR-expressing human T-lymphoid progenitors (CAR-HTLP). Altogether, the present data pave the way for the development of new immunotherapies relevant not only for transplanted patients but also for oncology and beyond.

Clinical Interests of mPB cells and CB cells

Our TNF α -supplemented immobilized DL-4 culture system was shown to generate high numbers of HTLPs from CB or mPB HSPCs. Our interest was focused on the differences existing between HSPCs isolated from these two sources.

For HSCT, mPB stem cells have progressively replaced bone marrow as a cell source as they don't require invasive aspiration and enable a more rapid neutrophil and platelet recovery as well as faster immune reconstitution [6]. Peripheral blood represented 70% of autologous transplants in 2018 [7]. Collection of peripheral blood HSPCs is still time-consuming and expensive, requiring several days of mobilization,

followed by often multiple apheresis sessions. Innovations in HSPC harvest tend to reduce the side effects of these protocols. G-CSF mobilization is progressively complemented or switched with other mobilization protocols, such as CXCR4 antagonists or VLA4 antagonists [8,9].

HSPCs isolated from CB have the major advantage of being rapidly available and requiring a markedly reduced stringency of HLA-matching compared with adult unrelated PBMC- or BM-isolated cells [10]. The use of CB has dramatically extended allograft access to racial and ethnic minorities [11]. Although CB unit use has been initially limited for the treatment of adult patients because of the low number of cells for transplantation, the combination of double CB units has enabled an increase in cell dose [12,13]. Recently, Yabe et al. demonstrated that even a low number of CB isolated CD34⁺ cells, as low as 75,000 cells/kg, could lead to satisfactory engraftment [14].

Treatments relying on the use of CD34⁺ HSPCs should explore differences existing in these two cell sources and their impact on cell cultures to produce cell and gene therapies.

Molecular and Cellular Comparison of mPB and CB HSPCs

Based on phenotypical characterization led by several teams [12-15], Wisniewski and colleagues carried out a comparison of expressed hematopoietic cell surface antigens by HSPCs isolated from CB and mPB [19]. Focusing on Lin⁻CD34⁺CD38⁻CD90⁺CD45RA⁻, they demonstrated that CB cells more frequently express HLA-DR compared with mPB cells ($75 \pm 3.6\%$ vs $40 \pm 18\%$), and similarly for expression of KIT ($50 \pm 7\%$ vs $10 \pm 4\%$). Conversely, mPB cells more frequently express IL-3R compared with CB cells ($43 \pm 14\%$ vs $19 \pm 4\%$). Furthermore, CB HSPCs were shown to contain more lymphoid progenitors than their adult counterparts, which exhibited a myeloid bias. To further evaluate differences in cell behavior, Forte and colleagues demonstrated the specific impact of different proinflammatory cytokines on 14 samples of mPB HSPCs and 14 samples of CB HSPCs, suggesting that CD34⁺ cells from CB were more actively responsive to inflammatory cues than their mPB counterparts [20].

Progress made in single-cell transcriptomics created new insights into the comparison between CB and mPB HSPCs. In particular, Dong and colleagues performed scRNAseq of fresh and cultured CD34⁺ cells from CB and mPB, enabling them to delineate distinct stemness-related genes associated with cell sources and culture conditions for both sources [21].

Generation of T Lymphoid Progenitors from mPB and CB HSPCs

To further evaluate the possible impact of the HSPC source as starting material of Human T lymphoid progenitors'

generation, we initiated a comparative study based on 3 samples obtained from differentiated CB cells (HTLP-CB) and 3 other samples obtained from differentiated mPB cells (HTLP-mPB). All samples were analyzed through a complementary approach involving mass cytometry and scRNAseq, in addition to classical flow cytometry (Figure 1A).

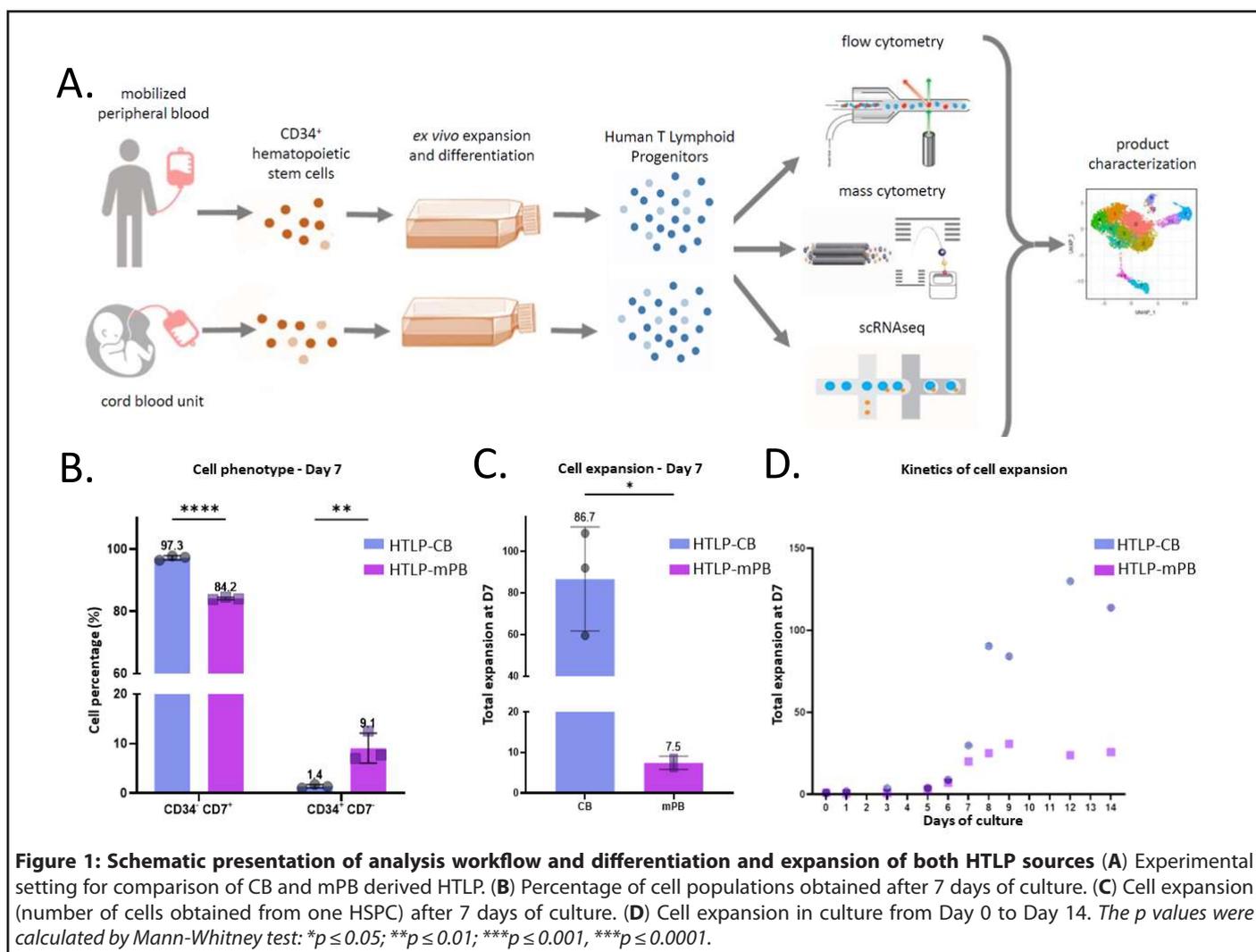
Extending the observation of specific cell expansion patterns by [3], HTLP obtained from CB CD34⁺ HSPCs were associated with a higher CD7⁺ subset percentage (Figure 1B). The remaining CD34⁺CD7⁻ fraction was larger in samples originating from mPB.

We confirmed superior CB cell expansion over mPB in the DL-4-based culture supplemented with TNF α . CB CD34⁺ HSPCs demonstrated on average an 11.5-fold higher expansion than the mPB counterparts (Figure 1C). One CD34⁺ cell from CB was able to give rise to 86.7 CD7⁺ cells (vs 7.5 CD7⁺ cells for mPB CD34⁺ cells). Superior cell expansion of CB HSPCs compared with mPB HSPCs was also confirmed through culture extension up to 14 days after seeding (Figure 1D).

Single Cell Transcriptomic Analysis of HTLP Obtained from mPB and CB HSPCs

The vast majority of HTLP were associated with the expression of transcription factors that are usually identified as transcriptional drivers of the T-cell lineage program, *GATA3*, *TCF7*, and *BCL11B* (Figure 2). *TCF7* is a direct Notch target [22], consistent with sustained activation dependent on DL-4 of NOTCH1 activation exercised during cell culture. Although our analysis demonstrated overall homogeneity of cell populations, specifically for T cell lineage markers, we highlighted some specificities of HTLP-CB and HTLP-mPB.

Interestingly, *IGF2BP1* and *IGF2BP3*, coding for insulin-like growth factor 2 mRNA-binding proteins putatively associated with survival and proliferation [23], appeared to be signatures of CB samples (51.2% (CB) vs 0.6% (mPB) and 24.5% (CB) vs 0.1% (mPB) of total sample respectively). Further investigation is required to link the expression of these growth factors with superior cell expansion from CB HSPCs.



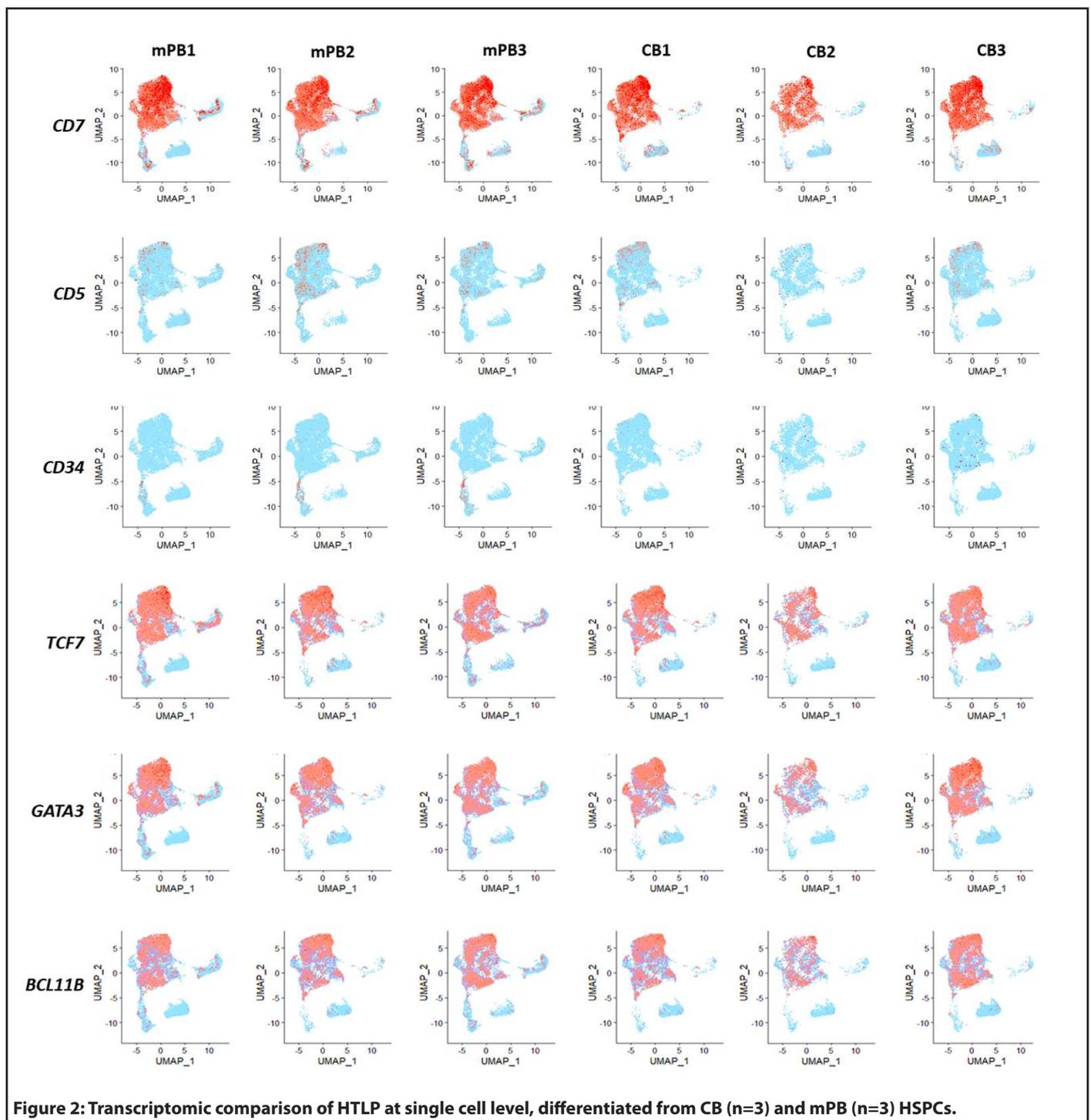


Figure 2: Transcriptomic comparison of HTLP at single cell level, differentiated from CB (n=3) and mPB (n=3) HSPCs.

Single Cell Mass Cytometry Analysis of HTLP Obtained from mPB and CB HSPCs

To complete the transcriptomic approach for comparison of the two cell products, we employed mass cytometry to delineate specific cell populations and associated markers, confronting specific transcript expressions detailed earlier.

Mass cytometry analysis highlighted the overall homogeneity

of markers expressed at the cell membrane of both CB and mPB-derived cells. CCR9 expression was found to be higher in HTLP-CB (43.5% (CB) vs 9.4% (mPB) of total sample) (**Figure 3B**).

In cell products obtained from both sources, we also highlight the high expression levels of the adhesion molecule PSGL-1 and the chemokine receptor CXCR4 (**Figure 3C-3D**), alongside a significant portion of cells expressing L-selectin (CD62L)

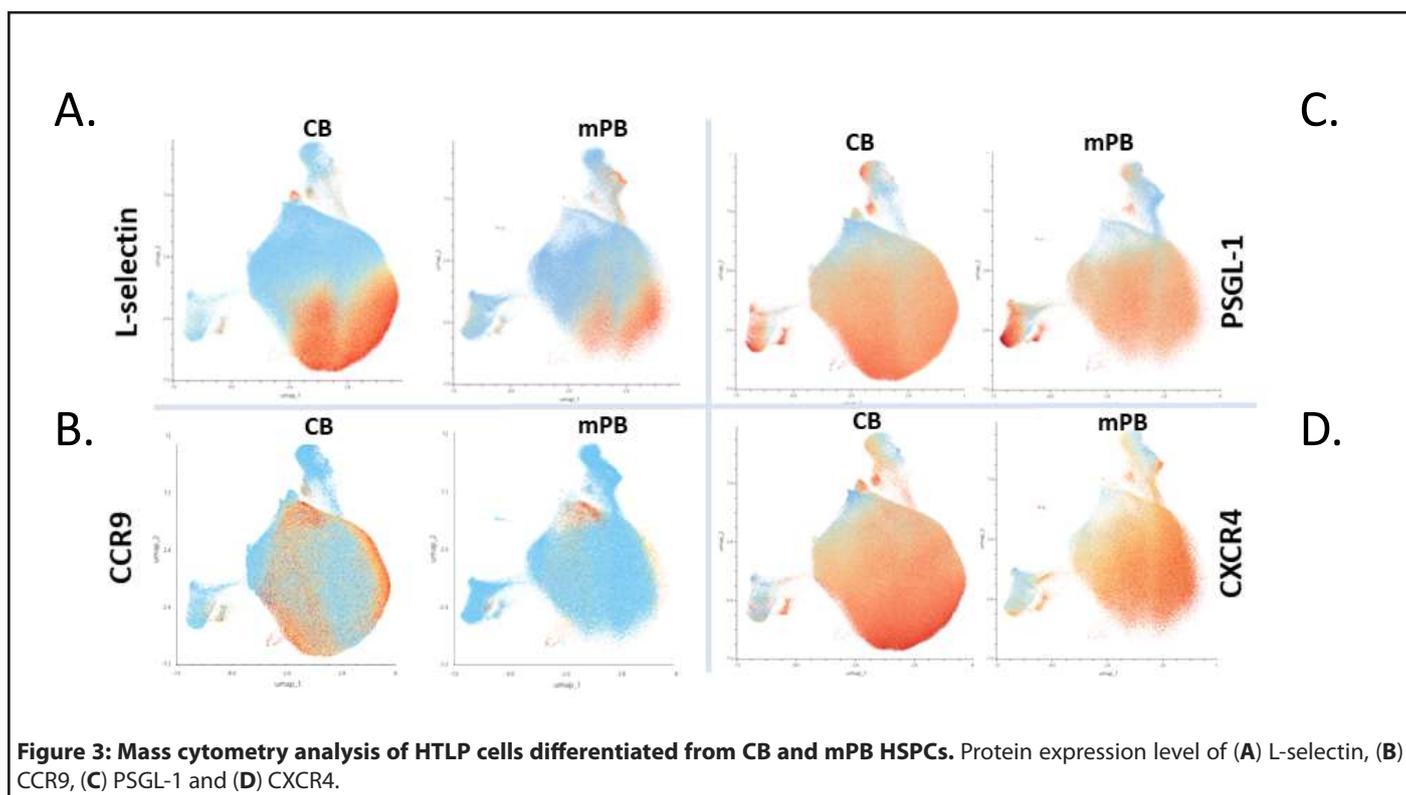


Figure 3: Mass cytometry analysis of HTLP cells differentiated from CB and mPB HSPCs. Protein expression level of (A) L-selectin, (B) CCR9, (C) PSGL-1 and (D) CXCR4.

(Figure 3A). These proteins may balance the impact of distinct levels of CCR9 expression among cell products. In the murine model, Calderon and Boehm demonstrated the cooperative use of multiple chemokine receptors enabling homing of hematopoietic progenitors into the embryonic thymus [24]. Among those, CXCR4 was found in the majority of HTLP differentiated from both CB and mPB HSPCs. Furthermore, the expression of PSGL-1, as a complement of L-selectin, could be involved in the efficient thymus homing of HTLP cells [25,26]. Mechanisms of thymus homing by lymphoid progenitors are still to be clarified in humans and thus far have been predicted from results obtained in murine models. Identification of which “facilitating” factors among cell phenotypes are associated with a better outcome for transplanted patients in clinical trials may shed light on unknown elements involved in lymphoid progenitors’ behaviors in the organism.

Gene Modification of Human T Lymphoid Progenitors and Clinical Perspectives

CD19 CAR-T cells have proven to be highly effective for treating several types of B-cell malignancies such as acute B- lymphoblastic leukemia (B-ALL) and B- Non-Hodgkin Lymphoma (B-NHL). However, disease relapse remains still an issue with progression-free survival rates currently below 50% [24-26], and event-free survival 3 years post-treatment at only 16.1% [30] for relapsed/refractory B cell malignancies. In addition, side effects such as cytokine release syndrome (CRS) and immune-effector cell-associated neurotoxicity syndrome

(ICANS), still need to be considered without impacting the efficacy of CAR-T cells [31,32].

Several reasons account for the failure to achieve durable complete remissions among which early loss, failure of expansion or exhaustion of CAR-T cells, as well as hampered reconstitution of the naïve T cell compartment, are important factors [33]. The delayed CD4⁺ T cell recovery with less than 50% of patients having >200 cells per μ L CD4⁺ T cells at 1 year following CAR-T cell therapy [34-36] represents another significant challenge, as immunodeficiency not only increases the chances of relapse but also leads to a significant rate of infectious complications [30,34-36].

Finally, the cost and complexity of manufacturing CAR-T cells are significant barriers to their general use [37]. In this context, an allogeneic CAR-HTLP strategy could bypass some of those main challenges, such as immediate availability, efficient expansion, and sustained anti-tumor response. This strategy could accelerate the reconstitution of a polyclonal and naïve, host-thymus-educated and tolerized, mature T cell compartment, thus providing durable and efficient anti-tumor responses without the risk for graft versus host disease (GvHD) [38]. In addition, naïve status and activation at the physiological level of HTLP-derived T-cells may impact cell fitness and potentially reduce the incidence of CRS and ICANS [39].

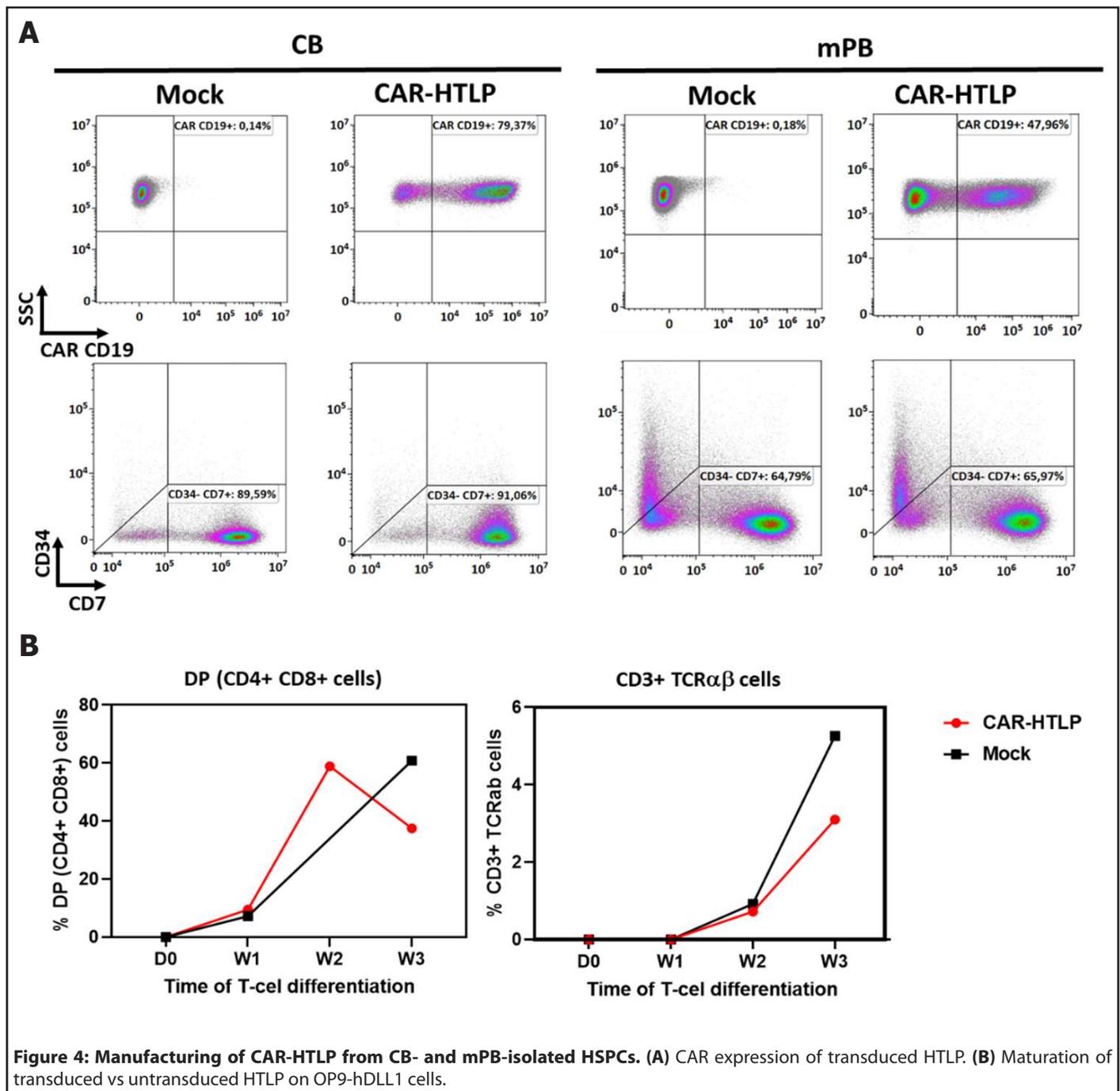
Recently we showed the feasibility of gene modification of our HTLPs [3]. Here we further show that our HTLP

production platform is compatible with using a lentiviral vector expressing a CD19 CAR molecule (19BBz). CB or mPB-derived HSPCs were subjected to a 7-day HTLP production process with an additional step of lentiviral transduction. The day-7 product was analyzed by flow cytometry for the expression of CD19 CAR molecule at cell surface. The result showed a high expression level of the CAR molecule in both CB and mPB derived products without altering the differentiation of stem cells toward HTLP (CD7⁺CD34⁺) (Figure 4A). Further differentiation of the day-7 CB-derived CAR-HTLP on the OP9-hDLL1 stromal cells demonstrated their efficient

differentiation into double positive (CD4⁺CD8⁺) and CD3⁺ mature T-cells as compared to non-transduced HTLP (Figure 4B). This observation, as well as the high efficacy of our HTLP production platform to manufacture a large number of gene-modified cells, offers a new opportunity to develop new immunotherapy cell products with the potential of improving the therapeutic index of current CAR-T cell therapies.

Concluding Remarks

Altogether, our work indicates that a T-lymphoid-based



cell product has the strong potential not only in the field of immune reconstitution after transplant but also in the onco-immunotherapy field, especially in an allogeneic setting. Compatible with both CB and mPB-derived HSPCs with high production efficacy, this platform can ensure widespread therapy accessibility. A pool of naïve, polyclonal, thymus-educated, and thus tolerant products can overcome certain hurdles of T-cell-based and HSPC-based cell and gene therapy as discussed above for leukemia. In particular, the homogeneity of the final cell product despite the different initial material sources, as we demonstrated, can reduce although not eliminate patient-to-patient variability, allowing for better comparison of outcomes in different clinical trials.

Besides these advantages, the possibility of producing a large number of gene-modified cells offers an opportunity to develop new immunotherapy cell products with the potential of improving the safety and efficacy of current CAR-T cell therapies. However, it remains to be investigated, in preclinical settings, whether the CAR-HTLP product is superior to approved mature CAR-T cell products.

Material and Methods

Transduction and differentiation of CD34⁺ HSPCs, as well as OP9-hDL1 culture and flow cytometry, were performed according to methods described by Moirangthem et al. [3]. scRNAseq libraries were generated with Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1, following the manufacturer's instructions. Transcriptomic datasets were analyzed using R [40]. Mass cytometry analysis was performed on the Helios mass cytometer and CyTOF software version 6.7.1014 (Fluidigm, Inc Canada) at the Cytometry Platform of La Pitié-Salpêtrière Hospital (CyPS). Mass cytometry standard file analysis was done using OMIQ data analysis software (www.omiq.ai). Statistical analysis of results was conducted using GraphPad Prism 9.4.0.673.

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Author Contributions

PG and TSS wrote the manuscript. PG, RDM, PR, and HS conducted cell culture experiments and analyzed the data. FC and ML took part to the scRNAseq experiments and their

analysis. TT discussed the data and reviewed the manuscript for critical content. TSS, IA, and ON designed and supervised the overall research and reviewed the manuscript for critical content.

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Conflict of Interest

FC, ML and TT did not declare any conflict of interest. PG, PR, HS, ON, and TSS work at Smart Immune. IA is a co-founder and holds equity in Smart Immune. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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