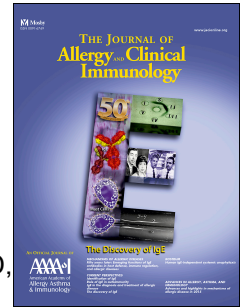


Accepted Manuscript

Generation of adult human T cell progenitors for immunotherapeutic applications

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PII: S0091-6749(17)31874-2

DOI: [10.1016/j.jaci.2017.10.034](https://doi.org/10.1016/j.jaci.2017.10.034)

Reference: YMAI 13140

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 19 June 2017

Revised Date: 12 September 2017

Accepted Date: 3 October 2017

Please cite this article as: Simons L, Ma K, de Chappedelaine C, Moirangthem RD, Elkaim E, Olivré J, Susini S, Appourchaux K, Reimann C, Sadek H, Pellé O, Cagnard N, Magrin E, Lagresle-Peyrou C, Taghon T, Rausell A, Cavazzana M, André-Schmutz I, Generation of adult human T cell progenitors for immunotherapeutic applications, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2017.10.034.

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“To the editor”**Generation of adult human T cell progenitors for immunotherapeutic applications**

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Conflicts of Interest: none

Sources of funding:

The study was funded by the French National Institute of Health and Medical Research (INSERM), a European Research Council grant (ERC Regenerative Therapy, 269037), a European Union FP7 grant (CELL-PID, 261387), a European Union H2020 grant (SCIDNet, 666908), Imagine Institute, an INCA-Plan Cancer grant (2009-2013) and a public grant overseen by the French National Research Agency (ANR) as part of the program “Investissements d’Avenir” (reference: ANR-10-IAHU-01). K.M. was funded by a China

Scholarship Council (CSC). E.E. was funded by an INSERM-Plan Cancer fellowship. C.R. was funded by postdoctoral scholarships from the Deutsche Forschungsgemeinschaft (DFG). T.T. was funded by the Fund for Scientific Research Flanders (FWO Vlaanderen research projects G0B2913N). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Capsule summary:

- Stroma-free *in vitro* generation of CD7⁺ T cell progenitors from adult HSPCs capable of generating a polyclonal T cell repertoire in NSG mice.
- Potential clinical use of T cell progenitors to shorten T cell recovery after HSCT.

Keywords: hematopoietic stem cell transplantation, T lymphocytes, translational research, human, lymphoid progenitor cells, immunity, thymus, cell culture, immunotherapy

Abbreviations:

HSCT	hematopoietic stem cell transplantation
HSPCs	hematopoietic stem and progenitor cells
CB	cord blood
ETP	Early Thymic Progenitor
mPB	mobilized peripheral blood
DL-4	Delta-like-4 Notch ligand
NSG	NOD/SCID/ γ c ^{-/-}
TCR	T-cell receptor

Prolonged T cell deficiency with subsequent high risk of infection and relapse is a major complication of non-HLA identical hematopoietic stem cell transplantation (HSCT).¹ Complete restoration of a polyclonal T cell repertoire takes up to 2 years and may never reach pre-transplant levels.² Even in T cell replete HSCT, homeostatic cytokines trigger a first peripheral wave of CD8⁺ T cells leading to a skewing of the TCR repertoire,³ and reconstitution of a fully functional T cell compartment relies on the production of new naïve T cells. Adoptive transfer of *in vitro* generated T lymphoid precursors provides a promising approach to overcome this hurdle and ameliorate T cell reconstitution after HSCT.⁴ Considering the known role of Notch signaling in T cell lineage differentiation in mouse and human,⁵ we have implemented a feeder cell-free culture system based on the immobilized Notch ligand DL-4 in combination with a specific set of cytokines.⁶ Compared to previously described feeder-free systems, our DL-4 culture is more efficient and highly reproducible when applied to cord blood (CB) HSPCs.⁷ Moreover, our system is currently in the process of being approved for clinical application.

Nevertheless, mobilized peripheral blood (mPB) is currently the main source of HSPCs in allogeneic HSCT as adult HSPCs are available in large quantity and exhibit several advantages over CB grafts in the clinical setting.⁸ However, their intrinsic properties differentiation, survival and proliferation have been little investigated. Here we demonstrate the production of T cell precursors from adult mPB HSPCs in a feeder- cell free DL-4 based *in vitro* system. All experimental procedures were performed as described in this article's Online Repository at www.jacionline.org.

Peripheral blood- derived CD34⁺ cells from G-CSF mobilized healthy donors were cultured with DL-4 protein and the phenotype of emerging cell populations was analyzed. As early as day 3 in cultures of adult HSPCs (Fig.1A), expression of CD34 and CD7 marked the presence of an ETP-like population not detected in control Fc cultures (Suppl. Fig. 1). In concordance with further T lineage commitment, precursors derived from adult HSPCs downregulated CD34 at day 7 giving rise to a CD34⁻ CD7⁺⁺ cell proT1-like cell population (Fig.1A and B). At day 7, the total number of CD7⁺ cells was 2.5 fold higher than at day 3 (Fig 1C).

We also quantified T lineage potential by culture of day 0, day 3 and day 7 DL-4 progenitors on OP9/DL1 in limiting dilution assays (Fig. 1D). The frequency of T cell precursors assessed by the presence of CD4⁺CD8⁺ DP cells or CD3⁺ cells reached a mean $\pm$ SEM of $1/13.5 \pm 1/3.5$ at day 3 and $1/18.9 \pm 1/6.6$ at day 7, similar to the one observed in day 7 CB DL-4 cultures and much higher than the one of uncultured CD34⁺ cells ($1/9,000$).⁶

To further characterize adult- derived T cell precursors, we performed gene expression analysis of day 0 mPB HSPCs and sorted CD7⁺ fractions on day 3 and day 7 of DL-4 cultures focusing on genes implicated in the Notch pathway, early lymphocyte development, thymus homing and T lineage differentiation. Major Notch target genes such as *DTX1*, *NRARP* and *HES1* were induced upon 3 days of DL-4 culture and remained stable thereafter (Fig.1E). Gene expression analysis revealed that exposure of mPB HSPCs to DL-4 led to (i) the gradual downregulation of multipotency and early lymphoid development genes like *CD34*, *IKAROS*, *MYB* and *KIT*, (ii) the silencing of *PAX5* and (iii) the induction of crucial T lineage differentiation genes such as *BCL11B*, *IL7Ra* and *TCF7*, all of which confirmed the proper induction of a T lineage program in CD7⁺ cells, not detected in CD7⁻ cells (Fig.1F, G and H).⁹ Regarding expression of homing molecules, *CXCR4* was already expressed in adult CD34⁺ HSPCs before culture (data not shown). After 3 and 7 days of DL-4 culture, major chemokine receptors potentially implicated in human thymus homing such as *CCR7*, *CCR9* and *CXCR4* were expressed significantly higher in the CD7⁺ population when compared to the CD7⁻ fraction (Fig. 1I).

Finally, the *in vivo* T cell potential of adult DL-4 precursors was tested by transplantation into non-irradiated newborn NSG mice. Human thymic engraftment was greatly accelerated occurring at only 4 weeks in 3 out of 3 mice injected with day 7 DL-4 cells and persisting thereafter (Fig 2A and Suppl. Table 1). In contrast, we did not observe engraftment prior to 6 weeks post-transplantation in recipients of untreated CD34⁺ cells or day 3 DL-4 cells (Fig 2A and Suppl. Table 1). Active human thymopoiesis was further demonstrated by the presence of human CD4⁺CD8⁺ DP cells and enlarged thymic lobes as compared to recipients of uncultured adult HSPCs (Fig. 2B and C). Mature CD3⁺ TCRαβ⁺ T cells displaying a polyclonal TCR repertoire were detected in the thymus and the spleen of recipient mice 8-week post-transplantation (Fig. 2C and D). Moreover, we detected a small population of CD25⁺CD4⁺Foxp3⁺CD127⁻ regulatory T cells in the spleen of injected mice (Suppl. Fig.2).

Overall, our results show that adult HSPCs provide an effective source of *in vitro* cultured T cell precursors harbouring all the necessary requirements for the *in vivo* reconstitution of a functional T cell compartment. Based on these results, we have established a clinical grade protocol to guide immunotherapeutic strategies employing T cell precursors to fasten T cell recovery in the adult transplantation setting, where HSPCs from mPB are more available and more frequently used than CB HSPCs.

Acknowledgments

We thank the animal house staff in the Transgenesis and Animal Experimentation Laboratory at SFR Necker (Paris, France) for careful handling of the mice. We thank Antoine Toubert, Emmanuel Clave and Ellen Rothenberg for fruitful discussions.

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Figure legends

Figure 1: Generation of T cell progenitors from mPB CD34⁺ HSPCs. (A, B, C) Phenotype, frequency and cell numbers of day 3 and day 7 DL-4 cultures. (D) Quantification of T-cell potential by T-cell differentiation on OP9/DL1 cells in limiting dilution conditions. Gene expression profile of day 0 CD34⁺ cells, day 3 and day 7 CD7⁺ cells by qRT-PCR (E, F, G) or day 3 and day 7. CD7⁻ and CD7⁺ cells (H, I). * $p < .05$, ** $p < .01$.

Figure 2: *In vivo* reconstitution potential of T cell precursors derived from mPB

(A) Human thymic engraftment evaluated by the frequency of human CD45⁺ (hCD45) and mouse CD45⁺ (mCD45) lymphocytes, in NSG recipients 6 weeks post-transplantation with CD34⁺ cells before (day 0) or after 3 or 7 days of culture in DL-4 conditions. (B) Representative photography of the thymi of recipients of day 7 DL-4 cultured progenitors and day 0 HSPCs, 8 weeks post-transplantation. (C) Presence of mature CD4⁺CD8⁺ DP cells in the thymus and CD3⁺TCR $\alpha\beta$ ⁺ T cells in the thymus and the spleen of recipients injected with day 7 DL-4 cultured progenitors (gated on hCD45⁺ cells, 8 weeks post-transplantation). (D) TCR β VJ diversity in splenocytes from NSG recipient mice of day 7 DL-4 cultured cells (8 weeks post-transplantation).

Figure 1

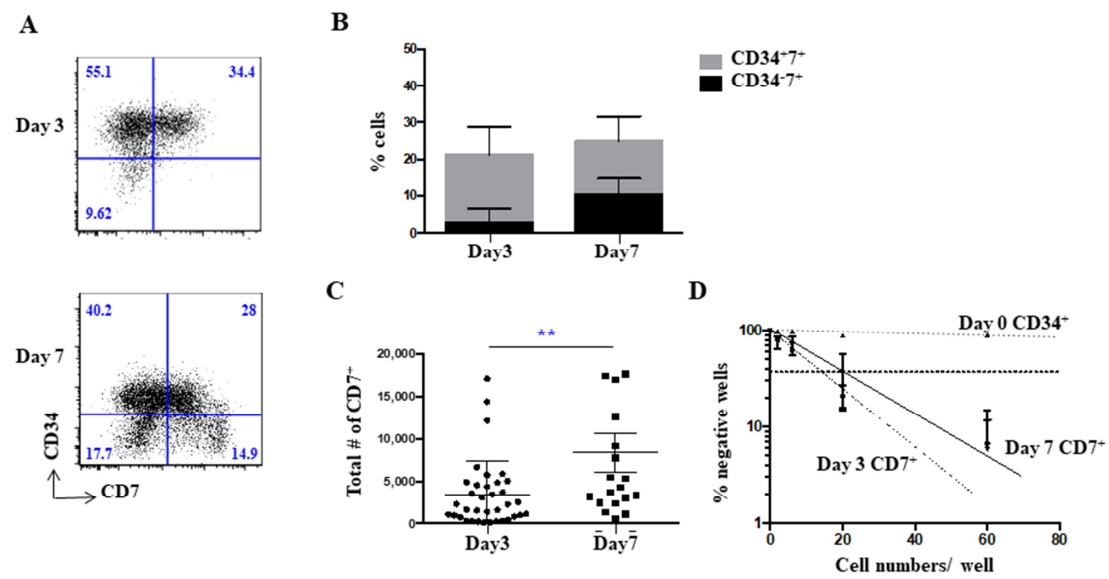


Figure 2 consists of three bar charts showing mRNA levels of Hprt, Hsp70, and Hsp90 in H9c2 cells at Day 0, Day 3, and Day 7. The y-axis for all charts is labeled (mRNA/Hprt) and represents relative mRNA levels. Hprt levels are used as a loading control and are relatively stable across all time points. Hsp70 levels show a significant increase at Day 3 and Day 7 compared to Day 0. Hsp90 levels also show a significant increase at Day 3 and Day 7 compared to Day 0. Error bars represent standard deviation.

Gene	Day 0	Day 3	Day 7
Hprt	~0.020	~0.004	~0.004
Hsp70	~0.008	~0.024	~0.024
Hsp90	~0.005	~0.008	~0.008

Figure 1

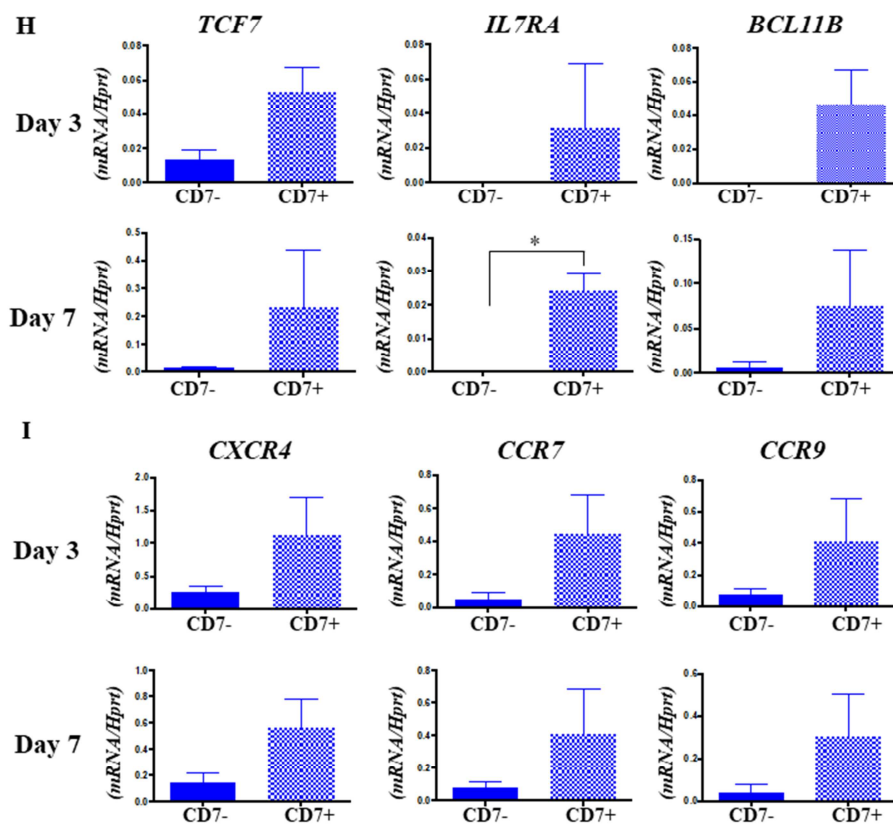
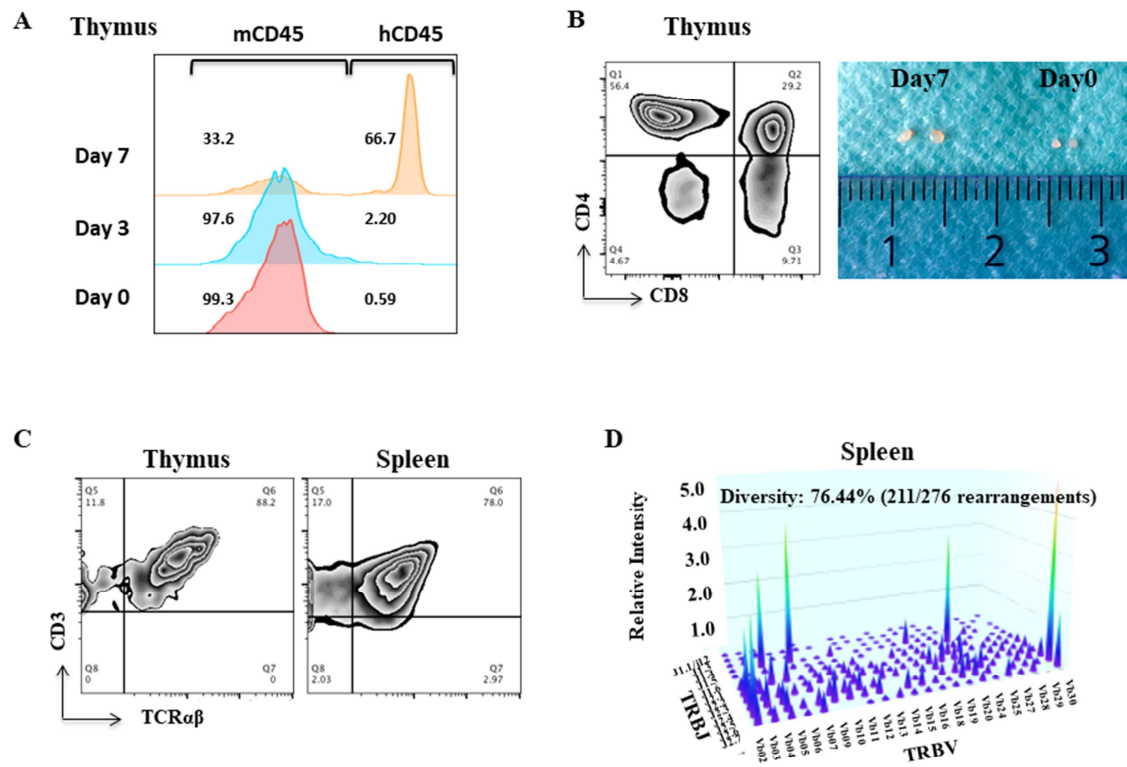


Figure 2



Supplementary Material and Methods

Human samples

Mobilized PB (mPB) samples used in the study corresponded to the unused fraction of grafts from 23 healthy donors mobilized with G-CSF, who had given their informed consent for research use. mPB samples are part of a collection authorized by the French Ministry of Research (DC-2014-2272, 23rd March 2015).

CD34⁺ hematopoietic stem and progenitor cells (HSPCs) were magnetically sorted from mPB as previously described.¹ Purity was > 93%.

Cultures

CD34⁺ cells were cultured in α -MEM medium (Gibco, Life Technology, Carlsbad, CA) in 24-well plates or 6-well plates that had been coated with RetroNectin® (25 μ g/ml, Ozyme, Saint-Quentin-en-Yvelines, France) and DL-4 (5 μ g/ml, PX³Therapeutics, Grenoble, France) as described previously.²

The T-lymphoid potential of naive CD34⁺ cells and T cell precursors generated by exposure to DL-4 was assessed via limiting dilution assays (LDAs) in OP9/DL-1 co-cultures, as previously described in n=3 independent experiments.¹

Flow cytometry analysis and cell sorting

Cells obtained after 3 days (n=36) or 7 days (n=19) of culture were analysed by flow cytometry. Monoclonal antibodies against human CD34 (AC136), CD3 (BW264/56), CD45 (5B1), CD4 (VIT4) and CD8 (BW135/80) were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany), and CD4 (SK4), CD7 (M-T701), CD25 (M-A251), CD127 (HIL-7R-M21), 7-aminoactinomycin D (7AAD) were from BD Biosciences (San Jose, CA). Anti-human CD8 (RPAT8) was from Sony Biotechnology (San Jose, CA), and Foxp3 (PCH101) and CD3 (UCHT1) were purchased from eBioscience (San Diego, CA). TCR alpha/beta (IP26A) and TCR gamma/delta (IMMU510) were from Beckman Coulter (Brea, CA).

Cells from xenogenic murine recipients were analyzed on a MACSQuant® apparatus (Miltenyi Biotech, Bergisch Gladbach, Germany).

The data were analyzed using FlowJo software (version 10.2, Treestar, Ashland, OR) after gating on viable, 7AAD-negative cells. Cell subsets were sorted on an ARIA II system (BD Biosciences).

Cultured cells were sorted by fluorescence-activated cell sorting (FACS) after 3 and 7 days of culture on DL-4 respectively to exclude CD34⁺/CD7⁺ myeloid cells from subsequent analysis. Cell subsets were sorted on BD FACSAria II SORP system using a highly pure sorting modality (4-way purity) achieving a purity of >98%. The viability of sorted CD7⁺ cells measured by 7AAD staining was 99.22 +/- 0.17% (mean ± SEM).

Quantitative, real-time polymerase chain reactions using RT² Profiler array

Total RNA of n=4 uncultured CD34⁺ HSPCs and sorted CD7⁺ cell fractions from n=6 day 3 and n=7 day 7 was isolated with the Rneasy Micro Kit (Qiagen, Courtaboeuf, France). RT² Profiler PCR arrays for the Human Notch Pathway and a custom- designed array including lymphocyte and T- lineage differentiation genes were performed following the protocol detailed in the *RT2 Profiler PCR Array Handbook* (SA Biosciences, Frederick MD). Briefly, from a total RNA eluate volume of 16µl, 8µl were subjected to genomic DNA elimination, cDNA synthesis and preamplification using the RT2 PCR System (PreAMP system, SA Biosciences, Frederick MD). Preamplified cDNA samples were reverse transcribed using the RT2 First Strand Kit (SA Biosciences, Frederick MD), which includes an additional step of DNase digestion to prevent contamination of cDNA with any genomic DNA. cDNA was mixed with RT² SYBR Green Mastermix and RNase-free water to make the PCR components mix, which was then distributed in 25µl aliquots across the RT² Profiler PCR Array. For the custom array, primers were synthesized by the manufacturer (SA Biosciences, Frederick MD) based on the list of gene names, symbols, UniGene and Genbank IDs. The plate was sealed and centrifuged to remove bubbles then run on an ABI Prism 7300 system (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. All transcript levels were normalized against the results for human hypoxanthine-guanine phosphoribosyltransferase (*HPRT*). Normalization to the *HPRT* was performed by calculating the ΔC_t for each gene of interest (GOI) in the plate (C_t value of GOI- C_t value of *HPRT*). Any C_t value >35 was considered to be a negative call. If the C_t value of the genomic DNA control was >30, then no genomic DNA was detectable. Negative controls remained unamplified throughout the study.

Data were processed using R packing by the value Log2 2-dCt. The non-significant different values based on Pearson's correlation coefficient were excluded. Heat map was created by Java Treeview software (version 1.1, <https://sourceforge.net/>).

Adoptive transfer of *in vitro*-generated T cell precursors derived from adult HSPCs into NSG neonates

The NOD/SCID/ γ c^{-/-} (NSG) mice (obtained from the Jackson Laboratory, Bar Harbor, ME, <http://www.jax.org>) were kept in a pathogen-free facility. Progeny derived from mPB CD34⁺ HSPCs in 3 or 7 day DL-4 culture (3×10^5 or 1×10^6) were injected intrahepatically into NSG neonates (0-4 days old) (n=5 and n=7 recipients transplanted with day 3 and day 7 cell products, respectively, in 3 independent experiments). Control mice were injected with either 3×10^5 non-cultured mPB CD34⁺ cells (n=9) or 100 μ l PBS (n=13).

Human engraftment rates were determined from 4 to 16 weeks post-transplantation. Flow cytometry analysis was performed on fresh cells collected from femur bone marrow, thymus and spleen. Cells were treated with 1x red blood cell lysis buffer (Biolegend, US) and washed before stained by antibodies.

T cell repertoire diversity was evaluated by determination of VDJ gene rearrangements using the ImmunTraCkeR kit (ImmuneID Technologies, Grenoble, France) on genomic DNA extracted from splenocyte of NSG recipient mice 8 weeks post-transplantation with human DL-4 induced T cell precursors. Signals were measured by semi-quantitative evaluation on fluorescence intensity and analyzed by ConstelID software (ImmuneID Technologies, Grenoble, France).

The injection of human progenitors in NSG mice has been approved by the Ministry of Higher Education and Research (APAFIS 2101-2015090411495178v4, 2nd November 2015).

Statistical analysis

Unpaired t- test was used to compare differences of gene expression between mPB HSPCs and DL-4 induced T cell precursors using Prism software (version 6, GraphPad Software Inc., La Jolla, CA).

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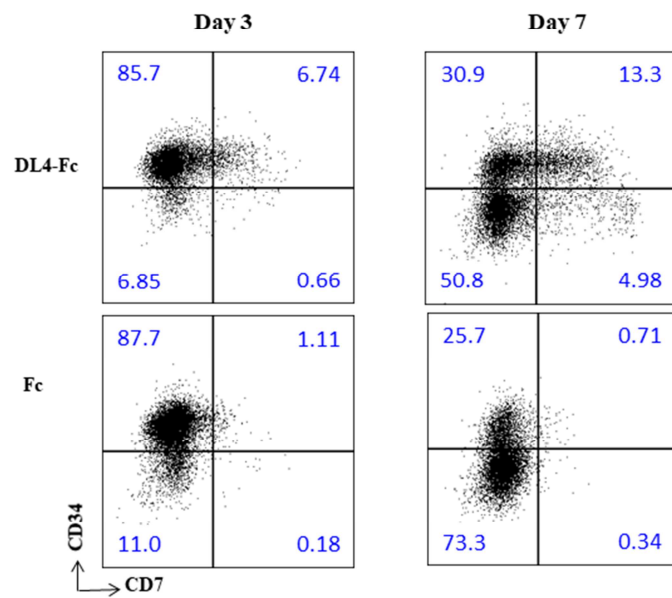
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Supplementary Data

Suppl. Table 1: Kinetics of thymic engraftment of DL-4 cultured progenitors after transplantation in NSG mice

Transplant (number of recipients)	Weeks post transplantation			
	4-5	6-7	8-10	11-16
Day 0 CD34 ⁺ cells (n=9)	0/1	0/2	1/2	4/4
Day 3 DL-4 precursors (n=5)	nd	1/2	0/1	4/4
Day 7 DL-4 precursors (n=7)	3/3	1/2	1/1	1/1

Suppl. Fig 1 : Phenotype of adult CD34⁺ HSPCs exposed to 3 or 7 days of DL4- Fc fusion protein compared to Fc alone.



Suppl. Fig 2: Presence of human CD25⁺CD4⁺Foxp3⁺CD127⁻ Treg cells in the spleen of NSG recipients 8 weeks after transplantation of adult Day 7 DL-4 precursors. Cells were first gated on 7AAD⁻ alive human CD45⁺ cells (left panel) and then on CD4⁺ CD25⁺ cells (right panel).

