purified natural Amb a 1.01 is capable of inducing strong T_H2 immune responses in mice in the absence of (extrinsic) adjuvants. In contrast, under the same conditions, the nonallergenic bacterial homologue induced a T_H1-biased response. Our data on Amb a 1.01 and DC-pelC are in agreement with previous findings for fold-stabilized Bet v 1 mutants.[8] Thus, we propose a model in which the intrinsic stability/susceptibility of a protein to proteolysis can directly influence its processing and determines the intensity and polarization of the ensuing immune response (Fig 2, *C*).

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Generation of adult human T-cell progenitors for immunotherapeutic applications

To the Editor:

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 might never reach pretransplantation levels.[2] Even in T cell–replete HSCTs, homeostatic cytokines trigger a first peripheral wave of $CD8^+$ T cells, leading to a skewing of the T-cell receptor (TCR) repertoire,[3] and reconstitution of a fully functional T-cell compartment relies on production of new naive 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 mice and human subjects,[5] we have implemented a feeder cell–free culture system based on the immobilized Notch ligand delta-like (DL) 4 in combination with a specific set of cytokines.[6] Compared with previously described feeder-free systems, our DL-4 culture is more efficient and highly reproducible when applied to cord blood (CB) hematopoietic stem and progenitor cells (HSPCs).[7] Moreover, our system is currently in the process of being approved for clinical application.

Mobilized peripheral blood (mPB) is currently the main source of HSPCs in allogeneic HSCTs because adult HSPCs are available in large quantities and exhibit several advantages over CB grafts in the clinical setting.[8] However, their intrinsic properties of differentiation, survival, and proliferation have been investigated poorly thus far. 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 the Methods section in this article's Online Repository at www.jacionline.org.

Peripheral blood–derived $CD34^+$ cells from granulocyte colony-stimulating factor–mobilized (G-CSF) healthy donors were cultured with DL-4 protein, and the phenotype of emerging cell populations was analyzed. As early as day 3 in adult HSPC cultures (Fig 1, *A*), expression of CD34 and CD7 marked the presence of an early thymic progenitor (ETP)-like population not detected in control Fc cultures (see Fig E1 in this article's Online Repository at www.jacionline.org). In concordance with further T-lineage commitment, precursors derived from adult HSPCs downregulated CD34 at day 7, giving rise to a $CD34^-CD7^{++}$ cell pro-

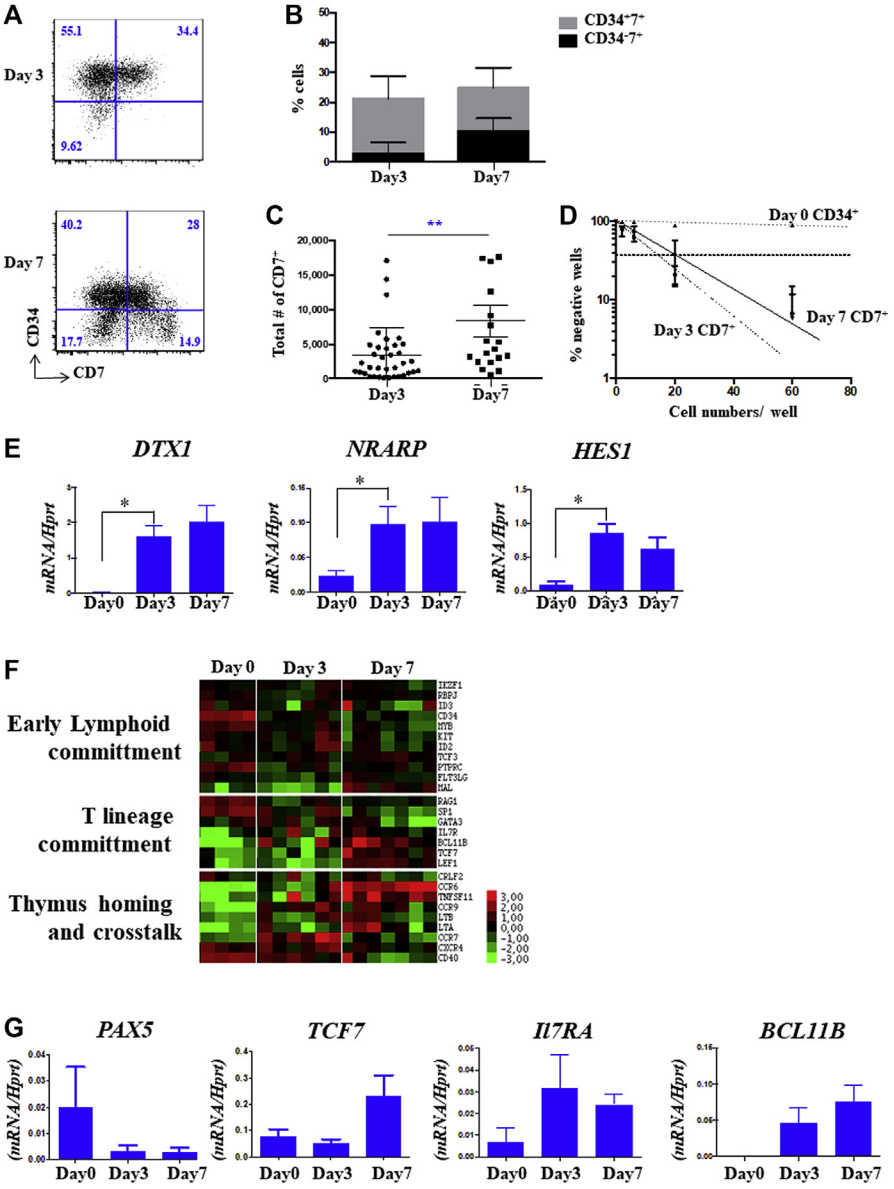

FIG 1. Generation of T-cell progenitors from mPB CD34$^+$ HSPCs. **A-C,** Phenotype, frequency, and cell numbers of day 3 and day 7 DL-4 cultures. **D,** Quantification of T-cell potential by T-cell differentiation on OP9/DL-1 cells in limiting dilution conditions. **E-I,** Gene expression profile of day 0 CD34$^+$ cells and day 3 and day 7 CD7$^+$ cells by using quantitative RT-PCR (Fig 1, *E-G*) or day 3 and day 7 CD7$^-$ and CD7$^+$ cells (Fig 1, *H* and *I*). *P < .05 and **P < .01.

T1–like cell population (Fig 1, *A* and *B*). At day 7, the total number of CD7$^+$ cells was 2.5-fold greater than at day 3 (Fig 1, *C*).

We also quantified T-lineage potential by culture of day 0, 3, and 7 DL-4 progenitors on OP9/DL-1 in limiting dilution assays (LDA) (Fig 1, *D*). Based on the presence of CD4$^+$CD8$^+$ double-positive cells or CD3$^+$ cells, the frequency of T-cell precursors assessed by LDA reached mean ± SEM values of 1/13.5 ± 1/3.5 at day 3 and 1/18.9 ± 1/6.6 at day 7. These values were similar to values observed in day 7 CB DL-4 cultures and much greater than the value in uncultured CD34$^+$ cells (1/9000).[6]

To further characterize adult-derived T-cell precursors, we performed gene expression analysis of day 0 mPB HSPCs and sorted CD7$^+$ fractions on days 3 and 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 after 3 days of DL-4 culture and remained stable thereafter (Fig 1, *E*). Gene expression analysis revealed that exposure of mPB HSPCs to DL-4 led to (1) gradual downregulation of multipotency and early lymphoid development genes, such as *CD34, IKAROS*, *MYB*, and *KIT*; (2) silencing of *PAX5*; and (3) 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 1, *F-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 at significantly higher levels in the CD7$^+$ population when compared with the CD7$^-$ fraction (Fig 1, *I*).

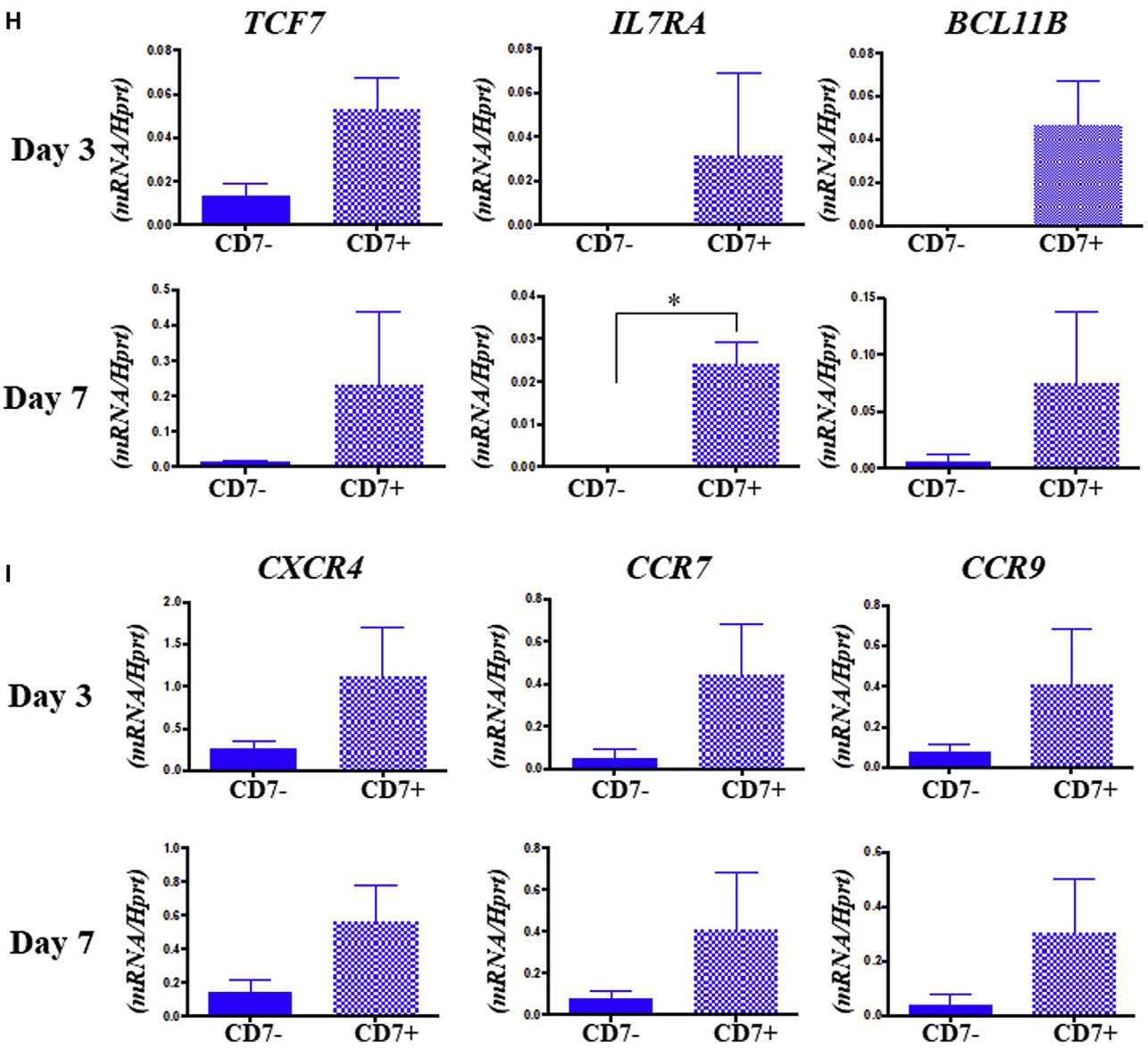

FIG 1. (*Continued*)

Finally, the *in vivo* T-cell potential of adult DL-4 precursors was tested by means of transplantation into nonirradiated newborn NOD/SCID/$\gamma c^{-/-}$ (NSG) mice. Human thymic engraftment was accelerated greatly, occurring at only 4 weeks in 3 of 3 mice injected with day 7 DL-4 cells and persisting thereafter (Fig 2, *A*, and see Table E1 in this article's Online Repository at www.jacionline.org). In contrast, we did not observe engraftment before 11 weeks after transplantation in recipients of untreated $CD34^+$ cells or day 3 DL-4 cells (Fig 2, *A*, and see Table E1). Active human thymopoiesis was further demonstrated by the presence of human $CD4^+CD8^+$ double-positive cells and enlarged thymic lobes compared with recipients of uncultured adult HSPCs (Fig 2, *B* and *C*). Mature $CD3^+TCR\alpha\beta^+$ T cells displaying a polyclonal TCR repertoire were detected in the thymi and spleens of recipient mice 8 weeks after transplantation (Fig 2, *C* and *D*). Moreover, we detected a small population of $CD25^+CD4^+Foxp3^+CD127^-$ regulatory T cells in the spleens of injected mice (see Fig E2 in this article's Online Repository at www.jacionline.org).

Overall, our results show that adult HSPCs provide an effective source of *in vitro*–cultured T-cell precursors harboring all the necessary requirements for *in vivo* reconstitution of a functional T-cell compartment. Based on these results, we have established a clinical grade protocol to guide immunotherapeutic strategies using 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 HSPCs from CB.

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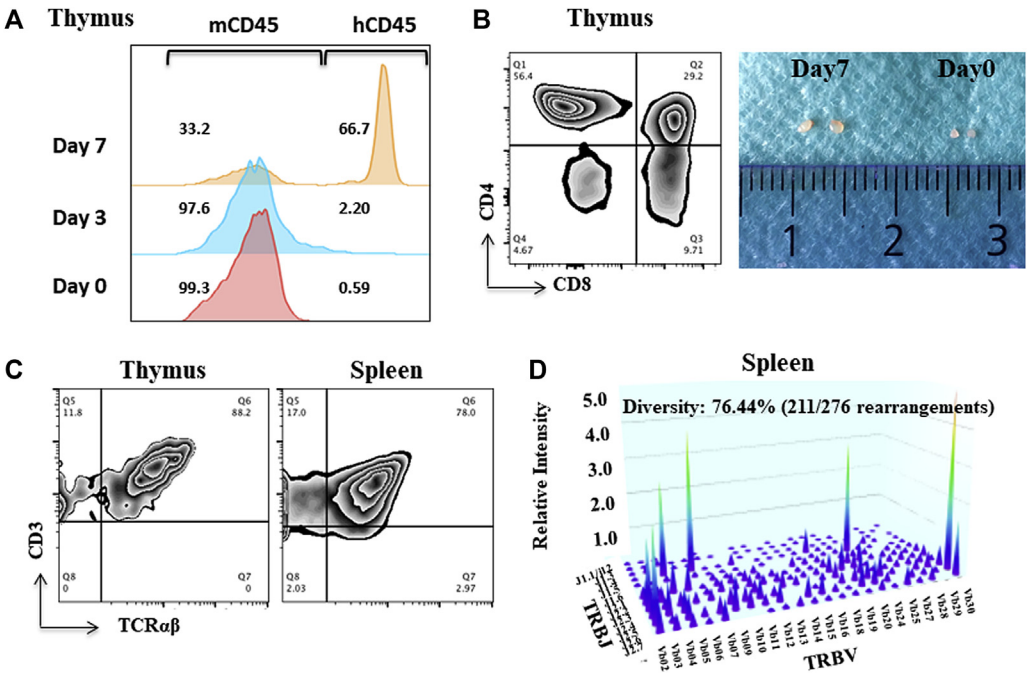

FIG 2. *In vivo* reconstitution potential of T-cell precursors derived from mPB. **A,** Human thymic engraftment evaluated by the frequency of human CD45$^+$ *(hCD45)* and mouse CD45$^+$ *(mCD45)* lymphocytes in NSG recipients 6 weeks after transplantation with CD34$^+$ cells before (day 0) or after 3 or 7 days of culture in DL-4 conditions. **B,** Presence of mature CD4$^+$CD8$^+$ double-positive cells in thymi of recipients transplanted with day 7 DL-4–cultured progenitors. The picture shows a representative photograph of recipient thymi injected with day 7 DL-4 cultured cells and day 0 HSPCs 8 weeks after transplantation. **C,** Presence of CD3$^+$TCRαβ$^+$ T cells in thymi and spleens of recipients injected with day 7 DL-4 cultured progenitors (gated on hCD45$^+$ cells 8 weeks after transplantation). **D,** TCRβ VJ diversity in splenocytes from NSG mice transplanted with day 7 DL-4–cultured cells (8 weeks after transplantation).

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METHODS

Human samples

mPB samples used in the study corresponded to the unused fraction of grafts from 23 healthy donors mobilized with granulocyte colony-stimulating factor who had provided 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).

$CD34^+$ HSPCs were sorted magnetically from mPB, as previously described.[E1] Purity was greater than 93%.

Cultures

$CD34^+$ cells were cultured in α-MEM medium (Gibco, Life Technology, Carlsbad, Calif) in 24- or 6-well plates coated with RetroNectin (25 μg/mL; Ozyme, Saint-Quentin-en-Yvelines, France) and DL-4 (5 μg/mL; PX'Therapeutics, Grenoble, France), as described previously.[E2]

The T-lymphoid potential of naive $CD34^+$ cells and T-cell precursors generated by exposure to DL-4 was assessed by using a limiting dilution assay in OP9/DL-1 cocultures, as previously described in 3 independent experiments.[E1]

Flow cytometric analysis and cell sorting

Cells obtained after 3 (n = 36) or 7 (n = 19) days of culture were analyzed by using 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, Calif). Anti-human CD8 (RPAT8) was from Sony Biotechnology (San Jose, Calif), and Foxp3 (PCH101) and CD3 (UCHT1) were from eBioscience (San Diego, Calif). TCRα/β (IP26A) and TCRγδ (IMMU510) were from Beckman Coulter (Brea, Calif).

Cells from xenogenic murine recipients were analyzed on a MACSQuant apparatus (Miltenyi Biotech). Data were analyzed with FlowJo software (version 10.2; TreeStar, Ashland, Ore) after gating on viable $7AAD^-$ cells. Cell subsets were sorted on a FACSAria II system (BD Biosciences).

Cultured cells were sorted by using fluorescence-activated cell sorting 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 with a highly pure sorting modality (4-way purity), achieving a purity of greater than 98%. The viability of sorted $CD7^+$ cells measured by using 7AAD staining was 99.22% ± 0.17% (mean ± SEM).

Quantitative real-time PCRs with the RT^2 Profiler array

Total RNA of 4 uncultured $CD34^+$ HSPCs and sorted $CD7^+$ cell fractions from 6 day 3 and 7 day 7 samples was isolated with the RNeasy Micro Kit (Qiagen, Courtaboeuf, France). RT^2 Profiler PCR arrays for the Human Notch Pathway and a custom-designed array, including lymphocyte and T-lineage differentiation genes, were performed according to the protocol detailed in the *RT2 Profiler PCR Array Handbook* (SA Biosciences, Frederick, Md). Briefly, from a total RNA eluate volume of 16 μL, 8 μL was subjected to genomic DNA elimination, cDNA synthesis, and preamplification by using the RT^2 PCR System (PreAMP system; SA Biosciences). Preamplified cDNA samples were reverse transcribed with the RT^2 First Strand Kit (SA Biosciences), which includes an additional step of DNAse digestion to prevent contamination of cDNA with any genomic DNA. cDNA was mixed with RT^2 SYBR Green Mastermix and RNase-free water to make the PCR components mix, which was then distributed in 25-μL aliquots across the RT^2 Profiler PCR Array. For the custom array, primers were synthesized by the manufacturer (SA Biosciences) based on the list of gene names, symbols, and UniGene and Genbank IDs. The plate was sealed and centrifuged to remove bubbles and then run on an ABI Prism 7300 system (Applied Biosystems, Foster City, Calif), according to the manufacturer's instructions. All transcript levels were normalized against the results for human hypoxanthine guanine phosphoribosyltransferase *(HPRT)*. Normalization to the *HPRT* gene was performed by calculating the Δ cycle threshold (Ct) for each gene of interest in the plate (Ct value of the gene of interest Ct value of *HPRT*). Any Ct value of greater than 35 was considered a negative call. If the Ct value of the genomic DNA control was greater than 30, then no genomic DNA was detectable. Negative controls remained unamplified throughout the study.

Data were processed by using the R package with the value $\log_2 2-\Delta Ct$. The nonsignificant different values based on the Pearson correlation coefficient were excluded. The heat map was created with 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

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 cultures (3×10^5 or 1×10^6) were injected intrahepatically into NSG neonates (0-4 days old; n = 5 and n = 7 recipients undergoing transplantation with day 3 and day 7-cell products, respectively, in 3 independent experiments). Control mice were injected with either 3×10^5 noncultured mPB $CD34^+$ cells (n = 9) or 100 μL of PBS (n = 13).

Human engraftment rates were determined from 4 to 16 weeks after transplantation. Flow cytometric analysis was performed on fresh cells collected from femur bone marrow, thymus, and spleen. Cells were treated with 1× red blood cell lysis buffer (BioLegend, San Diego, Calif) and washed before antibodies staining.

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

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

Statistical analysis

The unpaired *t* test was used to compare differences in gene expression between mPB HSPCs and DL-4–induced T-cell precursors by using Prism software (version 6; GraphPad Software, La Jolla, Calif).

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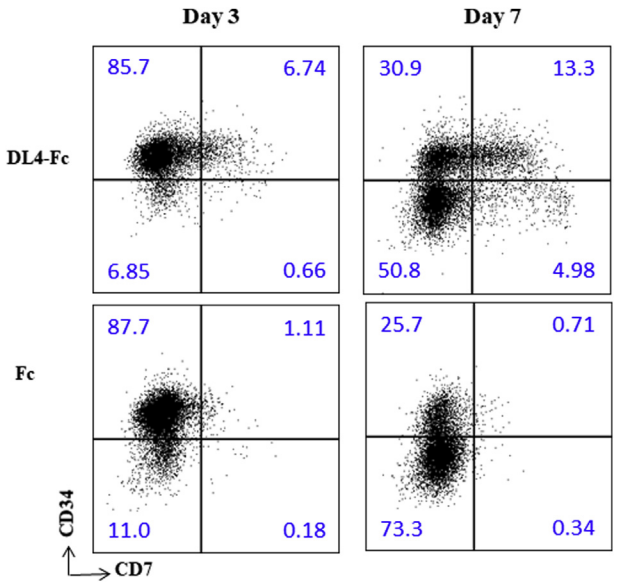

FIG E1. Phenotype of adult CD34^{+} HSPCs exposed to 3 or 7 days of DL-4–Fc fusion protein compared with Fc alone.

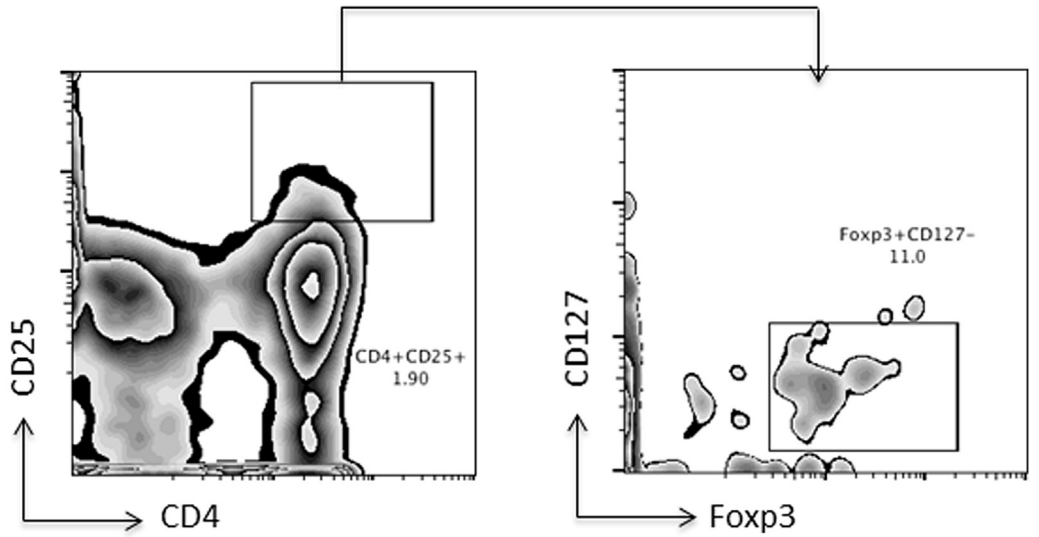

FIG E2. Presence of human $CD25^{+}CD4^{+}Foxp3^{+}CD127^{-}$ regulatory T cells in the spleens of NSG recipients 8 weeks after transplantation of adult day 7 DL-4 precursors. Cells were first gated on $7AAD^{-}$ live human $CD45^{+}$ cells *(left panel)* and then on $CD4^{+}CD25^{+}$ cells *(right panel)*.

TABLE E1. Kinetics of thymic engraftment of DL-4–cultured progenitors after transplantation in NSG mice

	Weeks after transplantation			
Transplantation (no. of recipients)	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

ND, Not determined.