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TECHNICAL ADVANCE

Technical Advance: **Ascorbic acid induces development of double-positive T cells from human hematopoietic stem cells in the absence of stromal cells**

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ABSTRACT

The efficacy of donor HSCT is partly reduced as a result of slow post-transplantation immune recovery. In particular, T cell regeneration is generally delayed, resulting in high infection-related mortality in the first years post-transplantation. Adoptive transfer of in vitro-generated human T cell progenitors seems a promising approach to accelerate T cell recovery in immunocompromised patients. AA may enhance T cell proliferation and differentiation in a controlled, feeder-free environment containing Notch ligands and defined growth factors. Our experiments show a pivotal role for AA during human in vitro T cell development. The blocking of NOS diminished this effect, indicating a role for the citrulline/NO cycle. AA promotes the transition of proT1 to proT2 cells and of preT to DP T cells. Furthermore, the addition of AA to feeder cocultures resulted in development of DP and SP T cells, whereas without AA, a preT cell-stage arrest occurred. We conclude that neither DLL4-expressing feeder cells nor feeder cell conditioned media are required for generating DP T cells from CB and G-CSF-mobilized HSCs and that generation and proliferation of proT and DP T cells are greatly improved by AA. This technology could potentially be used to generate T cell progenitors for adoptive therapy. *J. Leukoc. Biol.* 96: 1165–1175; 2014.

Introduction

HSCT is one of the current treatments of hematological malignancies. However, slow post-transplantation immune recovery may result in high infection-related mortality, especially if the donor source comes from haplo-identical or CB donors [1, 2]. In particular, immune reconstitution is hindered by a delayed T cell regeneration. Important reasons for this delay are age-related thymic involution, impaired thymus function, and a decreased repopulation of the thymus by stem/progenitor cells [3–6]. Only de novo T cell generation in the thymus ensures a broad T cell repertoire that guarantees complete immune recovery after HSCT.

T cell development is a highly regulated process that starts with HSCs in the bone marrow and proceeds in the thymus. Maturation of T cell progenitors in the thymus can be divided into several stages. Based on the expression of CD4 and CD8, T cell development can be separated into DN, DP, and SP stages [7]. The DN stages can be subdivided based on the expression of early T cell markers, with CD7 as the first marker expressed (proT1) and subsequent CD5 expression (proT2, previously described as DN2) [8–11]. CD1a is present on T-lineage-committed precursors (preT, previously described as DN3) and is followed by expression of CD4 (iSP) [11–13]. In the subsequent DP stage, CD3 and TCR$\alpha\beta$ are expressed, and after positive and negative selection in the thymic microenvironments, cells enter the periphery as mature, naive SP T cells. Crucial for T cell development is the interaction of the Notch receptor on progenitor cells with Notch ligands provided by the thymic stroma [14]. Both Notch ligands DLL1 and DLL4 are able to drive T-lineage development in vitro [15], whereas in vivo, only DLL4 gives the instructive signal to the thymus-seeding cells [16, 17]. As a result of the impor-

Abbreviations: AA=ascorbic acid, APC=allophycocyanin, CB=cord blood, CD=cluster of differentiation, DLL=Delta-like ligand, DN=double-negative, DP=double-positive, FLt3-L=FMS-like tyrosine kinase 3 ligand, HSC=hematopoietic stem cell, HSCT=hematopoietic stem cell transplantation, iCD=intracellular cluster of differentiation, iSP=immature single-positive, L-NMMA=N^G-monomethyl-L-arginine-monoacetate salt, mCD34$^+$=G-CSF-mobilized CD34$^+$ cells, NAC=N-acetyl-L-cysteine, ph-AA=2-phospho-L-ascorbic acid, PSGL-1=P-selectin glycoprotein-1, SCF=stem cell factor, SP=single-positive, TPO=thrombopoietin

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tance of Notch signaling during T-lineage development, DLL-expressing feeder cells have been used in vitro to generate T cell progenitors for adoptive immunotherapy.

Adoptive transfer of in vitro-generated (progenitor) T cells has evolved as a promising approach to accelerate T cell recovery in immune-compromised patients. In recent years, methods to generate T cell progenitors in vitro have been developed [9, 11, 18]. It is shown that these progenitors can enhance T cell reconstitution in vivo [9, 11, 19]. We have reported that mCD34$^+$ cells in coculture with thymic stroma-derived TSt-4/DLL feeder cells differentiate into T/NK progenitors [11]. Similar results have been obtained with CB- and bone marrow-derived CD34$^+$ cells in coculture with the bone marrow-derived OP-9/DL1 feeder cells [9, 18]. Together with some other stromal cell lines, such as S17/DL1 [20], the DLL-expressing feeder cell lines have the unique capacity to support T cell development in contrast to other stromal cells, for example, NIH3T3-expressing DLL [21]. Additionally, OP-9/DL1 can support T cell development in vitro to the DP/SP stage in contrast to TSt-4/DLL [22]. In vivo immune reconstitution experiments showed that TSt-4/DLL-derived progenitor cells from mCD34$^+$ and CB-derived proT cells from OP-9/DL1 cocultures home to the thymi of immune-deficient mice and complete their development into mature T cells faster than unmanipulated stem cells [9, 11]. Additionally, Eyrich et al. [19] detected extrathymic, mature T cells in mice after injection of T cell progenitors derived from OP-9/DL1 coculture.

Efforts have been made to generate in vitro clinical-grade T cell progenitors without the use of feeder cells [23, 24]. Ohishi et al. [23] showed that expansion of CB CD34$^+$ cells in the presence of immobilized DLL1 and cytokines resulted in the development of a CD34$^+$CD7$^+$ lymphoid progenitor population capable of CD3$^+$ T cell reconstitution in the thymus of immune-deficient mice. Moreover, a phase 1 clinical study with the transfer of CB CD34$^+$ DLL1 culture-derived progenitors showed enhanced engraftment and myeloid reconstitution [25]. However, the lack of improvement in T cell reconstitution in these patients indicates the need for more efficient methods for in vitro generation of T cell progenitors.

To extend on our previous work [11, 26], we searched for factors to enhance T cell differentiation and proliferation and investigated the role of AA in the current study. AA plays a multitude of roles in the immune system and is abundantly present in T cells [27], but its role during human T cell development is not known. In our present study, we have investigated the role of different forms of AA on T cell maturation and proliferation from HSCs and show the influence of AA in feeder and feeder-free conditions.

MATERIALS AND METHODS

Isolation and purification of CD34$^+$ cells

CB CD34$^+$ and mCD34$^+$ cells were obtained at the Maastricht University Medical Center (the Netherlands) after informed consent, in accordance with the Declaration of Helsinki and with approval of the local Medical Ethical Committee (METC 12-2-044). mCD34$^+$ cells were obtained from healthy volunteers treated with G-CSF (Neupogen; Amgen, Thousand Oaks, CA, USA). Mononuclear cells were isolated from fresh 1:1 PBS (PAA, Pasching, Austria)-diluted CB by Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation. CD34$^+$ cells were enriched using immunomagnetic beads, according to the manufacturer's instructions (CD34$^+$ microbead kit and CliniMACS CD34$^+$ selection kit for CB CD34 and mCD34$^+$, respectively; Miltenyi Biotec, Bergisch Gladbach, Germany). CD34$^+$CD38$^{-/dim}$ cells were purified further and depleted from contaminating T and NK cells by FACS using FACSAria (Becton Dickinson, Erembodegem, Belgium). For cell sorting, the following antibodies were used at proper dilutions: FITC-anti-CD38; PE-anti-CD34; PerCP-anti-CD4, -CD8, and -CD3; and APC-anti-CD56 or horizon V450-anti-CD56 (all Becton Dickinson). To obtain a sufficient number of starting cells, CB units from different healthy donors were obtained and pooled. Purity of mobilized and CB CD34$^+$ cells was ≥98% CD34$^+$ cells and ≤0.1%-contaminating CD3/CD4/CD8 or CD56$^+$ cells.

Cocultures with feeder cells

TSt-4 cells, an earlier gift of Professor Dr. H. Kawamoto (Kyoto University, Kyoto, Japan), were grown in standard RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 5% FBS (Greiner Bio-One, Kremsmuenster, Austria), 1% penicillin-streptomycin, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, and 5×10^{-5} M 2-ME (all from Invitrogen, Paisley, UK). OP-9 cells were cultured in αMEM medium, reconstituted from powder (Life Technologies, Carlsbad, CA, USA), containing 20% FBS (Hyclone; Thermo Scientific, Logan, UT, USA), 5 g/L sodium bicarbonate (Sigma-Aldrich), and 1% penicillin-streptomycin (Invitrogen). Liquid αMEM medium (Life Technologies) without AA was used when indicated. L-AA (Sigma-Aldrich) was supplemented to RPMI 1640 and liquid αMEM medium when indicated. OP-9 cells were a kind gift of Drs. R. Schotte and B. Blom (AMC, Amsterdam, the Netherlands). mCD34$^+$ cells were seeded at a density of 2.6×10^3 cells/cm^2 on monolayers of TSt-4 cells expressing Notch ligands DLL1 or DLL4 or monolayers of OP-9/DL1. After addition of CD34$^+$ cells, media were supplemented with human IL-7, SCF, and FLt3-L (Miltenyi Biotec). Cocultures were refreshed by half-medium change, three times/week. During the 1st week, 50 ng/ml, and from the 2nd week onward, 5 ng/ml of each cytokine were added to the cultures. Differentiating cells were transferred to fresh monolayers every week. For transfer, cells were separated from the monolayers by disruption of the monolayers using cell scrapers (Becton Dickinson), resuspension by pipetting, and filtration through a 70-μm mesh (Becton Dickinson) directly onto the new monolayer. Cocultures were maintained for 7 weeks.

Feeder-free culture

Nontissue culture-treated culture plates (Falcon; Becton Dickinson) were incubated for 1 h at room temperature with 10 μg/ml rabbit anti-human IgG Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and subsequently with DLL4:Fc (Sino Biological, Beijing, China), diluted in PBS for 1 h. For the second protocol, nontissue culture-treated culture plates (Falcon; Becton Dickinson) were incubated overnight at 4°C with DLL4:Fc diluted in PBS, together with 5 μg/ml RetroNectin (r-fibronectin fragment CH-296; Takara Shuzo, Otsu, Japan), washed extensively, and incubated further for at least 1.5 h with 2% HSA diluted in PBS. CD34$^+$CD38$^{-/dim}$ cells were seeded at a density of 3.9×10^3 cells/cm^2 and cultured in RPMI 1640, αMEM, or StemSpan Serum-Free Expansion Medium II (Stemcell Technologies, Grenoble, France), with or without L-AA or ph-AA (Sigma-Aldrich) supplementation. Cocultures were refreshed by half-medium change, three times/week and weekly exposed to fresh DLL4:Fc and fibronectin. All cultures were supplemented with human IL-7, SCF, FLt3-L (all 50 ng/ml), and 100 ng/ml TPO (all Miltenyi Biotec). Cultures were maintained for 5 weeks.

For some experiments (see those depicted in Fig. 4A), cultures were supplemented with 1 mM NAC, 20 nM sodium selenite, or 200 μM αtocopherol acetate (all Sigma-Aldrich). Concentrations were selected based on Esteban et al. [28].

For some experiments (see those depicted in Fig. 4D and E), cultures were supplemented with 5 mM L-NMMA (Abcam, Cambridge, UK).

Flow cytometry

In vitro-cultured cells were stained at indicated time-points. Cells were incubated with antibodies at proper dilutions for 20 min at room temperature. Staining for cytoplasmic CD3 was performed by first permeabilizing cells using Perm/Wash (Becton Dickinson).

The following antibodies were used: CD4 (SK3), CD7 (M-T701), CD45 (HI30), and TCR$\alpha\beta$ (IP26; eBioscience, San Diego, CA, USA); CD38 (HB7) and CD197 (CCR7, 150503; R&D Systems, Minneapolis, MN, USA), all FITC labeled; CD1a (HI149), CD7 (M-T701), CD8 (RPA-T8), and CD18 (integrin β2, MEM-48; ImmunoTools, Friesoythe, Germany); CD34 (8G12), CD45 (HI30), CD127 (M21), CD135 (4G8), CD162 (PSGL-1, KPL-1), and CD183 (CXCR3, 205410; R&D Systems), all PE labeled; CD3 (SK7), CD4 (SK3), CD8 (SK1), CD45 (2D1), and CD196 (CCR6, 53103; R&D Systems), all PerCP labeled; CDw199 (CCR9, 112509), Alexa Fluor 647 labeled, CD3 (UCHT1), CD5 (UCHT2), CD45 (2D1), and CD110 (REA250; Miltenyi Biotec); CD117 (A3C6E2; Miltenyi Biotec); CD184 (CXCR4, 12G5) and CD197 (CCR7, 150503; R&D Systems), all APC labeled; CD29 (integrin β1, TS2/16; BioLegend, San Diego, CA, USA), APC-Cy7 labeled; CD8 (RPA-T8), CD56 (B159), and CD45 (HI30), all Horizon V450 labeled. For apoptosis detection, a PE-Annexin V apoptosis detection kit was used. All antibodies, materials, and equipment were obtained from BD Biosciences (San Jose, CA, USA), unless stated otherwise. A minimum of 20,000 cells/sample was measured with the flow cytometer FACSCanto II (Becton Dickinson). Flow cytometric analysis was performed with BD FACSDiva software, version 6.1.2 (Becton Dickinson) or FlowJo software, version 10.0.6 (Treestar, Ashland, OR, USA). Living cells were gated on forward- and side-scatter patterns with subsequent doublet removal.

PCR

Genomic DNA was extracted from cells using a QIAamp DNA mini kit, according to the manufacturer's protocol (Qiagen, Venlo, the Netherlands). PCR was performed in 20 μl reaction volume containing 60 ng DNA from progenitors cultured in the presence of DLL4:Fc, T cells from peripheral blood, CD34$^+$ cells, or CD34$^+$ cells cultured with RetroNectin (Takara Shuzo) only. All PCR components were used, according to the manufacturer's instructions (iTaq; Bio-Rad Laboratories, Hercules, CA, USA), together with 500 nM of each primer (Eurogentec, Liege, Belgium). Dδ2-Jδ1 and Vδ1-Jδ1 primers are described in Dik et al. [12]. Annealing temperature of 65°C with 3 mM $MgCl_2$ and 35 cycles were used for both primer sets. EZ Load 100-bp molecular ruler (Bio-Rad Laboratories) was used as a marker. Dβ1.1-Jβ1.3 primers are described in Kato et al. [15]. Annealing temperature of 60°C with 1.5 mM $MgCl_2$ and 35 cycles were used. Gene ruler 100 bp plus DNA ladder (Fermentas; Thermo Scientific) was used as a marker.

Statistics

Data are presented as median. All statistical analyses were performed using the Prism program (GraphPad Software, San Diego, CA, USA). Differences between experimental conditions were evaluated for statistical significance with nonparametric Wilcoxon matched pairs test or with Mann-Whitney *U*-test. Significance was accepted at the level of $P < 0.05$: $*P = 0.01–0.05$; $**P = 0.01–0.001$; $***P < 0.001$.

RESULTS

Generation of CD7$^+$ proT cells from human CD34$^+$ cells in a feeder-free system

To evaluate the T cell-differentiation potential of CD34$^+$ cells in a system without feeder cells, optimal culture conditions were first determined. CB CD34$^+$ cells were cultured initially in the presence of αIgG-Fc and surface-immobilized DLL4:Fc in RPMI medium, as was established by Ikawa et al. [24]. Phenotype and fold expansion of T cell progenitors were defined after 3 and 4 weeks of culture. The optimal concentration of DLL4:Fc was found to be 5 μg/ml, resulting in the highest percentage and number of CD7-expressing cells (**Fig. 1A** and Supplemental Fig. 1). To improve further maturation of T cell progenitors, different coating protocols were tested. Coating with r-fibronectin fragment CH-296 (fibronectin, 5 μg/ml) [29] and DLL4:Fc resulted in higher CD7 expression (93%) compared with the combination of rabbit-anti-human IgG-Fc antibody with DLL4:Fc (61%; Fig. 1B). Additionally, fold expansion in the fibronectin DLL4:Fc condition was five times higher than in the IgG condition. Therefore, in further experiments, 5 μg/ml DLL4:Fc, in combination with 5 μg/ml fibronectin, was used.

The stromal cell lines TSt-4/DLL4 [15, 30] and OP-9/DL1 [31] are known to support T cell differentiation and are always cultured in RPMI and αMEM, respectively. We therefore decided to compare the influence of these media on human T cell differentiation in the feeder-free culture (Fig. 1C and D). Although similar percentages of CD7$^+$ and iCD3$^+$ cells were present in both conditions, CD5$^+$ proT2 cells were only abundantly present in αMEM (median 88%). Furthermore, CD7$^+$CD1a$^+$ preT cells were only present in αMEM medium (median 7%). In addition, fold expansion of T cell progenitors was 10 times increased in αMEM compared with RPMI after 3 weeks of culture (Fig. 1E). Fold expansions varied among different donors, but similar trends were observed in all experiments. Taken together, our data indicate that T cell maturation and proliferation were improved in αMEM medium compared with RPMI.

AA promotes proT1 to proT2 differentiation in the feeder-free system

As proT2 (CD7$^+$iCD3$^+$CD5$^+$) cells could develop in a feeder-free system in αMEM but were almost absent in RPMI medium, the composition of both media was compared to reveal compounds possibly accounting for this discrepancy. The addition of different types or higher percentages of FBS to RPMI medium did not result in the expression of CD5 or CD1a on progenitor cells (data not shown). Another difference in medium composition is the presence of L-AA in αMEM and its absence in RPMI. The natural-occurring L-AA is highly unstable to atmospheric oxygen, pH, light, and temperature [32]. Therefore, the more stable form of AA, ph-AA, in which a hydroxyl group is substituted by a phosphate group, was also tested [32]. To determine the influence of AA on T cell development, different concentrations of L-AA and ph-AA were added to RPMI medium and compared with αMEM (containing 284μM L-AA). CD7 expression was influenced slightly by the addition of AA, as almost all progenitors expressed CD7 to a similar extent in L-AA and ph-AA conditions after 3 weeks (**Fig. 2A** and Supplemental Fig. 2B). In contrast to CD7, the iCD3 expression on cells differed substantially between αMEM and RPMI conditions (85% vs. 41% iCD3$^+$ cells, respectively). Strikingly, addition of L-AA and ph-AA led to a concentration-dependent increase of CD5 expression on CD7$^+$ cells. The highest percentage of CD5 cells was present in the condition with 95μM ph-AA (75%). Cell proliferation substantially increased by the addition of AA to RPMI (Fig. 2B and Supple-

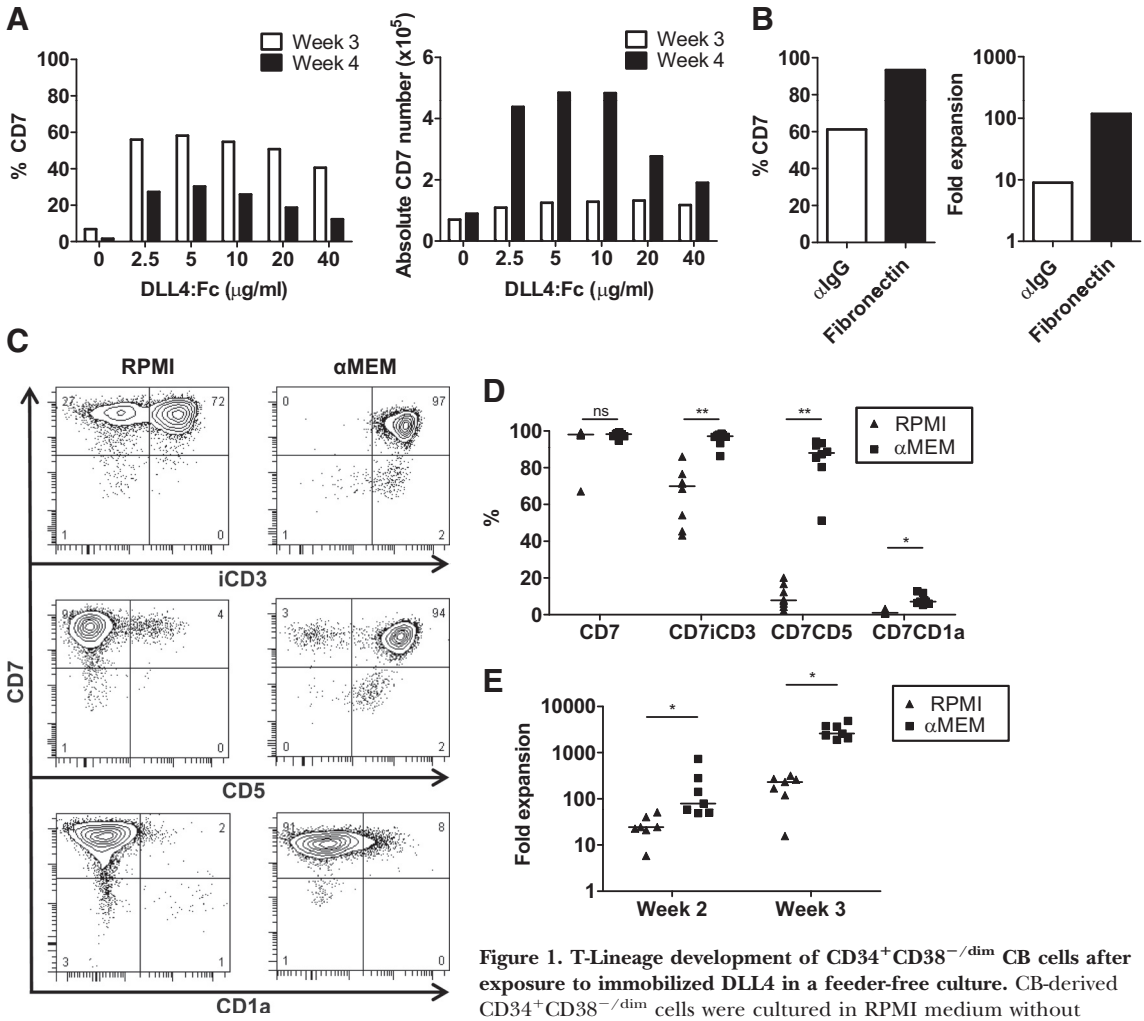

Figure 1. T-Lineage development of CD34$^+$CD38$^{-/dim}$ CB cells after exposure to immobilized DLL4 in a feeder-free culture. CB-derived CD34$^+$CD38$^{-/dim}$ cells were cultured in RPMI medium without feeder cells. Culture plates were coated with different concentrations of DLL4:Fc (μg/ml) and αIgG. (A) Percentage and absolute number of CD7$^+$ cells after 3 and 4 weeks of culture. (B) Percentage of CD7$^+$ cells and expansion after 3 weeks of culture with 5 μg/ml DLL4:Fc and αIgG or 5 μg/ml DLL4:Fc and fibronectin in RPMI medium. Representative results of two experiments are shown. Representative flow cytometry plots (C) and percentages of n = 8 experiments (D) show early T cell marker expression after 3 weeks of culture in the DLL4:Fc fibronectin culture in RPMI or αMEM. Wilcoxon matched pairs test; CD7 (P=ns), CD7iCD3 (**P=0.0078), CD7CD5 (**P=0.0078), and CD7CD1a (*P=0.014). (E) Fold expansion of total cells in the feeder-free culture in both media in the presence of DLL4:Fc fibronectin. Wilcoxon matched pairs test, Week 2 (*P=0.0156) and Week 3 (*P=0.0156; n=7).

mental Fig. 2B), without influencing cell viability (Supplemental Fig. 3). Interestingly, L-AA revealed a higher toxicity compared with ph-AA, as all cells in the presence of the highest concentration died within 1 week. Taken together, 95μM ph-AA was the most optimal-tested concentration for maturation and proliferation in the feeder-free cultures, leading to high expression of iCD3, CD5, and CD7.

Characterization of feeder-free-derived T cell progenitors

The chemokine receptor and integrin profile of generated T cell progenitors was determined to gain insight into the thymus-homing capacity of these cells. Expression of selected markers on feeder-free-generated T cell progenitors was compared with TSt-4/DLL4-derived T cell progenitors after 3 weeks of culture. We have shown previously that the TSt-4/DLL4-derived progenitors are able to home to the thymus and complete T cell development in the thymi of immune-deficient mice [11]. Feeder-free-generated T cell progenitors expressed the chemokine receptors described to be necessary for thymus-homing capability. Most of the tested chemokine receptors had higher expression on feeder-free-derived progenitors than on TSt-4/DLL4 coculture-derived progenitors, especially CCR4, as well as the adhesion molecules needed for thymus entry, integrin β1 and β2, and PSGL-1 (Fig. 2C). In contrast, CCR7 was lower expressed on feeder-free progenitors than on coculture-derived progenitors.

Next, TCR rearrangement was analyzed to assess the maturation status of generated T cell progenitors in feeder-free-derived progenitors, cultured with αMEM or RPMI, supple-

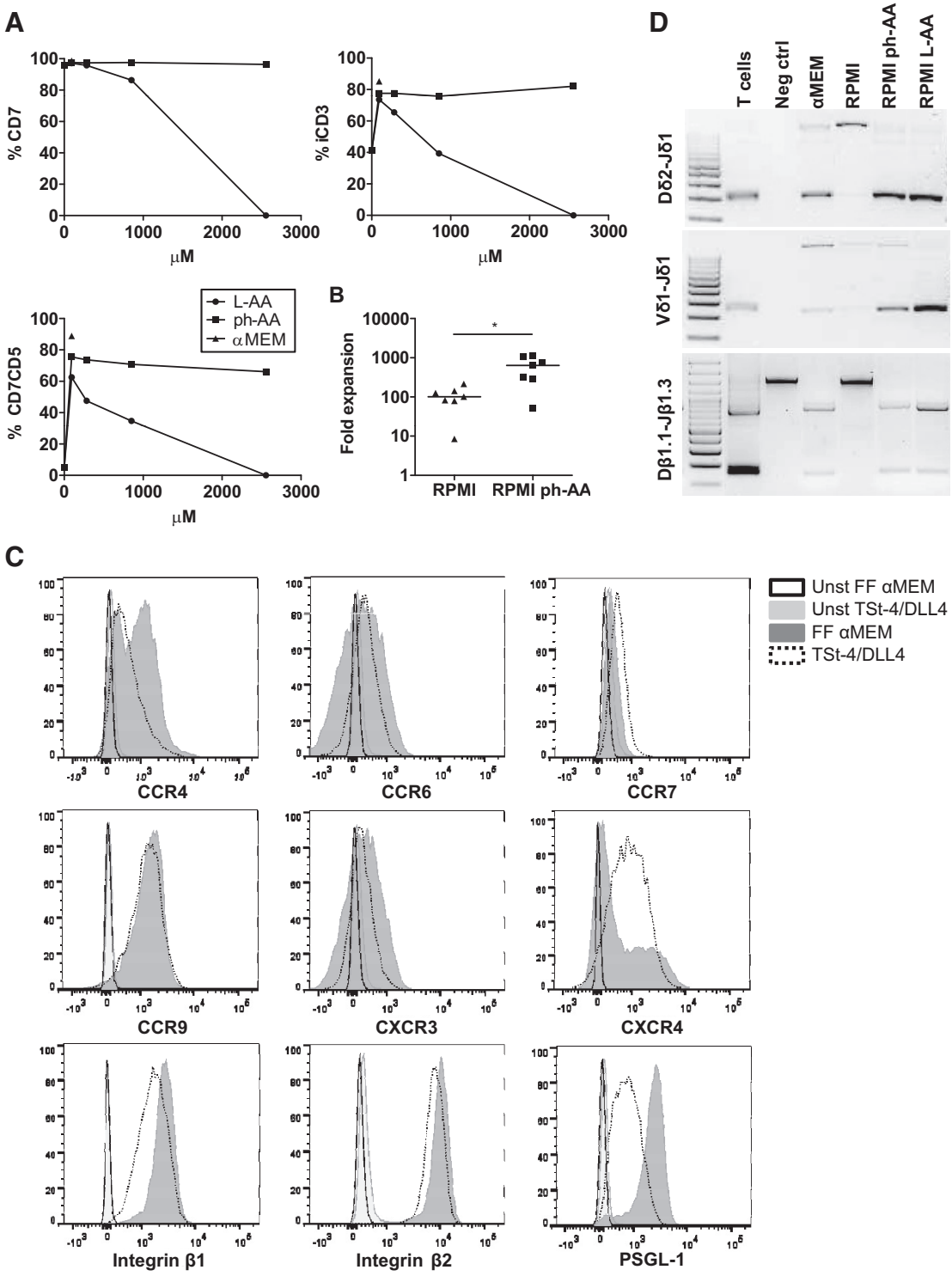

Figure 2. AA promotes preT cell development from CD34$^+$CD38$^{-/dim}$ cells in the feeder-free culture in the presence of DLL4:Fc and fibronectin. CD34$^+$CD38$^{-/dim}$ cells were cultured for 3 weeks in the presence of DLL4:Fc and fibronectin in RPMI medium, supplemented with ph-AA or L-AA in different concentrations and in αMEM. (A) Representative T-lineage marker expression (n=2) and fold expansion (B) of total cells (median of n=7, Wilcoxon matched pairs test; P<0.0156). (C) Chemokine receptor, integrin, and PSGL-1 profile of feeder cell-free-derived progenitor cells (FF) and TSt-4/DLL4 feeder-derived progenitors compared with unstained (Unst) corresponding cells after 3 weeks of culture, values are normalized to event count. Representative plots of feeder-free (n=2) and feeder culture (n=4) are shown. (D) D and B gene rearrangement of peripheral blood CD3$^+$ T cells (positive control), CB CD34$^+$CD38$^{-/dim}$ cells cultured with fibronectin [negative control (Neg ctrl)], or fibronectin and DLL4:Fc in αMEM, RPMI, RPMI ph-AA, or RPMI L-AA medium. Results for Dδ2-Jδ1,Vδ1-Jδ1, and Dβ1.1-Jβ1.3 are shown.

mented with L-AA or ph-AA. RPMI-cultured progenitors only showed a weak Dδ-Jδ rearrangement band and no subsequent Vδ-Jδ rearrangement (Fig. 2D), whereas αMEM-grown progenitors show Dδ-Jδ and Vδ-Jδ rearrangements. Upon addition of L-AA or ph-AA to RPMI, progenitors showed rearrangement of Dδ-Jδ and Vδ-Jδ, confirming the different maturation stage of T cell progenitors in the presence or absence of AA. In contrast to positive controls, two products were amplified from the DNA of progenitor cells. Sequence analysis had demonstrated previously that the larger products are partial rearrangements of the δ-locus [11]. Rearrangement of the TCRβ locus was present in cells cultured with αMEM and RPMI with AA; in contrast, in RPMI conditions, only a germline band was detected. Together, these data confirm that T cell development is advanced further in conditions with AA.

AA promotes T cell development in a GMP culture setting

Besides CB $CD34^+$, $mCD34^+$ cells are often used in clinical HSCT, as a result of the high numbers of cells that can be obtained, and as such, also available for adoptive (progenitor) T cell therapy. Therefore, $mCD34^+$ cells were cultured in the presence and absence of ph-AA. After 3 weeks of culture, the majority of $mCD34^+$ cells had up-regulated CD7 and iCD3 (**Fig. 3A**). Furthermore, a clear cell population coexpressing CD5 and CD1a was observed, whereas in culture without AA, cells expressed less CD7 (maximal 52%) and less iCD3 (maximal 22%) and were unable to up-regulate CD5 (Fig. 3A). Expansion of G-CSF-mobilized progenitors was only detected in the presence of ph-AA (median fourfold), albeit lower than of CB cells (Fig. 3B). Together, the positive influence of AA on T cell proliferation and maturation was clearly observed.

To examine T cell development in feeder-free GMP conditions, CB $CD34^+$ cells were cultured in serum-free medium (StemSpan), with or without 95 μM ph-AA. In general, less T cell progenitors ($CD7^+$) developed in StemSpan medium compared with the serum-containing media. The addition of ph-AA to the culture increased the percentage of CD7, iCD3, and CD5 expression (Fig. 3C). CD1a expression was hardly influenced. Furthermore, expansion of total cells was positively influenced by the addition of ph-AA (504 to 4648-fold; Fig. 3D). Thus, CB $CD34^+$ stem cells can be expanded in serum-free medium under the positive influence of AA.

Other antioxidants than AA lack the potential to drive preT cell development

To investigate whether the effect of AA on T cell maturation was a result of its antioxidant function, four other antioxidants were tested (**Fig. 4A**). CD7 and iCD3 expression was minimally influenced by the addition of the different antioxidants compared with RPMI. Interestingly, CD5 coexpression was also similar to standard RPMI and did not increase upon addition of different antioxidants other than ph-AA. Furthermore, antioxidants other than AA did not have a positive influence on expansion of the progenitor cells (Fig. 4B). Taken together, from the investigated antioxidants, only AA was able to drive preT cell development.

Cytokine receptor expression is not altered in the presence of AA

To examine whether the influence of AA on maturation and proliferation of T cell progenitors was a result of alterations of cytokine receptor expression, the receptors of cytokines provided to the culture were analyzed in the presence or absence of ph-AA. The expression of CD110 (TPOR), CD127 (IL-7Rα), and CD135 (Flt) was not differentially expressed upon addition of ph-AA to the culture (Fig. 4C). Although CD117 (SCFR) expression differed between the conditions, when evaluating ProT1 ($CD7^+CD5^-$) and ProT2 ($CD7^+CD5^+$) progenitors separately, no difference was observed (Fig. 4C). Therefore, the difference in expression of the whole population can be explained, as the RPMI culture contains more ProT1 cells that express more CD117.

Blockage of the citrulline/NO cycle results in less T cell progenitor development

As AA has been shown to increase NO production in endothelial cells [33] and as NO is involved in the differentiation of Th cell populations [34, 35], the role of the citrulline/NO

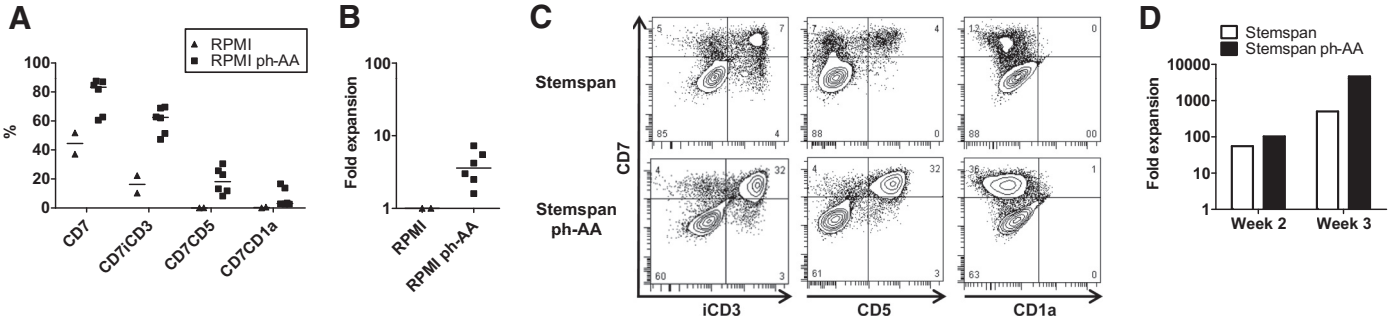

Figure 3. AA promotes T cell development in a GMP culture condition. (A) T Cell progenitor marker expression and fold expansion (B) of $mCD34^+CD38^{-/dim}$ cells after 3 weeks of DLL4:Fc fibronectin culture in RPMI or RPMI with 95μM ph-AA. Median of $n = 2$ and $n = 6$ with two and four different donors, respectively, for RPMI and RPMI ph-AA. (C) T Cell progenitor marker expression and expansion (D) of CB $CD34^+CD38^{-/dim}$ cells in StemSpan medium, with and without 95μM ph-AA. Representative results of $n = 2$ are shown.

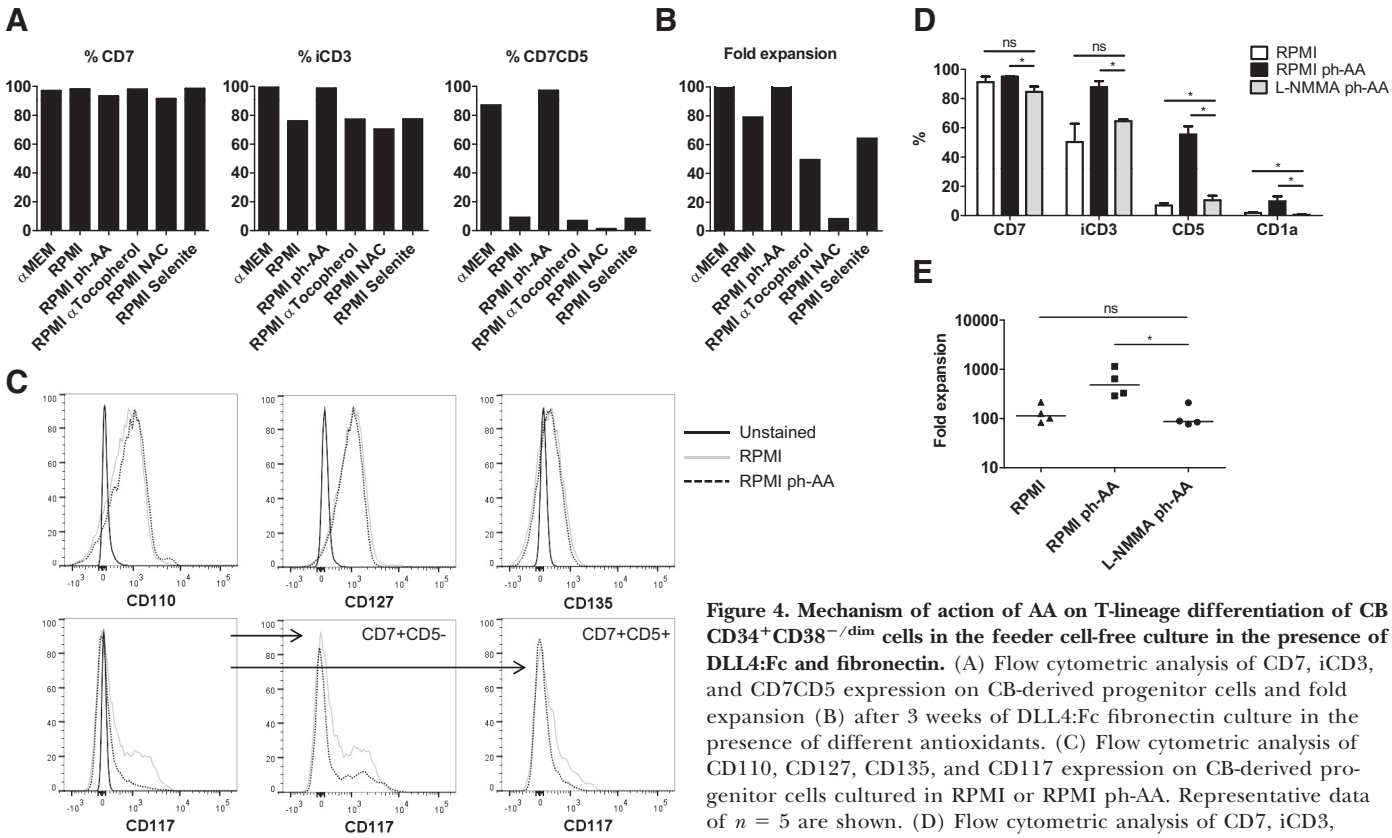

Figure 4. Mechanism of action of AA on T-lineage differentiation of CB CD34$^+$CD38$^{-/dim}$ cells in the feeder cell-free culture in the presence of DLL4:Fc and fibronectin. (A) Flow cytometric analysis of CD7, iCD3, and CD7CD5 expression on CB-derived progenitor cells and fold expansion (B) after 3 weeks of DLL4:Fc fibronectin culture in the presence of different antioxidants. (C) Flow cytometric analysis of CD110, CD127, CD135, and CD117 expression on CB-derived progenitor cells cultured in RPMI or RPMI ph-AA. Representative data of $n = 5$ are shown. (D) Flow cytometric analysis of CD7, iCD3, CD5, and CD1a expression on CB-derived progenitor cells in the presence of ph-AA and L-NMMA after 2 weeks of culture (Mann-Whitney *U*-test, n=4; *P<0.05) and corresponding fold expansion after 3 weeks of culture (E).

pathway [36] was investigated during early T cell development. L-NMMA, an L-arginine analog that competitively inhibits NOS, was added to the culture in the presence of ph-AA. Blocking of NO production resulted in less T cell development, indicated by significantly decreased percentages of CD7, iCD3, CD5, and CD1a expression compared with cells in the presence of ph-AA only (Fig. 4D). Furthermore, the positive effect on proliferation mediated by ph-AA was abolished in the presence of L-NMMA. These results hint for a role for the citrulline/NO pathway during T cell development driven by AA.

AA is a key factor in DP T cell development in feeder cell cocultures

We have reported previously that development of mCD34$^+$ cells is blocked at the pro/preT cell stage in coculture with TSt-4/DLL [11]. As AA results in the progression of T cell maturation in the feeder-free system, the effect of AA was also investigated in the TSt-4/DLL4 coculture system, which is normally performed in standard RPMI medium lacking AA. Our previously published results with mCD34$^+$ cells were confirmed with CB CD34$^+$ cells (**Fig. 5A**, upper). In the standard condition, CD7, iCD3, and CD5 were expressed, but CD4, CD8, TCR$\alpha\beta$, and CD3 were absent. Upon addition of L-AA, both CD5 and CD1a expression increased (Fig. 5A, lower). Interestingly, in contrast to the standard condition, iSP CD4 (CD3$^-$) cells were detected in the presence of L-AA (26%), together with a percentage of CD4 and CD8 DP cells (12%). TCR$\alpha\beta^+$CD3$^+$ cells were also present in the condition with L-AA (6%). Most of these cells were CD4$^+$CD8$^+$, as visualized after back-gating. Moreover, CD4 and CD8 SP cells were present in populations of 10% and 24%, respectively.

As DP cells could develop in the TSt-4/DLL4 feeder cell system, which never before showed support of DN to DP maturation, it was further studied whether DP/SP cells could develop in the OP-9/DL1 coculture system as a result of the presence of L-AA in αMEM medium. To this end, different formulations of αMEM were used. Liquid αMEM without AA was supplemented with fresh L-AA to the same concentration present in the standard powder αMEM. Without L-AA, CD7$^+$CD5$^+$ cells developed, although less efficiently than in αMEM with L-AA (Fig. 5B). Moreover, CD1a-expressing cells were almost absent in the condition without L-AA. Additionally, only with the addition of L-AA were iSP (69%) and CD4CD8 DP cells detected (27%). TCR$\alpha\beta^+$CD3$^+$ cells were also present (14%); most of these cells appear to be CD4CD8 DP (88%), and even minor populations of CD4 and CD8 SP cells were detected at 10% and 2%, respectively. Strikingly, in both feeder cell cultures, the full range of T cell development was only observed in the presence of AA, indicating that AA is the crucial component that promotes maturation of T cells in vitro.

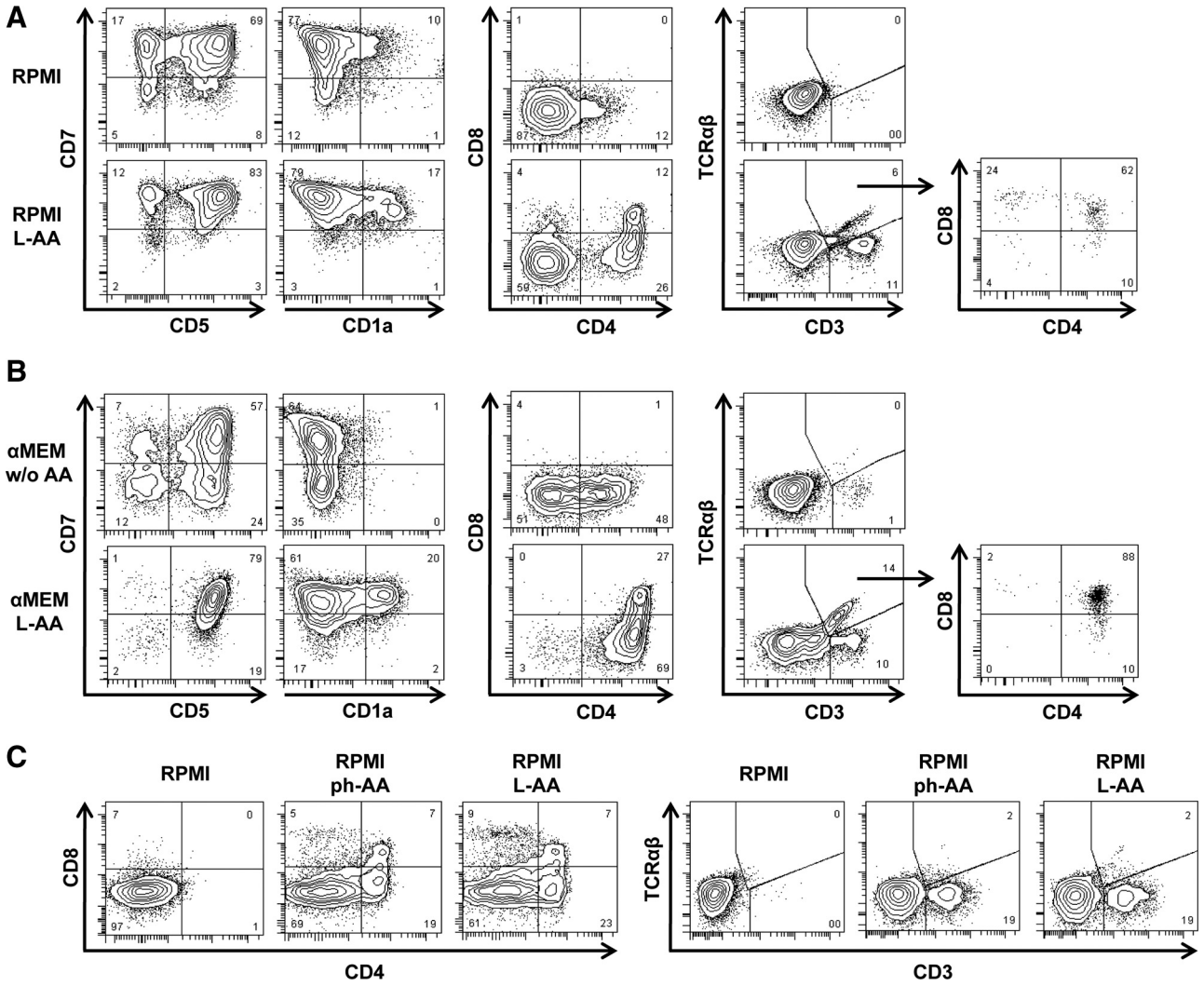

Figure 5. Effect of AA on late T cell development in feeder cell cocultures with TSt-4/DLL4 or OP-9/DL1 cells and feeder-free culture with CB $CD34^+CD38^{-/dim}$ cells. Flow cytometric analysis of T-lineage markers of progenitor cells after 7 weeks of culture on TSt-4/DLL4 (A) and OP-9/DL1 (B), with and without (w/o) AA. CD4, CD8, CD3, and TCR$\alpha\beta$ expression was analyzed on $CD7^+$ cells. The $TCR\alpha\beta^+CD3^+$ population is backgated on CD8 and CD4 expression. Representative plots of $n = 3$ are shown. (C) Flow cytometric analysis of CD4, CD8, CD3, and TCR$\alpha\beta$ expression on $CD7^+$ cells after 5 weeks of DLL4:Fc fibronectin culture of CB $CD34^+CD38^{-/dim}$ in different media: RPMI, RPMI 95μM ph-AA, and RPMI 285μM L-AA. Representative plots are shown (n=3 out of 9).

Feeder cells are not necessary to generate CD4CD8 DP T cells from HSCs

As the development of DP T cells seems to rely on AA and not on feeder cells, later stages of T cell development were investigated in the feeder-free culture. In standard RPMI conditions CD4CD8 DP, TCR$\alpha\beta$, or CD3-expressing cells could never be detected. In contrast, cells cultured in RPMI plus AA and αMEM did not arrest as preT cells expressing CD7, iCD3, and CD5 but were able to up-regulate CD4 and CD8 (7% for RPMI plus ph-AA or L-AA; Fig. 5C). Furthermore, a small population of $TCR\alpha\beta^+CD3^+$ cells was detected (2%). Together, these observations further indicate that feeder cells and feeder cell conditioned media are not necessary for generation of DP T cells from human stem cells.

DISCUSSION

This study shows a pivotal role for AA during in vitro human T cell development, as it promotes proT1 to proT2 cell transition, as well as progression from DN to DP T cells in both feeder- and feeder-free cultures. Furthermore, we show that feeder cells or feeder cell conditioned media are not required for in vitro generation of DP T cells. Finally, AA has a beneficial role in the differentiation of mobilized and CB HSCs in serum-free medium. These findings might be of great importance for the development of an efficient clinical method for in vitro generation of T cell progenitors.

The effect of AA on early T cell development was studied in the feeder-free DLL4:Fc culture. $CD34^+$ cells cultured in

RPMI showed a block in the very early $CD7^+$ proT1 cell stage. To our knowledge, only Reimann et al. [13] showed up to 10% CD5 proT2-expressing cells on immobilized DLL4 [23, 29, 37], and it is unknown if the medium used contained AA. Culture medium has a major effect on T cell differentiation. Six et al. [38] showed that fresh, reconstituted αMEM medium from powder resulted in more DP T cells in the OP-9/DL1 coculture system compared with ready-to-use αMEM. This observed effect could be a result of AA, which decays rapidly [39]. Fresh medium contains more AA that can have a beneficial effect on T cell development. Here, we confirm the influence of medium on T cell development, as the proT2/preT cell (CD7CD5CD1a) development occurred only in the presence of αMEM. By adding AA to RPMI medium, we show that AA is responsible for this effect. ph-AA results in more T cell development and proliferation than L-AA. ph-AA is taken up by the cells after phosphatases remove the phosphate group, after which, it enters the cells as L-AA. The removal of the phosphate group is the rate-limiting step in this process and therefore, ensures that ph-AA is available for the cells [32]. Therefore, the influence of AA on T-lineage development can be maintained longer. Our results thus show that addition of AA to the feeder-free culture results in more proliferation and improved T-lineage development. We are able to culture a large population of $CD7^+CD5^+CD1^+$ proT2/preT cells in the absence of feeder cells, whereas others observed mainly up-regulation of CD7.

Furthermore, we show that it is possible to generate T cell progenitors from $mCD34^+$ cells in the feeder-free system. The $mCD34^+$ population is a highly clinical, relevant population; cell numbers obtained via G-CSF mobilization and subsequent leukapheresis are much higher compared with CB $CD34^+$ cells (average $\sim 35\times10^6$ and $\sim 0.2\times10^6$ after $CD34^+CD38^{dim/neg}$ cell sorting, respectively). However, as CB cells can expand >1000-fold, both $CD34^+$ populations (patient-dependent) can be a suitable source for adoptive T-lineage therapy. However, preferred use of each population in the clinic remains to be proven, as the homing capacity, the maturation potential, and the number of cell divisions in each population are likely to be different and have not been determined in the current study.

The expression patterns of molecules important for thymus homing on feeder-free-generated T cell progenitors largely resemble the phenotype of TSt-4/DLL4-derived T cell progenitors. Feeder-free-generated T cell progenitors strongly expressed CCR4, CCR9, CXCR3, CXCR4, integrin $\beta1/\beta2$, and PSGL-1, whereas CCR6 and CCR7 were up-regulated slightly. As the feeder culture-derived cell population is able to migrate to the thymus and gives rise to fully mature T cells [11], it seems more than likely that also feeder-free-generated T cell progenitors possess this capacity. Chemokine receptors, integrins, and PSGL-1 are involved in migration and entry to and within the thymus and can be involved further in maturation of T cell progenitors [40]. CXCR4 has a role in cell egress from the bone marrow, the earliest step of migration to the thymus, as anti-CXCR4 antibodies reduce stem cell migration (reviewed in ref. [41]). The key molecules involved in murine thymic settling are CCR7, CCR9, and PSGL-1, although the precise mechanism is not yet unraveled. Thymus-seeding cells appear to require expression of CCR7, CCR9, or both for T-lineage development [40]. Therefore, it remains unknown whether the low CCR7 expression on our progenitor cells could be compensated by CCR9 or even by high PSGL-1 expression or other integrins. Additionally, in vivo experiments in immune-deficient mice need to confirm the potency of the feeder cell-free-derived progenitor cells to home to the thymus and complete their maturation.

Taken together, we are capable of generating proT2/preT cell precursors, confirmed with surface marker expression, chemokine receptor and integrin profile, and TCR rearrangement pattern, in the presence of AA and importantly, without the use of any feeder cells. We argued earlier that these cells are the ideal population to inject into patients, because of their retained thymus-homing capacity and further education in the thymus to prevent the emergence of autoreactive T cells [11].

Because of the effectiveness of AA in the early T-lineage stages, we also decided to investigate the role of AA in the later stages of T cell development. TSt-4/DLL and OP-9/DL1 feeder cells can support T cell development to a different extent. We argue, based on our results, that this is caused by different media used in these cultures and not because of unique properties of these feeder cells. Usually, cell lines and cultures are established in a particular medium without paying attention to its content. Indeed, the addition of AA to the TSt-4/DLL4 culture resulted in the generation of DP T cells, whereas the removal of AA from the OP-9/DL1 culture abrogated the capacity to produce DP and SP cells in these cultures. The effect of AA could be cell intrinsic on the feeder cells. However, we demonstrated that in the presence of AA, DP T cells could develop from both CB and $mCD34^+$ cells without any feeder cells. We thus show that feeder cells are not needed for differentiation but that simple cues, such as Notch signaling, cytokines, AA, and perhaps other fresh media components, are sufficient to drive in vitro generation of DP T cells. However, more signals seem to be necessary for proper TCR$\alpha\beta$ up-regulation and the development of SP T cells in the feeder-free culture, for example, the presence of MHC molecules. MHC-tetramers, anti-CD28, anti-CD3, and OP-9/DL1 conditioned medium have been required for the generation of antigen-specific cytotoxic $CD8^+$ cells in the feeder-free system [42]. This suggests that our current system could possibly be used for the generation of antigen-specific T cells. Altogether, these data suggest that the unique three-dimensional thymus structure is not necessary for complete T cell differentiation. However, the thymus microenvironments facilitate positive and negative selection of generated thymocytes and ensure a broad T cell repertoire [43].

Recently, Manning and colleagues [44] demonstrated a role for AA in murine DP T-lineage development. These data correlate well with our study using human cells, where we show an important role for AA, not only in DP T-lineage development but also in early T-lineage development. Manning et al. [44] show that the effect of AA is likely through epigenetic modulation of gene expression, possibly via the enhancement of enzyme activity of methyl marks on regulatory regions of

DNA. Recently, others have shown that besides transcriptional regulation, dynamic epigenetic regulations control T cell development in murine progenitor cells [45, 46]. Our findings indicate that it is indeed not the general antioxidant function of AA that is likely to drive T cell development. However, based on our L-NMMA NOS-blocking experiments, it seems likely that the citrulline/NO cycle plays a role during T cell development. To confirm this indication, extensive research has to be performed. As AA has many mechanisms of action, it would be likely that not one mechanism solely can account for the enormous influence of AA on T cell development. Currently, we are performing extensive molecular studies to elucidate further the mechanism by which AA induces T cell development; thus far, a definite conclusion cannot be drawn.

In conclusion, we unraveled an important role for AA during several steps of T cell development. CB- and mCD34$^+$ cells have the capacity to become preT cell progenitors in a well-defined, feeder-free culture in the presence of Notch ligands, defined cytokines, and AA. In this system, AA induces the transition from proT1 to proT2/preT cells and greatly enhances cell expansion. Moreover, DP cells develop as well. Furthermore, we show that only in the presence of AA, DP and even SP cells develop in coculture with OP-9/DL1 and TSt-4/DLL4. In the absence of AA in these feeder cultures, T cell progenitors arrest as CD4CD8 negative. Together, these findings indicate that feeder cells or coculture-derived conditioned media are not crucial to direct DP T cell development but that AA is a driving force in this process.

AUTHORSHIP

M.J.A.J.H. and M.W. provided the concept, design, collection, and assembly of data, data analysis and interpretation, and manuscript design and writing. N.K., J.J.B., B.L.M.G.S-G., and M.C.S. contributed the collection and assembly of data. G.M.J.B. provided financial support, data interpretation, and manuscript design. W.T.V.G. contributed the concept and design, financial support, data interpretation, and manuscript design and writing.

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DISCLOSURES

The authors declare no conflict of interest.

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