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A human thymic epithelial cell culture system for the promotion of lymphopoiesis from hematopoietic stem cells

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Abstract

Objective—A human thymic epithelial cell (TEC) line expressing human leukocyte antigen (HLA)-ABC and HLA-DR was engineered to overexpress murine Delta-like 1 (TEC-Dl1) for the purpose of establishing a human culture system that supports T lymphopoiesis from hematopoietic progenitor cells (HPCs).

Materials and Methods—Cord blood (CB) or bone marrow (BM) HPCs were co-cultured with either the parental TEC line expressing low levels of the Notch ligands, Delta-like 1 and Delta-like 4 or with TEC-Dl1 to determine if these cell lines support human lymphopoiesis.

Results—In co-cultures with CB or BM HPCs, TEC-Dl1 cells promote *de novo* generation of $CD7^{pos}CD1a^{pos}$ T-lineage committed cells. Most $CD7^{pos}CD1a^{hi}$ cells are $CD4^{pos}CD8^{pos}$ double positive (DP). We found that TEC-Dl1 cells are insufficient to generate mature $CD3^{hi}$ $CD4^{pos}$ or $CD3^{hi}$ $CD8^{pos}$ single positive (SP) T cells from the $CD4^{pos}CD8^{pos}$ DP T cells; however, we detected $CD3^{lo}$ cells within the DP and SP CD4 and CD8 populations. The $CD3^{lo}$ SP cells expressed lower levels of IL-2Rα and IL-7Rα compared to $CD3^{lo}$ DP cells. In contrast to the TEC-Dl1 line, the parental TEC-84 line expressing low levels of human Notch ligands permits HPC differentiation to the B-cell lineage.

Conclusions—We report for the first time a human TEC line that supports lymphopoiesis from CB and BM HPC. The TEC cell lines described herein provide a novel human thymic stroma model to study the contribution of HLA molecules and Notch ligands to T cell commitment and maturation and could be utilized to promote lymphopoiesis for immune cell therapy.

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Conflict of Interest Disclosure
The authors have no conflicting financial interests.

Keywords

lymphopoiesis; hematopoietic stem cell; stromal cells; umbilical cord blood

Introduction

The thymus is essential for the development of T cells from bone marrow-derived hematopoietic stem cells (HSCs) in animals and humans [1–3]. Genetic and functional studies show that thymic epithelial cells (TECs) play a critical role in T cell development [4–7]. Phenotype analyses of *ex vivo* human thymocytes have revealed distinct stages of T cell maturation. The stages of T cell development in the thymus have been defined as HSC ($CD34^{pos}CD7^{neg}CD1a^{neg}$), preT/natural killer ($CD34^{pos}CD7^{pos}CD1a^{neg}$), preT ($CD34^{pos/lo}CD7^{pos}CD1a^{pos}$), immature single positive ($CD1a^{pos}CD7^{pos}CD4^{pos}$), early double positive ($CD3^{neg}CD4^{pos}CD8^{pos}$), double positive (DP) ($CD3^{pos}CD4^{pos}CD8^{pos}$), and single positive (SP) ($CD3^{pos}CD4^{pos}CD8^{neg}$ or $CD3^{pos}CD8^{pos}CD4^{neg}$) [8].

Notch signaling is essential for T cell lineage commitment and differentiation [9, 10]; however, it is unclear which of the Notch ligands expressed by TEC trigger the physiological signal for T cell lineage commitment and/or maturation. Delta-like 4 (Dl4) and Delta-like 1 (Dl1) are both known to bind the receptor Notch-1 on HSC [11]. Murine BM stromal cell lines such as S17-DL1 and OP9-DL1 that overexpress the Notch ligand Dl1 and OP9-DL4 that overexpresses the Notch ligand Dl4 support T cell development from human cord blood (CB) HSCs [12–14] and bone marrow (BM) HSCs [15] as well as murine HSCs [16, 17].

Although BM stromal cells expressing Notch ligand support T lymphopoiesis *in vitro*, it is TECs that support T cell development *in vivo*. In this study, we describe human TEC lines that support human lymphopoiesis. Our data show that CB or BM hematopoietic progenitor cells (HPCs) co-cultured with a TEC line modified to overexpress the murine Dl1 (TEC-Dl1) give rise to T-lineage cells including $CD1a^{pos}$ cells that express both CD4 and CD8. In contrast, the parental TEC-84 line that expresses low levels of human DL1 and DL4 permits HPC commitment to the B cell lineage. We propose that the human TEC lines described herein provide a new model that can be used to define the contributions of HLA molecules to T cell development and to delineate the role of Notch ligands in human T lymphopoiesis.

Materials and Methods

Human blood and tissue sample collection

Collection of human TECs, CB, and adult BM was approved by Loyola University Chicago's Institutional Review Board and was done in accordance with the principles of the Helsinki Declaration.

Generation of the TEC-84 and TEC-Dl1 cell lines

TEC primary cultures were initiated by an explant technique as previously described [18]. TECs were immortalized by infection of the primary cultures with amphotropic retrovirus from a cell line, PA317 LXSN-16E6E7 [19] (American Type Culture Collection, Manassas, VA), containing the HPV E6E7 early genes and stable TEC lines were selected with G418 (800μg/ml). After two weeks in culture, the infected TECs were removed from selective media and expanded in TE media (3:1 DMEM:F12 medium with 5% FCS, 5.5μg/ml bovine insulin, 0.4μg/ml hydrocortisone, 9 ng/ml cholera toxin, 0.3% adenine hydrochloride, 1mM sodium pyruvate, 10ng/ml epidermal growth factor, 2.5μg/ml amphotericin B, and 55ng/ml

gentamicin sulfate). The selected TECs (TEC-84) were subjected to four rounds of electronic sorting and expansion for CD104 (integrin β4), CD29 (integrin β1), and CD49f (VLA6). The MigR1-GFP retroviral vector was engineered to express the murine Delta-like 1 gene 5' upstream of the internal-ribosomal entry site thereby allowing bicistronic expression of Dl1 and green fluorescence protein (GFP) [20]. Retrovirus containing Migr1-Dl1-GFP was generated using amphotropic Phoenix cells and supernatants were used to spinoculate TEC. Cells expressing high levels of Dl1 were sorted for GFP expression on a FACSARIA (Becton Dickinson (BD) Franklin Lakes, NJ, USA). Quantitative RT PCR was used to quantify the number of mouse and human Delta-like1 (DL1) and human Delta-like 4 (DL4) transcripts. RNA was extracted from TEC-84 or TEC-Dl1 cells using the RNeasy kit (Qiagen, Valencia, CA, USA). Primer sequences were as follows: Human DL1 (NM_005618): (5') ACACCATAAGCCCTGCAAGAA, (3') TCACAGATTTTGCCGTAGAAGC; Human DL4 (NM_019074): (5') AGTGGTCATTGCGCTTCTTG, (3') ACCTTCTCGCTCATCATCGAA; murine Dl1(NM_007865): (5') CTTCTTTCGCGTATGCCTCAA; (3') AGGCGGCTGATGAGTCTTTCT.

Flow cytometry

Cells were stained with fluorochrome labeled antibodies purchased from eBioscience, Biolegend, or BD Pharmingen (San Diego, CA, USA) or from Invitrogen (Carlsbad, CA, USA). Intracytoplasmic antibody staining was performed by fixing and permeabilizing cells with Cytofix/Cytoperm (BD Bioscience) prior to the addition of antibody. The percentage of positive cells for each marker was determined using a BD Canto or Canto II flow cytometer and FlowJo software (Tree Star, Ashland, OR, USA). Only cells within the lymphocyte gate were evaluated. Quadrants for positive and negative populations were based on cells stained with isotype control antibodies and fluorescence minus one (FMO) controls [21].

Co-culture of HPCs with TEC-84 or TEC-Dl1

$CD34^{pos}$ cells were enriched from CB or BM using the EasySep CD34 positive selection kit (StemCell Technologies, Vancouver, BC, Canada). For phenotyping experiments, $CD34^{pos}$ cells were stained with antibodies to lineage (Lin) specific markers: CD3, CD19, CD56, CD14, and CD15 (FITC); CD34 (PE); CD38 (PECy5 or APC); and CD45RA (PECy7 or APC). For co-culture experiments, $Lin^{neg}CD34^{pos}CD38^{neg}CD45RA^{pos}$ HPCs were sorted on a FACSARIA flow cytometer (purity >99%) and 3000 HPCs/well were co-cultured with confluent TEC-Dl1 in α-MEM media containing 15% FCS, 5% human serum, and 5ng/ml each of human IL-7, SCF, and Flt-3L (Peprotech, Rocky Hill, NJ, USA). For B lymphopoiesis studies, sub-confluent TEC-84 were co-cultured with 2000 HPCs/well in media containing 10% FCS, and 10ng/ml each of human IL-7, SCF, and Flt-3L, 6.6ng/ml IL-3, 20ng/ml HGF (Peprotech), and 5% CB plasma. The total number of live cells harvested from TEC-Dl1 or TEC-84 co-cultures was counted on a hemocytometer.

Ex vivo human thymocytes

Cryogenically preserved human thymocytes were stained with the same antibodies used to analyze T lymphoid progenitors from co-cultures.

Results

Expression of HLA and adhesion molecules on TEC-Dl1

To establish a TEC line capable of supporting T lymphopoiesis from human HPCs, we developed a primary TEC culture from pediatric thymus tissue as described in "Materials and Methods". By quantitative RT-PCR, the parental cell line, TEC-84 was found to express

low levels of the human Notch ligands, DL1 (<1000 transcript copies/μg RNA) and DL4 (1300 transcript copies/μg RNA) (data not shown). Since mouse and human DL1 are highly homologous and since OP9 cells overexpressing mouse or human DL1 support T lymphopoiesis [16, 22], we overexpressed the murine Dl1 by infecting the TEC-84 cells with retrovirus containing Migr1-Dl1-GFP to enhance the potential of the TEC-84 cell line to support T lymphopoiesis. The GFP^{pos} Dl1 expressing cells, TEC-Dl1, showed homogenous expression of GFP (Fig. 1A). By quantitative RT-PCR, we found that TEC-Dl1 express 1–1.3 $\times 10^6$ Dl1 transcripts/μg of total RNA which is comparable to the expression of Dl1 in OP9-DL1 cells (data not shown). The TEC-Dl1 cells uniformly express HLA-ABC and nearly half of the cells also express HLA-DR (Fig. 1B). The TEC-Dl1 cells also express the epithelial cell markers, CD29 (integrin β1), CD104 (integrin β4), and CD49f (VLA6) (Fig. 1C–F). While the parental TEC-84 cells uniformly express CD104 (data not shown), expression of CD104 is reduced in the TEC-Dl1 cells (Fig. 1D). We found that all TEC-Dl1 cells express CD106 (Fig. 1E) and one-half of them also express CD54 (Fig. 1F). These data verify that the phenotype of the TEC-Dl1 line is similar to the phenotype of *in vivo* human TECs.

TEC-Dl1 support of HPC differentiation to the T cell lineage

To determine if the TEC-Dl1 line could promote T cell lineage commitment and differentiation, $Lin^{neg}CD34^{pos}CD38^{neg}CD45RA^{pos}$ cells isolated from CB were co-cultured with TEC-Dl1 in media supplemented with human IL-7, SCF, and Flt-3L. We chose to isolate $CD45RA^{pos}$ cells because of their bias toward lymphopoiesis [13]. We first verified that HPCs were not already committed to the T cell lineage prior to culture. Enriched CB $CD34^{pos}$ cells were stained with antibodies to CD38, a marker of cells that have progressed beyond the HSC stage, the early T/NK cell marker CD7 and the T cell lineage commitment marker CD1a; flow cytometric analysis showed that the enriched $CD34^{pos}$ cells that were $CD38^{neg}$ displayed undetectable levels of CD1a or CD7 (Fig. 2A).

CB HPCs (3×10^3 cells) were cultured with TEC-Dl1 and after two weeks, the total number of cells was counted and these cells were analyzed by flow cytometry. On average, the cell number increased 60-fold (range= 1–3 $\times$ 10^5, n=7; data not shown) demonstrating that cell proliferation was occurring in these co-cultures. By the end of the culture period, a modest expansion of $CD34^{pos}CD7^{neg}CD1a^{neg}$ cells had occurred suggesting that some $CD34^{pos}$ cells may undergo proliferation without differentiation (Fig. 2B). These data are similar to a previous study showing an initial expansion of $CD34^{pos}$ cells cultured on OP9-DL1 [14] and are consistent with the role of Notch signaling in the maintenance and survival of hematopoietic progenitors [23].

The acquisition of CD1a expression and downregulation of CD34 expression by HSC has been used to assess T cell lineage commitment [22, 24]. CD1a is one of the earliest markers of T cell commitment with studies showing that $CD1a^{pos}$ cells no longer have natural killer or dendritic cell potential [25]. In the HPC-TEC-Dl1 co-cultures, between 10^4 and 5 $\times 10^4$ cells were $CD34^{neg}CD7^{pos}CD1a^{pos}$ demonstrating the *de novo* generation of approximately ten T-lineage committed cells from each HPC (Fig. 2B). Only cells that had downregulated CD34 expression ($CD34^{lo}$ or $CD34^{neg}$) expressed the T-lineage marker CD1a; the $CD1a^{pos}$ cells were also $CD7^{pos}$ and within this population, a distinct subset of $CD1a^{hi}$ cells was identifiable (Fig. 2C). Analysis of *ex vivo* human thymocytes showed the presence of similar cell subsets (Fig. 2D). No T-lineage cells were observed in our cultures when HPCs were cultured alone or in media supplemented with cytokines (data not shown), demonstrating that TEC-Dl1 cells were required to promote T lymphopoiesis.

We further examined the phenotype of CD1a expressing cells (as shown in Fig. 2C–D) from CB HPCs (Fig. 3A) or BM HPCs (Fig. 3B) co-cultures for the expression of CD4 and CD8.

Downregulation of CD1a expression correlates with maturation toward the SP stage, whereas CD1a expression is completely lost on mature SP cells and in recent thymic emigrants [26]. Similar to *ex vivo* human thymocytes (Fig. 3C), the majority of cells expressing CD1a in both CB and BM co-cultures were $CD4^{pos}CD8^{pos}$ DP. Some $CD4^{pos}CD8^{neg}$ immature single positive (ISP) cells that had not yet progressed to the DP stage were observed in the $CD1a^{hi}$ subset (Fig. 3A, lower right quadrant). We observed that BM cultures compared to CB cultures had higher percentages of DP cells within the $CD1a^{pos}$ and $CD1a^{neg}$ subsets. It is unclear whether this difference is related to the age of the donating individuals or to the source of the HPCs; further study is required to explore these possibilities. Similar to *ex vivo* thymocytes, the $CD1a^{neg}$ subset from HPC co-cultures contained few DP cells. In contrast to the $CD1a^{neg}$ subset of *ex vivo* human thymocytes which contained a distinct population of $CD4^{lo}CD8^{neg}$ and $CD8^{pos}CD4^{neg}$ SP cells, only a small percentage of cells from the HPC co-cultures were $CD4^{lo}CD8^{neg}$ or $CD8^{lo}CD4^{neg}$; the expression of CD8 on SP cells was also lower in the co-cultures compared to *ex vivo* thymocytes (Fig. 3A–C). Taken together, these data demonstrate that *de novo* expression of CD1a and generation of DP T cells in co-cultures of HPCs with TEC-Dl1 parallels human thymocyte development; however SP T cell development in the co-cultures may be diminished.

Maturation of the $CD4^{pos}CD8^{pos}$ DP cells in co-cultures of HPCs with TEC-Dl1

We hypothesized that the TEC-Dl1 should support T cell maturation of DP T cells to the SP stage because TEC-Dl1 express HLA Class I and Class II as well as the adhesion molecules CD106 (VCAM) and CD54 (ICAM) that are known to interact with developing thymocytes [27, 28] and function as co-stimulators of positive selection *in vitro* [29]. Positive selection results in the downregulation of either CD4 or CD8 co-receptors and increased expression of CD3 [30, 31]. To determine if T cells generated in the co-cultures of BM or CB HPCs with TEC-Dl1 were capable of maturation beyond the early DP stage, we analyzed these cells for expression of cell surface CD3, CD4, and CD8. In cultures of both CB and BM HPCs, a small percentage of $CD3^{lo}$ cells was detected (Fig. 4A–B). Co-cultures could not be continued beyond two to three weeks even when cells were transferred to fresh TEC-Dl1 cells due to a decrease in cell viability (data not shown). The majority of the $CD3^{lo}$ cells from TEC-Dl1 co-cultures were DP and a small fraction was either $CD4^{lo}$ or $CD8^{lo}$ SP cells. We found that >90% of the $CD3^{lo}$ DP cells express IL-7Rα(CD127) and the activation marker IL-2Rα (CD25). However, in comparison to the expression of these molecules on the $CD4^{pos}CD8^{pos}$ DP cells, the expression of CD127 and CD25 was downregulated on some $CD3^{lo}$ $CD8^{lo}$ and $CD3^{lo}$ $CD4^{lo}$ SP cells (Fig. 4A–B). We conclude that the majority of $CD3^{lo}$ cells are unable to progress through the DP stage to the SP stage since they retain expression of CD25, which is lost on DP T cells. Consistent with these data, TCRαβ or TCRγδcell surface expression was undetectable in the $CD3^{lo}$ population (data not shown).

TEC-84 cells that express low levels of DL1 and DL4 promote CB HPC differentiation to the B cell lineage

Several murine studies have demonstrated that thymic stroma cells can support B lymphopoiesis if Notch signaling is attenuated. $CD4^{neg}CD8^{neg}TCR^{neg}/CD3^{neg}$ thymocytes can differentiate into B cells when cultured with a murine thymic stromal cell line [32] and murine thymic stroma cell monolayer cultures that express low levels of the Notch ligands, Dl1 and Dl4 support the differentiation of hematopoietic progenitor cells to the B-cell rather than the T-cell lineage [20]. It has also been demonstrated that CB HSCs cultured in a mouse fetal thymic organ culture develop into B cells when cultured in the presence of a high concentration of a Notch signaling inhibitor [33]. Based on these data, we hypothesized that the TEC-84 cell line would permit HPC differentiation to the B cell lineage. CB HPCs (4×10^3) were co-cultured with TEC-84 and the cells were analyzed after one to two weeks

in culture. From a total of five independent experiments, we recovered on average, 92×10^4 live cells (range $50 \times 10^4 – 12 \times 10^5$) from the cultures (data not shown). By flow cytometry, we detected myeloid lineage cells and some common lymphoid progenitors (data not shown); however nearly half of these cells were $CD7^{neg}$ $CD79a^{pos}$ B-lineage cells (average of 39×10^4; range $26 \times 10^4 – 63 \times 10^4$). B-lineage cells were not generated in the absence of TEC-84 cells (data not shown). The majority of the B-lineage progenitors were preproB cells ($CD19^{neg}$cytoplasmic μ^{neg}) (range $13 \times 10^4 – 33 \times 10^4$ cells) or proB cells ($CD19^{pos}$cytoplasmic μ^{neg}) (range $5 \times 10^4 – 23 \times 10^4$). On average, only 10% of the B cell progenitors were preB cells ($CD19^{pos}$cytoplasmic μ^{pos}) (range $<1 \times 10^4 – 8 \times 10^4$) (Fig. 5A–B). These data demonstrate that TECs expressing a low density of Notch ligand permit the development of B cells from HPC.

Discussion

In these studies, we describe human TEC lines that support human lymphopoiesis. Although the TEC-Dl1 cells were homogenous for expression of Dl1, HLA-ABC, CD29, CD106, and CD49f, bimodal distribution of HLA-DR, CD104, and CD54 was observed. The appearance of the bimodal expression of HLA-DR, CD104 and CD54 was not due to heterogeneity of the TEC-Dl1 cell line because analysis of several TEC-Dl1 clones obtained by limiting dilution showed similar surface phenotype characteristics (data not shown). While the parental TEC-84 line was established based on homogenous expression of CD104, expression of CD104 was reduced in all subclones of TEC-Dl1 (data not shown). We have previously demonstrated that TEC express various isoforms of TGF-β *in vivo* and *in vitro* [18]. Expression of CD104 in epithelial cells is down modulated by TGF-β via epigenetic regulation [34]. We speculate that the interaction of murine Dl1 with human Notch on the TEC-Dl1 may lead to activation of TGF-β signaling resulting in a down modulation of CD104 expression.

In our co-culture system, TEC-Dl1 promoted commitment of CB or BM HPC to the T cell lineage as evidenced by the emergence of $CD34^{lo/neg}CD7^{pos}$ cells that are either $CD1a^{pos}$ or $CD1a^{hi}$. In agreement with a previous report, we found that the CB $CD34^{pos}CD45RA^{pos}$ cells that were $CD38^{neg}$ displayed undetectable levels of CD7 expression [13] indicating that the $CD7^{pos}CD1a^{pos}$ cells generated in co-cultures of HPCs with TEC-Dl1 were generated *de novo*. Importantly, the *de novo* CD1a cell subsets were phenotypically similar to those found in *ex vivo* human thymocytes, demonstrating that the TEC-Dl1 co-culture system mimics *in vivo* thymocyte development. Similar to our findings, a recent kinetic study showed that the emergence of CD1a expression correlates with the downregulation of CD34; however, CD7 expression in our TEC-Dl1 co-cultures (Fig. 2C) was less prominent than that seen in co-cultures of CB HSCs with OP9-DL1 [35]. CD7 expression may not be directly mediated by Notch signaling but rather by an unknown mediator(s) present in the murine OP9 cells because both the TEC-Dl1 and OP9-DL1 cell lines express similar levels of Notch (data not shown).

Currently, the exact role of Notch signaling in the maturation of DP cells to CD4 or CD8 SP cells remains controversial (reviewed in [36]). In mice, Notch expression is progressively downregulated at the $CD4^{pos}$ and $CD8^{pos}$ SP stage and is expressed at low levels in the peripheral T cells [37]. Although we observed $CD8^{lo}CD4^{neg}$ and $CD4^{lo}CD8^{neg}$ SP cells in co-cultures of HPC with TEC-Dl1, these cells have low expression of CD3, suggesting that Dl1 signaling is not sufficient and that additional developmental signals are required for upregulation of CD3 expression. It is possible that Notch signaling via DL4 is necessary for this developmental stage. Although the differential effect of DL1 and DL4 is not known in humans, mouse Dl4 is required for T cell development *in vivo*; while inactivation of *Dl1* does not inhibit T cell development, the absence of Dl4 signaling completely blocks T cell

development [38, 39]. To further investigate the roles of Dl1 and DL4 in T cell development, we will generate TEC lines with varying Dl1 and human DL4 expression and determine the ability of these cell lines to support HPC differentiation to the SP T cell stage.

Constitutive Notch signaling early in T cell development may lead to an irreversible block in maturation. It has been demonstrated that expression of *DELTEX1* (DTX1) and NRARP, genes that are normally downregulated during TCRαβ lineage differentiation [40], is induced in HPCs co-cultured with OP9-DL1 cells [17]. Although we did not assess the expression of these genes in lymphoid progenitors from our co-cultures, we did observe that >90% of the $CD3^{lo}$ DP T cells from our co-cultures express CD127/IL-7Rα. Notch signaling has been shown to induce constitutive expression of CD127/IL-7Rα on thymocyte precursors [41]. We attempted to address this by disaggregating the developing lymphocytes from the TEC-Dl1 stroma and transferring them to the parental TEC line that did not overexpress Dl1, however this resulted in reduced cell yield without further development (data not shown). It is possible that T cell progenitors may require a gradient of Notch ligand expression during development; we are currently in the process of generating multiple TEC lines varying in DL1 expression to test this hypothesis. We are also in the process of generating TEC lines expressing mouse and human DL4. Perhaps, signaling through DL4 is needed for maturation of DP cells to SP cells. The low level of DL4 expression in our TEC-Dl1 cells may not support DP T cell maturation toward the SP stage. Once the TEC-DL4 cell lines are developed, we will be able to determine the contribution of DL1 and DL4 to the development of human SP T cells.

While overexpression of Dl1 in TEC promotes T lineage commitment, our data demonstrating that HPCs co-cultured with TEC-84 that express low levels of DL1 and DL4 become B cell progenitors indicate that B cell development is permitted in the human thymic microenvironment in the absence of strong Notch signaling. However, maturation of B cell progenitors to surface IgM^{pos} B cells was not supported by the TEC-84 line, even when the culture period was extended to three weeks (data not shown). The lack of IgM^{pos} B cells may be due to the absence of a B cell maturation factor in the cultures. More likely, B cell maturation was inhibited by Notch signaling because even temporary activation of Notch signaling prevents the development of HSC beyond the proB stage [42] and leads to degradation of transcription factors and kinases essential for B cell development [43].

Taken together, these data demonstrate that TEC-Dl1 promote HPC differentiation to various T cell subsets with phenotypes similar to those observed with *ex vivo* thymocytes, whereas TEC-84 promote HPCs to develop into B-lineage cells. These human TEC lines represent a novel tool for studying human lymphopoiesis and have the potential to generate lymphocytes for *in vivo* therapy in patients with hematologic malignancies and immunodeficiency disorders.

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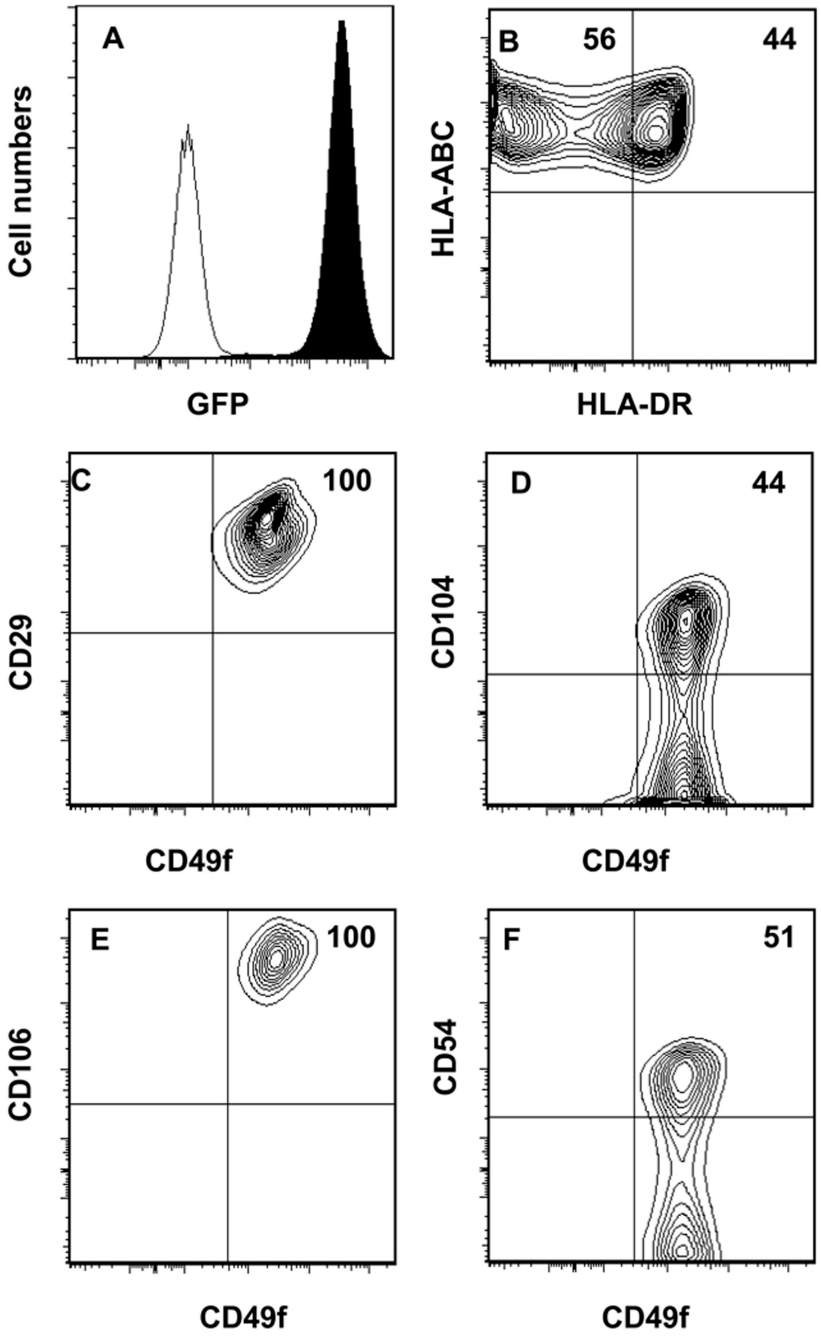

Figure 1. Cell surface marker characterization of the human TEC-Dl1 as determined by flow cytometric analysis

The established TEC-Dl1 cell line was analyzed for : (A) Expression of GFP as a measure for murine Dl1 expression; filled histogram is TEC-Dl1, unfilled histogram is control parental TEC-84; (B) human MHC class I (HLA-ABC) APC and MHC class II (HLA-DR) PE; (C) CD29 (integrin β1) FITC and CD49f (VLA6) APC; (D) CD104 (integrin β4) PE and CD49f APC; (E) CD106 (VCAM) PE and CD49f APC; and (F) CD54 (ICAM) PE and CD49f APC. All axes except the y axis of (A) display log10 fluorescence. Numbers shown in quadrants are percentages.

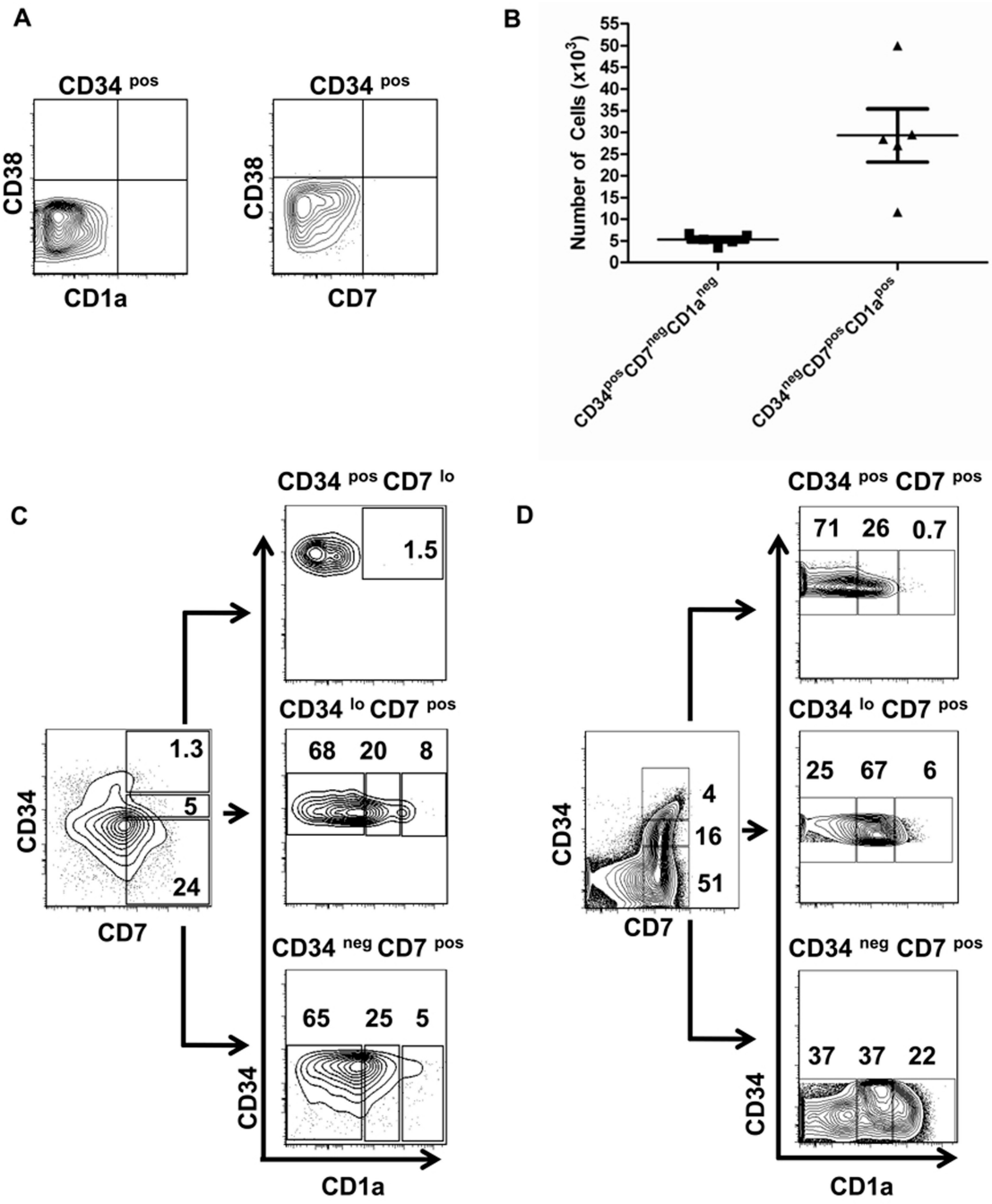

Figure 2. Expansion of CB HPCs and expression of T lineage surface markers on CB HPCs after co-culture with TEC-Dl1

(A) Lack of CD38 (APC), CD7 (biotin + SA-APC-Cy7) and CD1a (biotin + SA-APC-Cy7) expression on enriched $CD34^{pos}$ CB cells before culture; (B) CB HPCs (3000 cells) were co-cultured with TEC-Dl1 for two weeks; the total number of $CD34^{pos}CD7^{neg}CD1a^{neg}$ cells uncommitted to the T cell lineage (n=5) and the total number of $CD34^{neg}CD7^{pos}CD1a^{pos}$ cells committed to the T cell lineage (n=5) was determined. The bars indicate the means ± S.D.; C and D) CD1a expression within the $CD34^{pos}CD7^{lo}$, $CD34^{lo}CD7^{pos}$, and $CD34^{neg}CD7^{pos}$ subsets generated from CB HPC-TEC-Dl1 co-cultures (C) or cryopreserved *ex vivo* pediatric human thymocytes (D) as determined by flow cytometry. Numbers shown

are percentages. These data are representative of five CB HPC cultures and thymocytes from three human thymi. The scale for each axis is log10 fluorescence.

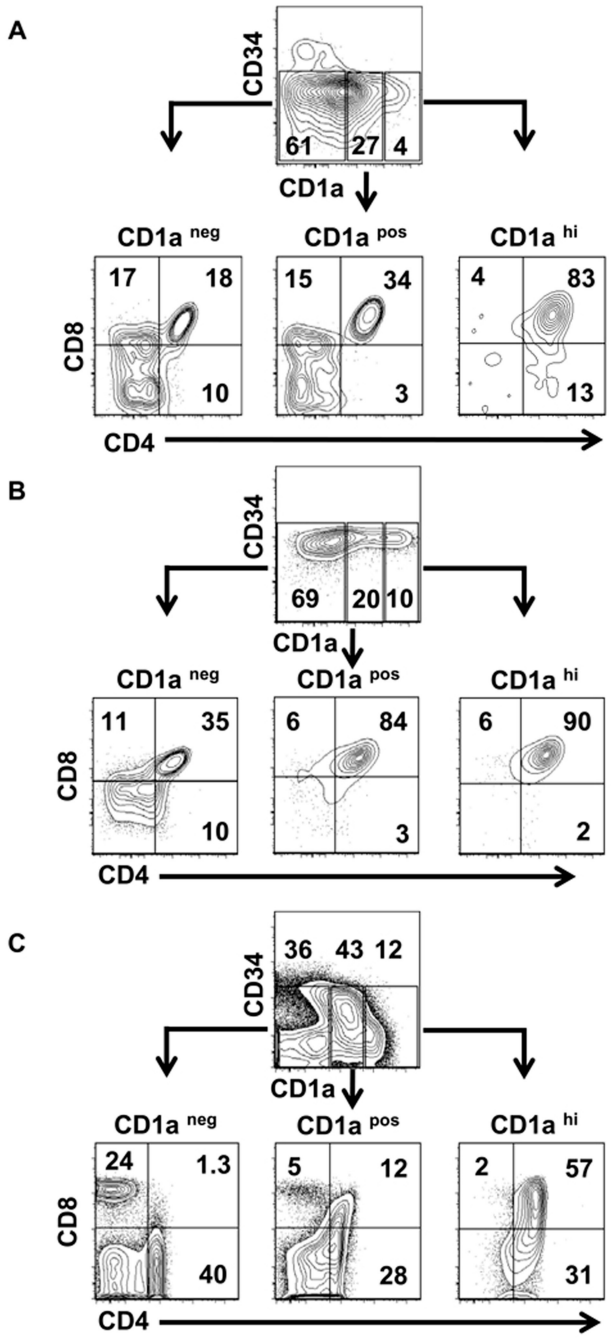

Figure 3. Flow cytometric analysis of CD4 and CD8 expression in three populations identified by the expression of CD1a

(A) Cells harvested from the CB HPC-TEC-Dl1 co-cultures after two weeks were analyzed for the expression of CD34 (PE), CD1a (APC), CD4 (AF750), and CD8 (PECy7). Expression of CD4 and CD8 was determined within the $CD34^{neg}$ $CD1a^{neg}$ (left), $CD1a^{pos}$ (middle), and $CD1a^{hi}$ (right) populations. Similar analyses were performed for cells generated from BM HPCs co-cultured with TEC-Dl1 cells (B) and cryopreserved *ex vivo* human thymocytes (C). Numbers shown are percentages within each region or quadrant. Data shown are representative of five CB HPC co-cultures, two BM HPC co-cultures, and three human pediatric thymi. Log10 fluorescence is shown on the axes.

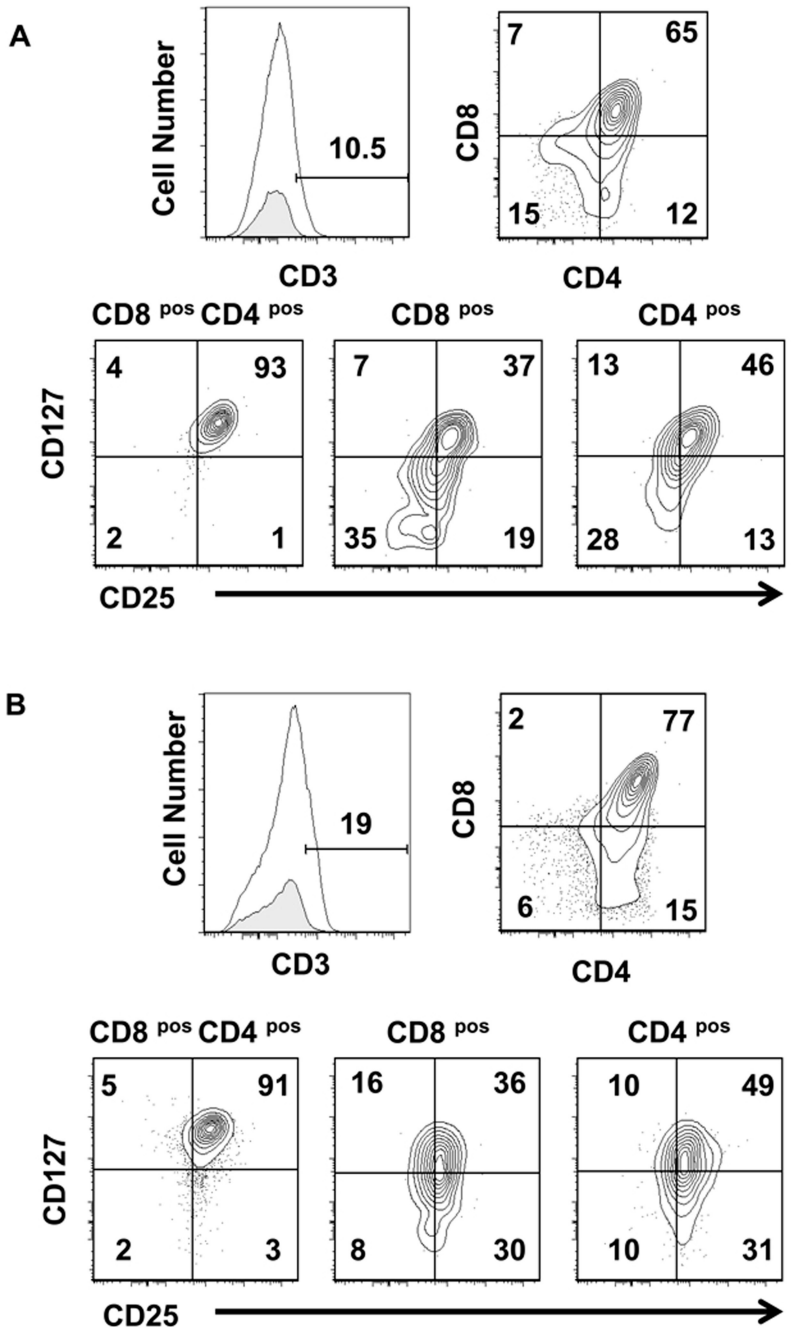

Figure 4. Expression of CD25 and CD127 within $CD3^{lo}$ $CD4^{pos}CD8^{pos}$ double positive and $CD4^{pos}$ or $CD8^{pos}$ single positive populations

(A) Cells were harvested from CB HPC-TEC-Dl1 co-cultures after two weeks and cell surface phenotype was analyzed by flow cytometry. The $CD3^{lo}$ cell population was determined by comparing cells stained with APC anti-CD3 (unshaded, top left panels) with an APC conjugated antibody that does not bind T cells (shaded, top left panels). The percentage of $CD3^{lo}$ cells is indicated. $CD3^{lo}$ cells were divided into subsets based on expression of CD4 (AF750) and CD8 (PECy7) (top right panels). These subsets were then analyzed for the expression of CD25 (PE) and CD127 (PECy5) (bottom panels). Numbers shown above markers or within quadrants are percentages. (B) Similar analyses were

performed for BM HPC-TEC-Dl1 co-cultures. Representative data of five independent CB HPC co-culture and two independent BM HPC co-culture experiments are shown. Axes show log10 fluorescence.

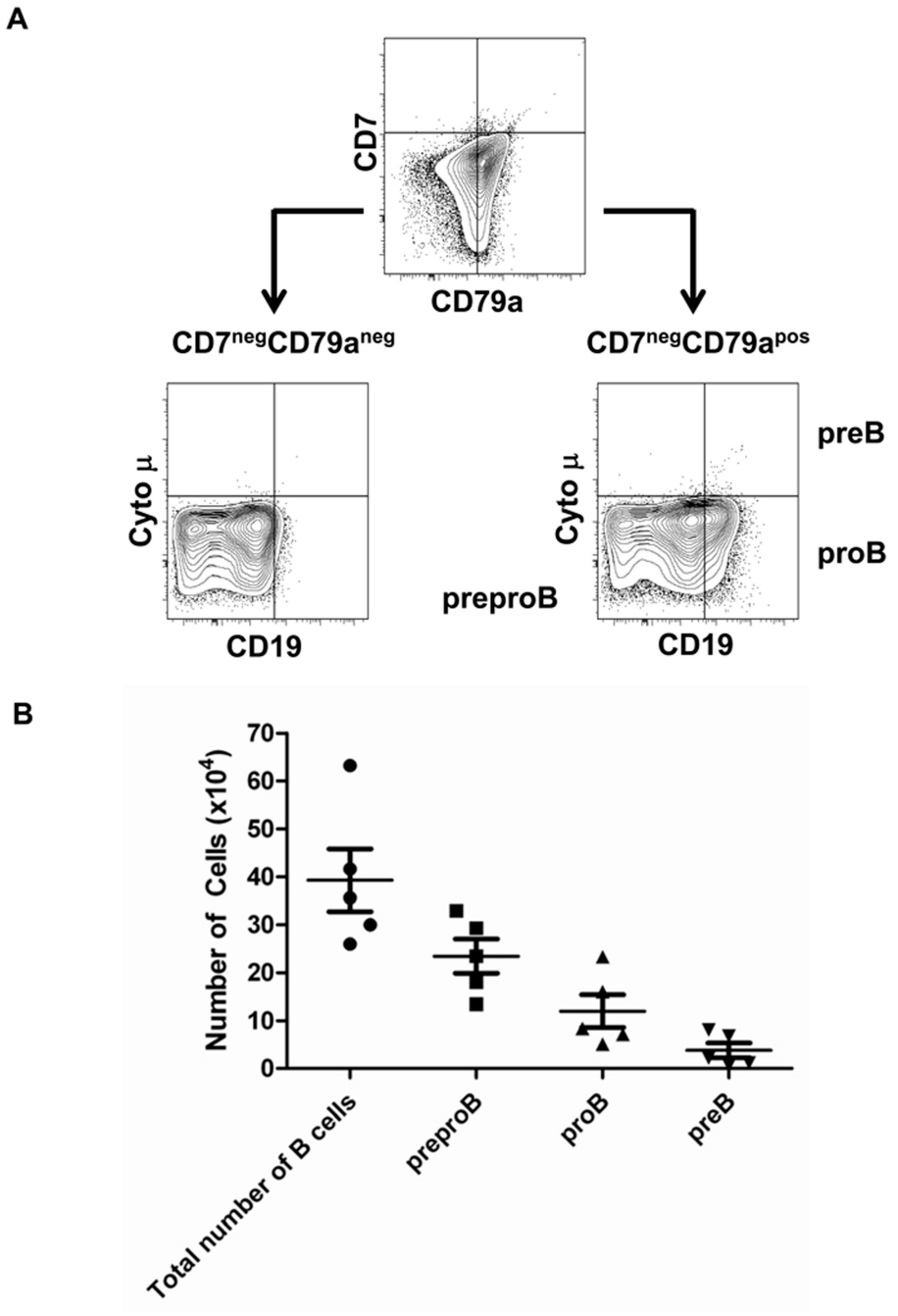

Figure 5. Characterization of B cells generated from CB HPCs co-cultured with TEC-84

CB HPCs (2 wells of 2×10^3) were cultured with TEC-84 for two weeks and analyzed by flow cytometry for the immature B cell subset markers CD79a (PE), CD7 (PECy5), CD19 (PECy7) and intracellular IgM (APC). (A) $CD79a^{neg}CD7^{neg}$ cells were not considered to be committed to the B-cell lineage and were negative for two other B cell markers, CD19 and cytoplasmic IgM (cyto μ) (bottom left). $CD7^{neg}CD79a^{pos}$ cells were considered committed to the B-cell lineage and were classified as preproB, proB, or preB cells based on the expression of CD19 and cyto μ (bottom right). The axes show log10 fluorescence. (B) The total number of B cells represents all cells that were positive for the B cell lineage marker, CD79a. The number of each B cell progenitor was calculated by multiplying the percentage

of cells with each B cell progenitor phenotype by the total number of CD79a^{pos} cells. The bars indicate the mean ±S.D. of five independent CB HPC cultures.