DL4-mediated Notch signaling is required for the development of fetal $\alpha\beta$ and $\gamma\delta$ T cells

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T-cell development depends upon interactions between thymocytes and thymic epithelial cells (TECs). The engagement of delta-like 4 (DL4) on TECs by Notch1 expressed by blood-borne BM-derived precursors is essential for T-cell commitment in the adult thymus. In contrast to the adult, the earliest T-cell progenitors in the embryo originate in the fetal liver and migrate to the nonvascularized fetal thymus via chemokine signals. Within the fetal thymus, some T-cell precursors undergo programmed TCR$\gamma$ and TCR$\delta$ rearrangement and selection, giving rise to unique $\gamma\delta$ T cells. Despite these fundamental differences between fetal and adult T-cell lymphopoiesis, we show here that DL4-mediated Notch signaling is essential for the development of both $\alpha\beta$ and $\gamma\delta$ T-cell lineages in the embryo. Deletion of the DL4 gene in fetal TECs results in an early block in $\alpha\beta$ T-cell development and a dramatic reduction of all $\gamma\delta$ T-cell subsets in the fetal thymus. In contrast to the adult, no dramatic deviation of T-cell precursors to alternative fates was observed in the fetal thymus in the absence of Notch signaling. Taken together, our data reveal a common requirement for DL4-mediated Notch signaling in fetal and adult thymopoiesis.

Keywords: Delta-like 4 · Fetal T-cell development · FoxN1Cre · Thymic epithelial cells

Introduction

T cells differentiate in the thymus following sequential developmental steps that constantly require interaction with thymic epithelial cells (TECs), the major cell type of the thymic stroma. TECs are organized as a 3D network that facilitates thymocyte migration, and provide important thymopoietic factors such as Kit ligand, FLT3L, IL-7, CCL25, CXCL12, CCL19, and CCL21. Anatomically, the adult thymus is divided into cortex and medulla, each comprised of specialized TECs, cTECs (cortical epithelial cells) and mTECs (medullary epithelial cells), with different phenotype and function [1–3]. This epithelial compartmentalization of the adult thymus is essential for the correct development of T cells, and epithelial cells require specific interactions with developing thymocytes in order to maintain the cTEC versus mTEC specification. Such a mutual interdependency between thymocytes and TECs has been called “thymic crosstalk” [4].
In the adult, BM-derived common lymphoid precursors continuously seed the thymus from the bloodstream [5]. Inside the thymus, these precursors adopt a T-cell fate through a non-cell autonomous process that strictly depends on Notch signaling mediated by interactions with cTECs. Notch signaling directs the lymphoid precursors towards a T-cell lineage differentiation program and blocks alternative lineage differentiation pathways [6–9].

Fetal thymocyte development differs from the adult in two main aspects. First, fetal thymocytes differentiate from fetal liver lymphoid precursors that, compared to adult BM lymphoid precursors, seem to have a more restricted or at least different lineage potential [10–12], as well as a very restricted and programmed TCR rearrangement [13, 14]. Second, in contrast to the compartmentalized adult thymus, the first waves of fetal lymphoid precursors colonize an undifferentiated and not yet vascularized thymic primordium (around E11.5) comprised of a very homogeneous population of TECs that will undergo sequential steps of differentiation throughout fetal development that ultimately lead to the emergence of cTECs and mTECs [15]. The first thymocyte progenitors enter the incipient thymus following CCR7 and CCR9 chemokine signals in contrast to subsequent waves of lymphoid precursors in late fetal stages and postbirth, which enter the thymus via the vascular system [16]. Interestingly, some T-cell subsets found in the adult are strictly fetal-dependent, such as IL-17-producing γδ T cells [17] or dendritic epidermal T cells (DETCs) [18], a unique type of γδ T cells that critically modulate innate immune surveillance and homeostasis in the adult skin.

Given the different nature of lymphoid progenitors and the unique temporal coincidence of epithelial and thymocyte differentiation, fetal thymic development might require different lymphoepithelial interactions and signaling pathways compared with that in the adult. In this regard, a recent study has revealed distinct mechanisms of fetal and adult crosstalk in the thymic medulla due to developmentally regulated availability of RANKL and CD40 ligand [19]. We have focused our attention on Notch signaling. In contrast to the adult, it has been suggested that fetal T-cell lineage commitment may occur in the liver, prior to intrathymic Notch signaling [20]. However, the role of Notch signaling during fetal T-cell development is poorly characterized due to the lack of mouse models that allow specific deletion of Notch receptors in fetal lymphoid precursors. To address this issue, we have taken advantage of DL4ΔFoxn1 mice (DL4 is delta-like 4) [7]. DL4ΔFoxn1 mice carry loxP sites flanking the first three coding exons of the DL4 gene, and the Foxn1Cre transgene that allows specific DL4 deletion on TECs. T-cell development is completely blocked in adult DL4ΔFoxn1 mice and B cells develop ectopically in the thymus, the same as in mice deficient for Notch1 in lymphoid precursors [8]. These results provided clear evidence that DL4 expressed on endothelial cells (defined by expression of them coexpressing UEA-1 (Fig. 2B and [26]). As shown in Fig. 1A, whereas DL4 is highly expressed on all TECs from control littermates, in DL4ΔFoxn1 embryos, we determined that DL4 expression on endothelial cells in the fetal liver was not affected in these mice (data not shown).

Importantly, DL4 deletion in embryonic TECs did not grossly affect epithelial cell differentiation, since B1 versus UEA-1 expression profile (Fig. 1B) and the pattern of keratin (K)5 and K8 staining [27] at E15.5 (Fig. 1C) were identical in control and DL4ΔFoxn1 embryos. Moreover, expression of IL-7 and IL-15, cytokines which are important for the development of both γδ and γδ T-cell lineages of both γδ and γδ T-cell lineages [28, 29], was identical in E15.5 TECs from control and DL4ΔFoxn1 embryos (Fig. 1D). Together, these results show that DL4 expression is efficiently and specifically abolished on TECs from DL4ΔFoxn1 embryos without grossly altering their early development. Therefore these mice are suitable for studying the role of DL4-mediated Notch signaling during fetal T-cell development.

Because FoxN1-dependent Cre expression is highly restricted to TECs and is active from very early stages of fetal development [23], we thought DL4ΔFoxn1 mice might be ideal for studying the intrathymic Notch signaling requirement for fetal T-cell commitment. We have first validated the DL4ΔFoxn1 mouse model for our study and subsequently analyzed in detail aβ and γδ T-cell differentiation as well as initial TEC development during the fetal stage in these mice.

Results and discussion

DL4 deletion on fetal TECs

We first verified whether DL4 expression is abolished on fetal DL4ΔFoxn1 TECs by making use of a mAb against DL4 developed in our laboratory [21]. In the adult thymus, cTECs are identified by the expression of BP1 (a transmembrane enzyme with aminopeptidase activity [24]), whereas mTECs are identified by means of their capacity to bind the lectin Ulex europaeus agglutinin 1 (UEA-1, [25]). At early stages of fetal thymic development, virtually all TECs express BP1 (cTEC phenotype) with a small proportion of them coexpressing UEA-1 (Fig. 2B). As shown in Fig. 1A, whereas DL4 is highly expressed on all TECs from control E15.5 embryos, as we have previously shown [21], DL4 expression is not detected in TECs from DL4ΔFoxn1 littermates. In order to confirm the specificity of DL4 deletion for TECs in DL4ΔFoxn1 embryos, we determined that DL4 expression on endothelial cells in the fetal liver was not affected in these mice (data not shown).

Importantly, DL4 deletion in embryonic TECs did not grossly affect epithelial cell differentiation, since BP1 versus UEA-1 expression profile (Fig. 1B) and the pattern of keratin (K)5 and K8 staining [27] at E15.5 (Fig. 1C) were identical in control and DL4ΔFoxn1 embryos. Moreover, expression of IL-7 and IL-15, cytokines which are important for the development of both γδ and γδ T-cell lineages [28, 29], was identical in E15.5 TECs from control and DL4ΔFoxn1 embryos (Fig. 1D). Together, these results show that DL4 expression is efficiently and specifically abolished on TECs from DL4ΔFoxn1 embryos without grossly altering their early development. Therefore these mice are suitable for studying the role of DL4-mediated Notch signaling during fetal T-cell development.

Lack of DL4 expression by TECs severely impairs fetal development of αβ T cells

In order to evaluate the effect of a DL4 null TEC compartment on fetal T-cell development, we first calculated the absolute number of thymocytes. Thymic cellularity increases rapidly as fetal development progresses. This is mainly due to the differentiation of the first wave of fetal αβ T-cell precursors, which is accompanied by cellular proliferation. We calculated the absolute number of thymic CD45+ cells and observed a strong reduction in DL4ΔFoxn1 mice compared to control littermates (Fig. 2A). CD3+ (T lineage) cells were also found to be dramatically decreased on E15.5 thymus sections (Fig. 2B).
As the great majority of normal thymocytes belong to the αβ T-cell lineage, we performed specific staining to identify different stages of αβ T-cell differentiation, in order to determine whether the decrease in fetal thymic cellularity observed in DL4<sup>FoxN1</sup> embryos is due to arrest at a specific developmental stage. Early development of CD<sup>4</sup><sup>-</sup>CD<sup>8</sup><sup>-</sup> (DN) thymocytes proceeds through DN1 (CD4<sup>+</sup>CD25<sup>-</sup>), DN2 (CD4<sup>+</sup>CD25<sup>+</sup>), DN3 (CD4<sup>-</sup>CD25<sup>+</sup>), and DN4 (CD4<sup>-</sup>CD25<sup>-</sup>) stages before differentiation into CD4<sup>+</sup>CD8<sup>-</sup> (DP) cells. We show in Fig. 2C two time points, one at a very early stage of fetal thymic development (E15.5, when normally the first wave of αβ thymocytes have differentiated to the DN3 stage) and another around birth (E18.5, when they have differentiated to the DP stage). As depicted in Figure 2C, we found that αβ T-cell differentiation does not progress normally in DL4<sup>FoxN1</sup> embryos, as they show a strong reduction in the DN2 and DN3 populations at both time points and, consequently, a much greater apparent DN1 population. The DP population is also strongly reduced at the E18.5 stage (from 85% in control littermate to 10% in DL4<sup>FoxN1</sup> embryos). These results demonstrate that, like in the adult [6, 7], DL4 mediated-Notch signaling is necessary for correct development of fetal αβ T cells. Whether the small residual population of αβ T lineage cells in the thymus of DL4<sup>FoxN1</sup> mice results from a failure to delete DL4 efficiently in a subset of TECs (which stain below the threshold of detection with our anti-DL4 mAb) or, alternatively, represents a minor pathway of DL4-independent T-cell development remains to be investigated.

In order to analyze whether the lack of Notch signaling in fetal lymphoid precursors results in the alternative differentiation of other hematopoietic cell lineages such as reported in adult mice [6, 7, 9], we further analyzed the apparent DN1 population depicted in Fig. 2C. This population was defined as CD4<sup>+</sup>Ter119<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>-</sup>TCR<sup>β</sup><sup>-</sup>TCR<sup>δ</sup><sup>-</sup> and therefore contains lineage positive CD4<sup>+</sup>CD25<sup>-</sup> cells (such as myeloid...
cells, NK cells, B cells, and DC) that were not excluded in the analysis. As shown in Fig. 2D, none of these cell lineages was dramatically increased or decreased in absolute number in DL4<sup>F<sub>x</sub></sup>/Fox<sup>N1</sup> embryos compared with those in control littermates, although B cells and myeloid cells were slightly increased in DL4<sup>F<sub>x</sub></sup>/Fox<sup>N1</sup> embryos. This result differs from the adult DL4<sup>F<sub>x</sub></sup>/Fox<sup>N1</sup> thymus where it has been shown that very large numbers of B cells (300-fold increase compared with that in controls) ectopically develop from BM lymphoid precursors in the absence of DL4-mediated Notch signaling [6, 7]. The absence of any major B-cell expansion in the embryonic DL4<sup>F<sub>x</sub></sup>/Fox<sup>N1</sup> thymus cannot be attributed to a lack of IL-7, since levels of this cytokine in TECs were normal (Fig. 1D).

Different alternative fates of fetal and adult T-cell precursors in DL4<sup>F<sub>x</sub></sup>/Fox<sup>N1</sup> thymus could be due to two main reasons. First, fetal lymphoid precursors may have intrinsically different cell lineage potential compared to adult as suggested by several groups [10–12]. Alternatively, fetal thymic stroma may not support the development of alternative non-T-cell lineages. We attempted to directly test the latter possibility by performing fetal thymus engraftment under the adult kidney capsule, thus allowing the potential development of adult BM lymphoid precursors in a fetal DL4<sup>F<sub>x</sub></sup>/Fox<sup>N1</sup> thymus. Unfortunately this approach failed, as fetal DL4<sup>F<sub>x</sub></sup>/Fox<sup>N1</sup> thymi did not engraft correctly whereas the control thymus did.
Fetal γδ T cells, including DETC precursors, strictly depend upon DL4-mediated Notch signaling

Unlike the adult, fetal γδ T-cell development is characterized by successive changes in TCRγ and TCRδ expression pattern [30, 31]. This is presumably due to specific intrinsic properties of the different lymphoid precursors that successively arrive in the fetal thymus [13]. The first fetal CD3+ thymocytes are observed around E14.5. They are γδ T cells, most of which express an invariant TCR composed of canonical TCRVγ3 and TCRVδ1 chains. These Vγ3/Vδ1 T cells are the progenitors of DETCs. Interestingly TCRVγ3 and TCRVδ1 chains are not expressed by adult thymocytes [32, 33], and it is believed that their expression is exclusive to the first wave of fetal lymphoid precursors that exhibit a very restricted preprogrammed TCR rearrangement [33]. We and others have previously shown that this fetal restricted γδ TCR specificity is necessary for correct DETC development [34, 35]. Furthermore, the differentiation of DETC progenitors depends on the highly specific selecting ligand Skint-1 expressed on the fetal thymic stroma [36].

To investigate DETC progenitors (Vγ3+ cells), and also other fetal γδ T-cell subsets, we analyzed the thymus at different gestational stages. As expected, we found a high proportion of γδ T cells expressing Vγ3 in E15.5 control thymus (0.7% in the total gated population, and more than 50% among CD3+ cells); Vγ3+ cells, however, were barely detected in E15.5 DL4ΔFoxn1 thymus (Fig. 3A). At E18.5, Vγ3+ cells normally represent a smaller proportion (around 10%) of total γδ T cells, as other TCRγ chains are more prominent at this fetal stage [30, 31]. At E18.5, a strong reduction in Vγ3+ cells is also observed in the DL4ΔFoxn1 thymus compared with that in control littersmates, but less pronounced in comparison with that at E15.5 (Fig. 3A). This is suggestive of the accumulation/proliferation of residual Vγ3+ cells developing in the DL4ΔFoxn1 thymus. We determined the maturation state of this residual Vγ3+ population by analyzing the expression pattern of CD24 and CD45RB [37]. Immature Vγ3+ cells express high levels of CD24 and intermediate levels of CD45RB (as in control E15.5 dot plot of Fig. 3B). Upon positive selection mediated by the thymic stromal factor Skint-1, Vγ3+ cells downregulate CD24 and upregulate CD45RB (mature phenotype, [38]). We found that a large proportion of the residual Vγ3+ cells found in E18.5 DL4ΔFoxn1 embryos exhibit a mature phenotype (Fig. 3B). Consistent with this observation, expression levels of Skint-1 were very high in fetal TECs from DL4ΔFoxn1 embryos, albeit slightly lower than in control TECs (Fig. 1D). Our data, thus, indicate that the process of thymic positive selection of residual Vγ3+ DETC progenitors is not impaired in DL4ΔFoxn1 embryos and further suggest that DL4-mediated signaling is critical prior to the maturation stage.

The proportion of Vγ3− γδ T cells was also strongly reduced in E15.5 and E18.5 DL4ΔFoxn1 thymus (Fig. 3A). The reduction in the percentage of both Vγ3+ and Vγδ− γδ T cells in DL4ΔFoxn1 fetal thymus is accompanied by a similar reduction in absolute numbers (Fig. 3A). These results show that fetal γδ T-cell development in general depends on DL4-induced Notch signaling, as shown previously for the adult thymus [6] where diverse γδ TCR is generated.

We next analyzed DETCs in adult mice to determine whether the few Vγ3+ cells observed in fetal DL4ΔFoxn1 thymus can migrate to the skin. As soon as 2 weeks after birth, it is possible to observe a population of DETCs (CD45+CD3+ cells) in the skin of control mice whereas DETCs are virtually absent in DL4ΔFoxn1 mice (Fig. 3C). However, the same analysis performed in 16-week-old mice did not show any difference in the percentage of DETCs in control and DL4ΔFoxn1 mice (Fig. 3C). As Vγ3+ cells do not develop in the thymus after birth, this result suggests that the residual Vγ3+ thymocytes that develop and mature in DL4ΔFoxn1 embryos migrate to the skin and undergo homeostatic proliferation in situ to recover normal DETC numbers. Consistent with this hypothesis, we found a gradual recovery of DETCs with age in DL4ΔFoxn1 mice (Fig. 3D) and a normal TCR repertoire of these DETCs, as shown by staining with anti-Vγ3 and the mAb 17D1 (which recognizes an epitope formed by the combination of Vγ3 and Vδ1, [39]; Fig. 3E). The same presumed homeostatic expansion of DETCs has been observed in CCR7/CCR9 double-deficient mice that show a diminished Vγ3+ population in the thymus and a significantly reduced DETC population in the skin of very young mice, but normal numbers of DETCs in the adult [16].

Concluding remarks

Taken together, our data show that the interaction of DL4 expressed by TECs with Notch receptors expressed by lymphoid precursors is a general requirement for commitment and/or development of all T-cell lineages, regardless of their TCR characteristics or fetal versus adult origin. In agreement with our conclusion, it has been recently reported that the development of fetal derived IL-17-producing γδ T cells requires the Notch-Hes1 pathway [40].

Despite the dramatic block in αβ and γδ T-cell development in the DL4ΔFoxn1 embryonic thymus, initial TEC differentiation appeared to be unaffected. Normal cTEC and incipient mTEC populations were detected at E16.5 both by flow cytometry (BP1 versus UEA-1 staining) and on cryosections (K5 versus K8 staining). In addition, DL4-deficient TECs expressed the DETC selecting molecule Skint-1 as well as thymopoietic cytokines such as IL-7 and IL-15 at levels similar to control TECs. These data strongly suggest that early embryonic TEC development occurs independently of lymphostromal interactions, in agreement with other studies [27, 41].

In contrast to the adult [6, 7], ectopic B-cell development in the embryonic DL4ΔFoxn1 thymus was minimal. In this context, several studies indicate that fetal T-cell progenitors are devoid of B-cell lineage potential but rather exhibit myeloid or NK-cell potential [10–12]. Nevertheless, neither myeloid nor NK cells were significantly increased in absolute numbers in the embryonic DL4ΔFoxn1 thymus. Thus it appears that fetal bipotent T/myeloid and T/NK progenitors fail to adopt their alternative fate in the absence of Notch signaling. Whether this reflects an intrinsic difference in Notch regulation of cell fate in fetal versus adult T-cell progenitors
Figure 3. Development of fetal \( \gamma \delta \) T cells in DL4\(^{\Delta FoxN1} \) mice. (A) Impaired fetal \( \gamma \delta \) T-cell development in DL4\(^{\Delta FoxN1} \) mice. Thymocyte suspensions from E15.5 or E18.5 control or DL4\(^{\Delta FoxN1} \) littermates were stained for CD45, CD3, lineage marker cocktail (which includes CD4, CD8, TCR\( \beta \), Ter119, F4/80, and Gr1) and V\( \gamma \)3. CD3 versus V\( \gamma \)3 dot plots correspond to the gate CD45\(^+ \) lineage marker cocktail\(^- \), therefore CD3\(^+ \) cells are \( \gamma \delta \) T cells in these dot plots. The percentages of V\( \gamma \)3\(^- \) and V\( \gamma \)3\(^+ \) \( \gamma \delta \) T cells in this gate are indicated for each dot plot as mean \( \pm \) SD of \( n \geq 3 \). The absolute numbers of thymic V\( \gamma \)3\(^- \) and V\( \gamma \)3\(^+ \) \( \gamma \delta \) T cells from control (grey bars) or DL4\(^{\Delta FoxN1} \) (black bars) littermates at the indicated fetal stages are also shown (right) as mean \( \pm \) SD of at least three mice per group. \( * \) \( p \leq 0.05 \), \( ** \) \( p \leq 0.01 \), unpaired Mann–Whitney test. (B) Analysis of V\( \gamma \)3\(^+ \) thymocyte maturation. Thymocyte suspensions were stained as in Fig. 3A, except that anti-CD24 and anti-CD45RB mAbs were also included. CD24 versus CD45RB dot plots correspond to CD3\(^+ \) V\( \gamma \)3\(^+ \) cells (discontinuous gate in Fig. 3A). The percentage of mature V\( \gamma \)3\(^+ \) cells (CD24\(^{low} \) CD45RB\(^{high} \)) is indicated as mean \( \pm \) SD, \( n \geq 3 \) mice. (C) Absence of DETCs in young but not in adult DL4\(^{\Delta FoxN1} \) mice. Dot plots show CD45 versus CD3 staining of total epidermal cell suspensions from control or DL4\(^{\Delta FoxN1} \) mice, at 2 or 16 weeks of age. The percentage of DETCs (CD45\(^+ \) CD3\(^+ \) cells) is indicated in each dot plot. Results are representative of three mice of each type analyzed independently with equivalent results. (D) Age-dependent expansion of DETCs in DL4\(^{\Delta FoxN1} \) mice. The percentage of CD3\(^+ \) V\( \gamma \)3\(^+ \) cells in the CD45\(^+ \) cell population of epidermal cell suspensions from control or DL4\(^{\Delta FoxN1} \) littermates at different ages is shown as mean \( \pm \) SD of at least four mice per group. (E) Analysis of DETC TCR repertoire. Epidermal cell suspensions were stained for CD45, CD3, V\( \gamma \)3, and the V\( \gamma \)3V\( \delta \)1 idiotype recognized by mAb 17D1. 17D1 versus V\( \gamma \)3 dot plots correspond to CD45\(^+ \) CD3\(^+ \) epidermal cells as gated in Fig. 3C. The percentage of cells in each quadrant is indicated. Results are representative of three mice of each type analyzed independently with equivalent results.
or a failure of the embryonic thymic microenvironment to support the development of ectopic lineages remains to be established.

Finally, it has been suggested that commitment of embryonic hematopoietic precursors to the T-cell lineage occurs via Notch signaling in the fetal liver, prior to migration to the thymus [20]. Although our data do not directly address this issue, they nevertheless demonstrate that continuous intrathymic Notch signaling via interactions of T-cell progenitors with DL4-expressing TECs is essential for fetal thymopoiesis.

Materials and methods

Mice

FoxN1-Cre $^{+}$ DL4$^\Delta$FoxN1 mice have been previously described [7]. Timed pregnancies were set up, designating the day of finding a vaginal plug as day 0.5 of embryonic development. Mothers were sacrificed at different stages of gestation and embryos were typed by PCR. Control (FoxN1-Cre$^{-}$ DL4$^\Delta$FoxN1 litters) and DL4$^\Delta$FoxN1 embryos were processed individually, with the exception of qPCR analysis where control and DL4$^\Delta$FoxN1 embryonic thymi were pooled for electronic sorting of TECs and CD45$^{+}$ populations. All animal experiments were conducted under the authorization and with approval of the Review Board of the Veterinary Service from Canton de Vaud, Lausanne, Switzerland.

Cell preparation, flow cytometry, and sorting

Thymocyte suspensions were prepared by pressing the thymus through a sieve. Enriched populations of TECs and epidermal cells were prepared as described previously [42, 43]. Cells were preincubated with 2.4G2 culture supernatant to block Fc$\gamma$ receptors and subsequently stained with combinations of mAb as indicated in the figure legends. Cells were analyzed on a FACSCantoTM flow cytometer using FACSDivatm software (Becton Dickinson, Franklin Lakes, NJ, USA). Sortings were performed on a FACSariaTM flow cytometer (Becton Dickinson). Dead cells were gated out by their forward and side scatter profile. Data were processed with FlowJo software (Tree Star, Ashland, OR, USA).

Histology and immunofluorescence microscopy

Unfixed E15.5 thymus was embedded in O.C.T. compound (Sakura), cut, fixed with acetone, and stained as previously described [44]. After a blocking step, sections were labeled with rat mAb anti-K8 (Troma1, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) and rabbit anti-K5 (Covance, Princeton, NJ, USA) followed by a Cy-3-conjugated donkey antirat IgG (Jackson Immunoresearch, West Grove, PA, USA) and an Alexa647-conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). The Armenian hamster Ab to CD3 (clone 145–2C11) was revealed with a biotinylated anti-Armenian hamster IgG secondary Ab followed by a Cy-3-conjugated streptavidin (both Jackson Immunoresearch). Images were acquired using a Leica DM5500 microscope and processed using Adobe Photoshop (brightness and contrast were adjusted equally).

Real-time RT-PCR

Skint-1, IL-7, IL-15, and FoxN1 expression in sorted TECs (CD45$^{+}$ MHC class II (MHCII)$^{+}$ EpCAM$^{+}$ BP1$^{+}$) and CD45$^{+}$ thymocytes were analyzed by quantitative RT-PCR and normalized as described [45]. Primer sequences are available upon request.

Statistical analysis

Statistical differences were calculated with an unpaired Mann–Whitney test, one tail (XLstat). Differences were considered significant when $p \leq 0.05$ (*), very significant when $p \leq 0.01$ (**), and extremely significant when $p \leq 0.001$ (***)

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References

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Abbreviations: cTEC: cortical epithelial cell · DETC: dendritic epidermal T cell · DL4: delta-like 4 · DN1: CD44+ CD25− · DN3: CD44− CD25+ · DP: CD4+ CD8+ · K: keratin · mTEC: medullary epithelial cell · TEC: thymic epithelial cell · UEA-1: Ulex europaeus agglutinin 1

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