Cutting Edge: Thymic Crosstalk Regulates Delta-Like 4 Expression on Cortical Epithelial Cells

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Interactions between Notch1 receptors on lymphoid progenitors and Delta-like 4 (DL4) ligands on cortical thymic epithelial cells (cTEC) are essential for T cell lineage commitment, expansion, and maturation in the thymus. Using a novel mAb against DL4, we show that DL4 levels on cTEC are very high in the fetal and neonatal thymus when thymocyte expansion is maximal but decrease dramatically in the adult when steady-state homeostasis is attained. Analysis of mutant mouse strains where thymocyte development is blocked at different stages indicates that lymphostromal interactions (“thymus crosstalk”) are required for DL4 down-regulation on cTEC. Reconstitution of thymocyte development in these mutant mice further suggests that maturation of thymocytes to the CD4<sup>+</CD8</sup><sup>+</sup> stage and concomitant expansion are needed to promote DL4 down-regulation on cTEC. Collectively, our data support a model where Notch1-dependent thymopoiesis by controlling DL4 expression levels on cTEC. The Journal of Immunology. 2008, 181: 8199–8203.

The earliest intrathymic progenitors are CD4<sup>−</sup>CD8<sup>−</sup> double negative (DN)<sup>3</sup> and they subsequently up-regulate CD4 and CD8 coreceptors to become CD4<sup>+</CD8</sup><sup>+</sup> double positive (DP) before undergoing positive and negative selection that results in the mature CD4<sup>+</CD8</sup><sup>+</sup> T cell repertoire. During the DN thymocyte stage the earliest precursors are CD44<sup>+</CD25</sup><sup>−</sup>DN1, which give rise sequentially to CD44<sup>+</CD25</sup><sup>+</sup>DN2, CD44<sup>+</CD25</sup><sup>+</sup>DN3, and finally CD44<sup>+</CD25</sup><sup>+</sup>DN4 before maturing to the DP stage (1).

It is now clear that the evolutionarily conserved Notch signaling pathway is critical for both T cell lineage commitment, proliferation, and differentiation. The earliest intrathymic progenitors are CD4<sup>−</sup>CD8<sup>−</sup> double negative (DN)<sup>3</sup> and they subsequently up-regulate CD4 and CD8 coreceptors to become CD4<sup>+</CD8</sup><sup>+</sup> double positive (DP) before undergoing positive and negative selection that results in the mature CD4<sup>+</CD8</sup><sup>+</sup> T cell repertoire. During the DN thymocyte stage the earliest precursors are CD44<sup>+</CD25</sup><sup>−</sup>DN1, which give rise sequentially to CD44<sup>+</CD25</sup><sup>+</sup>DN2, CD44<sup>+</CD25</sup><sup>+</sup>DN3, and finally CD44<sup>+</CD25</sup><sup>+</sup>DN4 before maturing to the DP stage (1).

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support a model in which thymic crosstalk quantitatively regulates the rate of N1-dependent thymopoiesis by controlling the expression level of DL4 on cTEC.

Materials and Methods

Mice

C57BL/6 wild-type (WT), TCRβ<sup>−/−</sup>, and RAG2<sup>γ<sub>−</sub>/−</sup> mice were purchased from Harlan Netherlands, The Jackson Laboratory, and Taconic Farms, respectively. This study has been reviewed and approved by the Service Vétérinaire Cantonal of État de Vaud.

Production of mAbs against DL1 and DL4

Anti-DL1 and anti-DL4 mAbs were generated by immunizing 8- to 10-wk-old male LOU/CNimOlaHsd rats (purchased from Harlan Netherlands) with chimeric proteins composed of either DL1 extracellular domain (aa 1-523) or DL4 extracellular domain (aa 1-527) fused with human IgG1 Fc domain (12). Splenocytes from immunized rats were fused with nonsecreting X63-Ag8.653 myeloma cells by standard procedures. Hybridoma supernatants from individual wells (2880 for DL1 and 6720 for DL4) were tested for specific reactivity against the chimeric DL1 or DL4 proteins in ELISA and subsequently assayed by FACS on OP9 stromal cells engineered to express GFP together with mouse DL1 or DL4. Cells from FACS-positive wells (three for DL1 and two for DL4) were cloned by limited dilution. The clones 30B11.1 (anti-DL1, rat IgG2α) and 9A1.5 (anti-DL4, rat IgG1k) were selected for further analysis. Purified Abs from these clones were biotinylated and revealed with streptavidin-allophycocyanin.

Cell preparation, flow cytometry, and sorting

Thymocytes and enriched populations of TEC were prepared as described previously (4, 13) and stained with combinations of mAbs as indicated in the figure legends. Cells were analyzed on a FACSVerse flow cytometer using FACS Diva software (BD Biosciences). Dead cells were gated out by their forward and side scatter profile. Sorting of TEC was performed on a FACSaria flow cytometer (BD Biosciences). Data were processed with FlowJo software (Tree Star).

Immunofluorescence microscopy

Unfixed E15 thymus were embedded in O.C.T. compound (Sakura), cut, fixed and stained as previously described (14). The labeling was performed using either rat anti-DL1 or rat anti-DL4 hybridoma supernatant followed by biotinylated anti rat IgG (Jackson Immunoresearch Laboratories) followed by streptavidin-HRP (Amersham). After a 1-h blocking step with 5% normal rat serum, sections were then labeled with rat mAb anti-keratin 8 (Troma1; Developmental Studies Hybridoma Bank) conjugated in house to Alexa Fluor 488 (Molecular Probes) and rabbit anti-keratin 5 (Covance) followed by a Alexa Fluor 647-conjugated donkey anti-rabbit IgG (Molecular Probes). Finally, HRP was visualized using a tyramide-Cy3 signal amplification system. Images were acquired using a Leica DM5500 microscope and processed using Adobe Photoshop (brightness and contrast were adjusted equally).

Bone marrow chimeras and in vivo anti-D3treatment

Eight-wk-old RAG2<sup>−/−</sup> mice received 0.3 Gy of gamma irradiation 4 h before i.v. injection of 5 × 10<sup>5</sup> T-cell-depleted C57BL/6 wild-type bone marrow (BM) (4). Five-wk-old TCRβ<sup>−/−</sup> mice were injected i.p. with 100 µg of anti-D3 mAbs (clone 145-2C11) per mouse.

Real-time RT-PCR

DL4 expression in sorted cTEC (CD45<sup>−</sup>B1<sup>hi</sup>) was analyzed by quantitative RT-PCR and normalized as described (15). Primer sequences are available upon request.

Results and Discussion

Generation of mAbs against DL1 and DL4

Several reports have analyzed the expression of Notch ligands, including DL1 and DL4, in thymocytes and thymic stromal cells (2, 3). To date, these studies have mainly relied upon detection of ligand expression in heterogeneous cell populations by RT-PCR and polyclonal antisera. To obtain more definitive expression data at the single cell level, we recently generated rat mAbs against the extracellular domains of DL1 and DL4 (see Materials and Methods for details). These mAbs specifically stained OP9 stromal cells that were retrovirally transduced with the appropriate ligand (5). Moreover, our mAb to DL4 (but not DL1) stained isolated TEC from control mice but not from mice where DL4 was conditionally ablated in TEC (5), further confirming in vivo the specificity of this reagent.

Regulation of DL4 expression on cTEC during thymus development

Given the critical importance of DL4 expression on TEC for T cell lineage commitment (5, 6) we investigated expression levels of DL4 on TEC at various stages of thymus ontogeny. Enriched populations of TEC were isolated from enzymatically digested thymus preparations by standard procedures (13). TEC were electronically gated as CD45<sup>−</sup> cells expressing panpsychokeratin (PanCK) (16) and mTEC were distinguished from cTEC based on B1P1 expression (17). Control experiments with TEC from adult thymus (Fig. 1A) confirmed that quantitatively similar results could be obtained using either PanCK (16) or MHC class II (18) to detect CD45<sup>−</sup> TEC, and either B1P1 (17) or the lectin UEA-1 (18) to distinguish cTEC from mTEC. As shown in Fig. 1B, DL4 was expressed at very high levels on the vast majority of PanCK<sup>−</sup>B1P1<sup>high</sup> cTEC on embryonic day 15 fetal (E15), a stage when hematopoietic precursors have recently colonized the thymus. DL4 expression on cTEC remained very high in the neonatal thymus (4 days old) but decreased progressively with age to become barely detectable in the adult (Fig. 1, B and C). As expected (17), the proportion of mTEC (PanCK<sup>−</sup>B1P1<sup>low</sup>) increased with age. At all developmental stages, DL4 expression by mTEC was very low and DL1 expression was undetectable on both mTEC and cTEC (Fig. 1B).

DL4 mRNA levels in sorted cTEC by quantitative RT-PCR indicated a strong reduction (5- to 10-fold) in the adult compared with the neonate or fetus (Fig. 1C), consistent with the flow cytometry data. Taken together, these results demonstrate that DL4 surface expression on cTEC is dramatically down-regulated during thymus development, most likely at the level of transcription.

DL4 expression on cTEC in situ at E15

Further in situ analysis of DL4 expression by immunostaining of E15 thymus sections (Fig. 1D) demonstrated clearly that DL4 was expressed by a major subset of TEC that coexpressed keratin 8, but not by the minor TEC population coexpressing keratin 5. It is generally accepted that keratin 8<sup>+</sup> TEC at E15 represent the major TEC population, whereas keratin 5<sup>+</sup> TEC correspond to a minor subset of emerging mTEC (19, 20). Thus, by using independent markers and staining techniques we confirmed the preferential expression of DL4 on cTEC. As expected from the FACS analysis (Fig. 1, B and C), DL1 expression was not detected on E15 thymus sections (Fig. 1D).

High DL4 expression in adult cTEC from RAG2<sup>γ<sub>−</sub>/−</sub> and TCRβ<sup>−/−</sup>-deficient mice

The strongly reduced expression of DL4 in adult vs fetal/neonatal cTEC could reflect a programmed decrease in DL4 with age intrinsic to this population. Alternatively, DL4 might be regulated on cTEC as a result of lympho-stromal interactions occurring during thymus ontogeny (7, 8), a concept often referred to as “thymic crosstalk” (9). One general approach that has been used frequently to distinguish between these possibilities is to analyze mutant mice in which thymocyte development is blocked at different developmental stages. We therefore
investigated DL4 expression levels on cTEC isolated from adult RAG2/γc−/− or TCRβ-deficient mice. RAG2/γc−/− mice have virtually no CD45+ hematopoietic cells in the thymus, whereas T cell development in TCRβ−/− mice is almost totally blocked at the DN3 stage because of the absence of a pre-TCR. As shown in Fig. 2, cTEC from both RAG2/γc−/− and TCRβ−/− adult thymuses expressed high levels of DL4 comparable to those found in fetal or neonatal WT thymus (Fig. 1B). These results establish that the decrease in DL4 expression observed in adult WT cTEC is not simply programmed with age but rather depends upon normal development of the thymic hematopoietic component. Moreover, the high DL4 levels in adult TCRβ−/− cTEC suggest that down-regulation of DL4 in adult WT cTEC requires progression of thymocytes beyond the DN3 stage.

**FIGURE 1.** Regulation of DL4 expression on TEC during thymus ontogeny. A, TEC extracted from 8-wk-old C57BL/6 WT mice were stained with anti-BP1-PE, anti-CD45.2-PECy5.5, anti-PanCK-FITC, anti-MHCII-allophycocyanin, and anti-UEA-1-biotin (revealed with streptavidin-PECy7) mAbs and analyzed by cytofluorometry. CD45+ cells were gated on PanCK+ (top left panel) or MHCII+ (top right panel). PanCK+ or MHCII+ cells were plotted vs BP1 (middle panels) or vs BP1 and UEA-1 (lower panels). By gating on either PanCK+ or MHCII+ TEC populations, the percentage of cTEC and mTEC was comparable. B, TEC extracted from C57BL/6 WT mice at different ages (as indicated) were stained with anti-BP1-PE, anti-CD45.2-PECy5.5, anti-PanCK-FITC, and either anti-DL1-biotin or anti-DL4-biotin (revealed with streptavidin-allophycocyanin) mAbs. CD45+ PanCK+ cells were gated according to their BP1 expression level as cTEC or mTEC (left panels). The middle and right panels, respectively, show DL1 and DL4 expression vs BP1 in CD45+ PanCK+ cells. DL1 staining was indistinguishable from an isotype control (data not shown). 4d, 4 days. C, The upper panel shows representative mean fluorescence intensity (MFI) of DL4 (black bars) and DL1 (gray bars) in WT cTEC (PanCK+ BP1high) at different ages indicated. The lower panel shows DL4 mRNA expression by real-time PCR (normalized with TATA-binding protein (TBP)) of sorted cTEC (CD45+ BP1high) at the same ages. D, Sequential E15 thymus cryosections were stained with anti-keratin 5 (K5; blue staining) and anti-keratin 8 (K8; green staining) together with either anti-DL1 or anti-DL4 (red staining). The staining combinations are indicated on top of each panel. Yellow cells (bottom right) are K8+ DL4+. The Journal of Immunology
DN3 stage (and concomitant cellular expansion), we used two independent protocols. First, we reconstituted adult RAG2/-/- mice with WT BM. As shown in Fig. 2A, development of all major thymocyte subsets could be detected 4 wk after BM reconstitution in this model and a dramatic (10-fold) increase in absolute thymocyte cell numbers occurred between 4 and 5 wk. Concomitant with thymocyte maturation and expansion, down-regulation of DL4 expression on a subset of cTEC was first observed at 4 wk and became more pronounced at 5 wk (Fig. 2A). Similarly, in adult TCRβ/-/- mice injected 8 days previously with purified anti-CD3 mAbs, progression of DN3 thymocytes to the DN4 and DP stages was accompanied by a 5-fold increase in thymocyte numbers and a clear decrease in cTEC expressing high levels of DL4 (Fig. 2B). Collectively these results indicate that maturation and expansion of thymocytes is required to down-regulate DL4 expression on cTEC.

Concluding remarks

In conclusion, our data strongly support a novel thymic crosstalk mechanism in which the presence of developing thymocytes leads to reduced DL4 expression on cTEC. Two possible scenarios could be envisaged to explain these results. The most straightforward hypothesis would be that a single lineage of cTEC is induced to down-regulate DL4 as a result of signals generated by thymus crosstalk. Alternatively, it cannot be excluded that adult cTEC expressing low levels of DL4 represents a separate lineage that is induced to expand and thereby replaces (or dilutes) fetal/neonatal cTEC expressing high DL4 levels. Irrespective of which explanation is correct, our data suggest that regulation of DL4 levels on cTEC via thymic crosstalk may be required to promote optimal N1-dependent thymopoiesis in the fetus and neonate while restraining T cell development in the adult to maintain thymic (and thus indirectly peripheral) homeostasis. At present, there is no direct evidence in vivo indicating that DL4 levels on cTEC regulate the rate of thymocyte development. Nevertheless, it is known that the rate of progression of early T cell progenitors to the DP stage is much faster in the fetal or neonatal thymus than in the adult, consistent with DL4 levels on cTEC measured here.

Additional experiments will be necessary to uncover the precise thymus crosstalk mechanism responsible for regulating DL4 expression on cTEC. In this regard, it is unlikely that N1:DL4 interactions per se play a significant role, because binding of N1 to DL4 is maximal on DN thymocytes (12) that are by themselves unable to down-regulate DL4 expression on cTEC (as for example in TCRβ/-/- mice).

Whatever the explanation, it seems clear that our novel mAbs will facilitate future investigations of Notch:Delta interactions within the immune system as well as in other developing tissues.

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Disclosures

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References


