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On the role of Eph signalling in thymus histogenesis; EphB2/B3 and the organizing of the thymic epithelial network

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ABSTRACT In the current study, we extend our own previous results on the thymocyte phenotype of EphB2 and/or EphB3 deficient mice by analyzing the phenotype and the histological organization of their thymic epithelial stroma. All studied adult EphB-deficient thymi showed profound alterations with respect to the wild-type (WT) ones. Each mutant exhibited a specific phenotype, but also showed common features including occurrence of $K5^+K8^+MTS10^+$ immature medullary epithelial cells, numerous $K5^-K8^-MTS20^+$ cells and $K5^+K8^+$ cells in the thymic cortex and cortical and medullary $K5^-K8^-$ areas devoid of epithelial cell markers. In addition, comparative analysis of WT and EphB-deficient embryonic and newborn thymi demonstrated that the observed adult phenotype was a consequence of the gradual accumulation of early phenotypic and morphological defects, becoming more severe at the end of embryonic life and in newborn animals. Together, these results confirm a role for EphB2 and EphB3 in thymus morphogenesis. The obtained data are discussed from the point of view of the recognized role played by these two Ephs in the homeostasis of other epithelia and their possible relationships with molecules known to be involved in thymic epithelial cell development.

KEY WORDS: *development, tyrosine kinase receptor, keratin, thymic epithelial cell, lymphoid organ*

Introduction

The thymus is a highly compartmentalized organ that is involved in the functional maturation of lymphoid progenitors, which migrate into the organ to accomplish a complex process of differentiation. Thymus organogenesis begins in mice at 10 days post-coitum as a result of the cell interactions between the epithelium of the third pharyngeal pouch and neural crest-derived mesenchyme (Blackburn and Manley, 2004). One day later, lymphoid progenitors surround the thymic primordium (Itoi *et al.*, 2001; Manley and Blackburn, 2003) whereas the activation of the transcription factor Foxn1 induces the maturation of thymic epithelial cell progenitors (Blackburn *et al.*, 1996; Su *et al.*, 2003). On day 13 post-coitum, epithelial cell precursors that express keratins K5 and K8, are scattered throughout the central area of thymic primordium. Two days later, the K8 expression is down-regulated resulting in the appearance of central, small groups of $K5^+K8^{-/lo}$ cells surrounded by $K5^-K8^+$ cells that constitute the cortical parenchyma. Thymic compartmentalization progresses in the following days and the neonatal thymus consists of a $K5^-K8^+$ cortical epithelium and small foci of $K5^+K8^-$ medullary epithelial cells that will converge to organize a unique, central medulla in the adult thymus. Among these two areas, the cortico-medullary border still contains $K5^+K8^+$ epithelial cells (Klug *et al.*, 1998; Klug *et al.*, 2002). Mechanisms involved in both early organogenesis and three-dimensional organization of this thymic epithelial network are, however, largely unknown. Eph kinases and their ligands, ephrins, reported to be key molecules for establishing the organization pattern of different tissues (Himanen *et al.*, 2007) are good candidates to be involved in the thymus histogenesis.

Abbreviations used in this paper: Eph, erythropoietin-producing hepatocellular; ephrin, Eph receptor interacting protein; K, keratin; MHC, major histocompatibility complex; MTS, mouse thymic stroma; TEC, thymic epithelial cell.

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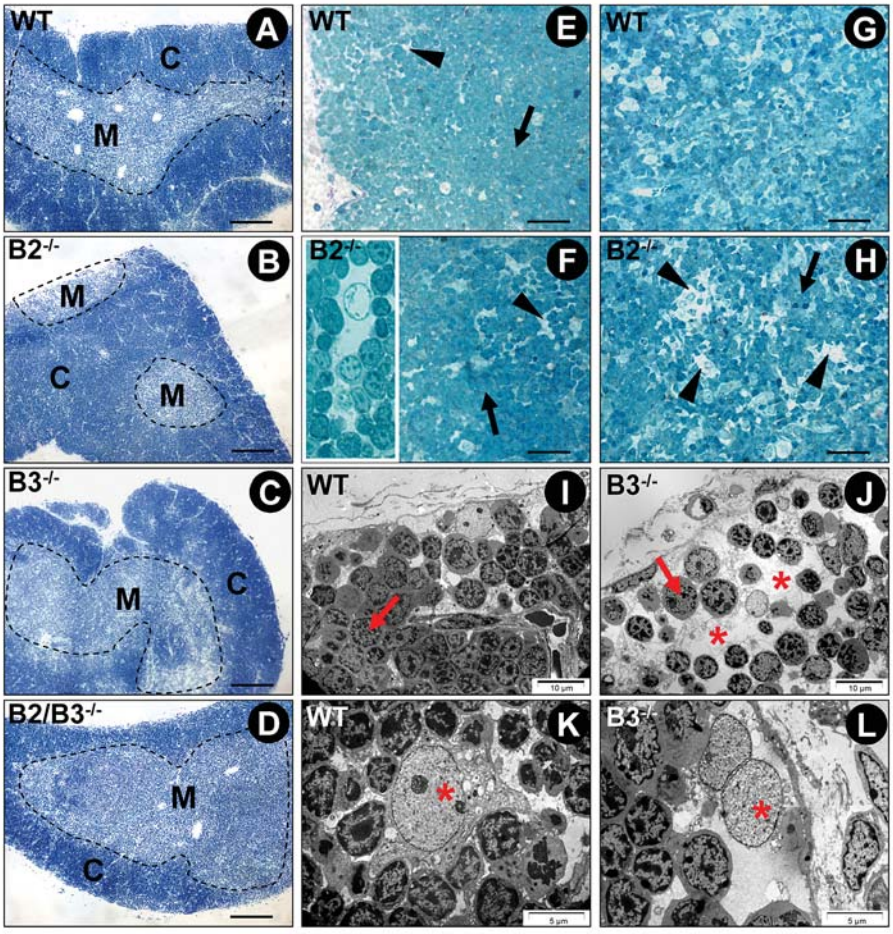


Fig. 1. Histological and ultrastructural organization of WT and EphB-deficient adult thymi. (A) *Histological section of a 2 month-old WT thymus. Notice the peripheral region, cortex (C) and the inner central one, medulla (M).* **(B,C,D)** *Histological sections of mutant thymi, respectively. EphB-deficient thymi show smaller size than WT thymi and profound alterations in the thymic histological organization. EphB2$^{-/-}$ thymi show fragmented, small medullary regions (M) dispersed throughout the thymic parenchyma whereas EphB3$^{-/-}$ ones exhibit a reduced cortical region and EphB2/B3$^{-/-}$ mice present an enlarged central medulla that, in some areas, invades a reduced cortical area (C).* **(E)** *At higher magnification, the WT cortex appears densely occupied by small thymocytes (arrow) that mask the TECs (arrowhead).* **(F)** *Detail of the thymic cortex of a EphB2$^{-/-}$ mouse showing few numbers of thymocytes (arrow) and large areas consisting of large, pale epithelial cells (arrowhead and insert).* **(G)** *Thymic medulla of a WT thymus containing lower numbers of thymocytes than the cortex.* **(H)** *Thymic medulla of an EphB2$^{-/-}$ mouse showing numerous pale TEC (arrowhead) and few thymocytes (arrow).* **(I)** *At electron microscopy the WT cortex appears fully occupied by thymocytes (arrow) making it difficult to identify the thymic epithelial meshwork.* **(J)** *Few cortical thymocytes occur in the EphB3$^{-/-}$ thymus (arrow). Note the presence of large areas devoid of TECs (asterisk).* **(K)** *Detail of a cortical epithelial cell (asterisk) in a WT thymus.* **(L)** *Detail of a degenerated cortical TEC (asterisk) in an EphB3-deficient thymus. Scale bars: (A,B) 200 μm; (E-H) 30 μm; (I-J) 10 μm; (K-L) 5 μm.*

Although studies on Eph/ephrins and the immune system have principally focused on their role in both T-cell maturation and activation of peripheral lymphocytes (Munoz *et al.*, 2002; Aasheim *et al.*, 2005; Wu and Luo, 2005; Freywald *et al.*, 2006; Munoz *et al.*, 2006; Alfaro *et al.*, 2007; Sharfe *et al.*, 2008), we recently demonstrated that EphA4-deficient mice showed profound blockade of T-cell differentiation due to the collapse of the cortical epithelium network (Munoz *et al.*, 2006). Furthermore, ephrinB1-Fc fusion proteins are able to *in vitro* disorganize the three-dimensional epithelial network established in reaggregates of fetal thymic epithelial cells and DP ($CD4^+CD8^+$) thymocytes (Alfaro *et al.*, 2007). Previously, Eph/ephrinsB have also been suggested to be involved in the cell positioning and development of different epithelia (i.e. intestinal, vascular, and urorectal) as well as in the coordination of migration and proliferation of epithelial stem cells (Batlle *et al.*, 2002; Dravis *et al.*, 2004; Foo *et al.*, 2006; Holmberg *et al.*, 2006). Other studies have reported no changes in the thymus of mice deficient for different Eph and ephrins, including EphA2 (Chen *et al.*, 1996), ephrinB3 (Wu and Luo, 2005) and EphB6 (Shimoyama *et al.*, 2002), although some authors reported compromised T-cell functions in the latter mice (Luo *et al.*, 2004) and over expression of EphB6 in transgenic mice results in alterations in both thymic cortex-medulla limits and splenic white pulp (Coles *et al.*, 2004). Moreover, the thymus phenotype has not been described in EphB2-deficient mice (Coles *et al.*, 2004) although ephrinB1, one of the main ligands of EphB2, has been reported to be critical for T-cell development (Yu *et al.*, 2006).

Because preliminary results (Alfaro *et al.*, 2007; Alfaro *et al.*, 2008) have demonstrated profound alterations in the maturation of thymic epithelial cells in EphB2 and/or EphB3 deficient mice, although changes in T-cell differentiation were less important, in this study we have combined morphology, electron microscopy and immunofluorescence to confirm the relevance of EphB2 and EphB3 in the maturation of thymic epithelial cell (TEC) network.

Results

Thymic epithelium was profoundly altered in adult EphB2 and/or EphB3 deficient mice

Firstly, we comparatively investigated the thymus morphology of the three studied mutants and wild type (WT), control, adult mice. Control thymus (Fig. 1A) exhibited the typical compartmentalization in a cortex fully occupied by small thymocytes (Fig. 1E) and a pale medulla mostly constituted by epithelial cells (Fig. 1G). EphB-deficient mice showed smaller thymi with profound alterations in the distribution and organization of its distinct histological compartments (Fig. 1 B,C,D). As previously demonstrated (Alfaro *et al.*, 2007; Alfaro *et al.*, 2008), mutant thymi contained reduced numbers of thymocytes in both cortex and medulla (Fig. 1 F,H) that allowed broad areas of large, pale epithelial cells to be identified (Fig. 1F insert). By electron microscopy cortical thymocytes appeared frequently isolated in “empty”

areas devoid of thymic epithelial cells (TEC) (Fig. 1J) or containing degenerated epithelial cells (Fig. 1L).

Changes in the TEC subpopulations of EphB2 and/or EphB3 deficient mice

Our immunoflourescence studies combining distinct markers specific for thymic epithelial cell subsets (keratins K5 and K8, MTS10, MTS20) were of special relevance to understand the changes occurring in the thymic epithelial stroma of EphB-deficient mice. As previously reported by other authors (Gill *et al.*, 2002; Klug *et al.*, 2002) the thymus of WT mice (Fig. 2A) consisted of a peripheral cortex constituted by $K5^-K8^+MTS10^-$ epithelial cells, perpendicularly oriented respect to the connective tissue capsule (Fig. 2E) and a central, unique medulla consisting of a heterogeneous population of epithelial cells (Fig. 2A). In the medulla, most TEC are $K5^+K8^-MTS10^+$ with a minor $K5^-K8^+MTS10^-$ cell population (Fig. 3A). There were also a few $K5^-K8^-$ areas and double stained $K5^+K8^+MTS10^-$ cells largely distributed in the cortico-medullary border (Fig. 3A). A few $K5^+K8^-MTS10^-$ and $K5^+K8^+MTS10^-$ cells also appeared throughout the cortical region (Fig. 2E; green and yellow cells, respectively).

The immunofluorescent study in EphB-deficient thymi confirmed the changes observed by light microscopy: reduced size of the organ and severe modifications in the topological distribution, organization and cell content of the two main thymic compartments, cortex and medulla (Fig. 2 B,C,D). The changes observed were different in the three studied mutants supporting that the functions of EphB2 and EphB3 receptors in the thymus are not overlapping but specific and non-redundant. Moreover, the lack of one receptor in the mutants was not compensated for by the presence of the other one. Both EphB3-deficient mice and EphB2/B3 double mutants showed one large, central unique thymic medulla (Fig. 2 C,D), but in the EphB2-deficient mice this was reduced to small medullary areas scattered throughout the thymic parenchyma, even under the connective tissue capsule (Fig. 2B). In these last mutants the cortical epithelial network appeared severely disorganized: the $K5^-K8^+$ TEC did not show the perpendicular arrangement with respect to the connective tissue capsule and their cell processes were extremely shortened (Fig. 2F). In addition, an increased expression of K5 occurred in the thymic cortex of these animals resulting in the appearance of high numbers of $K5^+K8^+$ cells and even $K5^+K8^-$ cells (Fig. 2F, yellow and green cells, respectively). In the EphB3-deficient thymi there was also a decreased K8 expression but, in general, the cortical

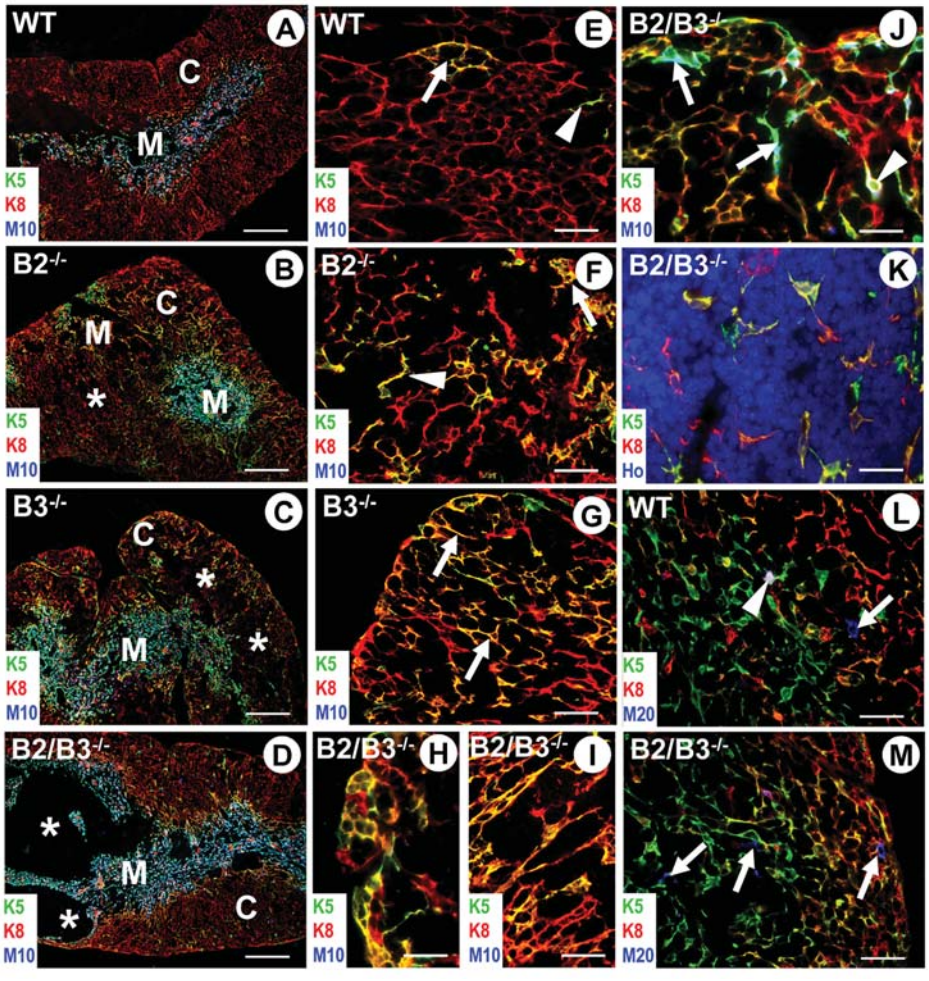


Fig. 2. Immunofluorescence study of the thymic cortex of adult WT and EphB-deficient mice. *The immunofluorescence analysis was carried out combining specific markers for TECs (keratin 5 (K5), keratin 8 (K8), MTS10 (M10) and MTS20 (M20)).* **(A-D)** *WT thymi present, as previously described by light microscopy, an outer cortex (C) and a central inner medulla (M). However, this organization was severely affected in EphB-deficient thymi that presented altered distribution and organization of these two compartments as well as important variations in their cell content, including the existence of large $K5^-K8^-$ areas (asterisks).* **(E)** *Detail of the cortical region of a WT thymus showing a majority $K5^-K8^+MTS10^-$ TEC population (red cells) and a few $K5^+K8^+MTS10^-$ (arrow) and $K5^+K8^-MTS10^-$ (arrowhead) cells.* **(F)** *Altered cortical TEC organization in an EphB2$^{-/-}$ thymus. Note the loss of the perpendicular organization of the epithelial network with respect to the WT condition (see 2E) and the shortened epithelial cell processes. In addition, these thymi presented numerous $K5^+K8^+$ (arrow) and $K5^+K8^-$ TEC (arrowhead).* **(G)** *Detail of the cortical epithelial organization of EphB3$^{-/-}$ thymi. Note the parallel columns of TEC with elongated cell processes (see the condition in WT – 2e - and EphB2$^{-/-}$ 2f – thymi) and high numbers of $K5^+K8^+$ TEC (arrow).* **(H,I)** *Cortical regions of EphB2/B3$^{-/-}$ thymi showing either shortened or elongated epithelial cell processes similarly to the situation observed in EphB2$^{-/-}$ and EphB3$^{-/-}$ thymi, respectively.* **(J)** *Both $K5^+K8^-MTS10^+$ (arrow) and $K5^+K8^+MTS10^+$ (arrowhead) medullary TEC in the cortex of an EphB2/B3$^{-/-}$ thymus.* **(K)** *Detail of a $K5^-K8^-$ area in EphB2/B3$^{-/-}$ thymus which contains numerous cells (blue) as revealed by Hoechst 33342 (H) staining.* **(L)** *Presence of both $K5^+K8^+MTS20^+$ cells (arrowhead) and $K5^-K8^-MTS20^+$ (arrow) in WT thymus.* **(M)** *Numerous $K5^-K8^-MTS20^+$, but not of $K5^+K8^+MTS20^+$, cells (arrows) in an EphB2/B3$^{-/-}$ thymus. Scale bars: (A-D) 200 μm; (E-M) 25 μm.*

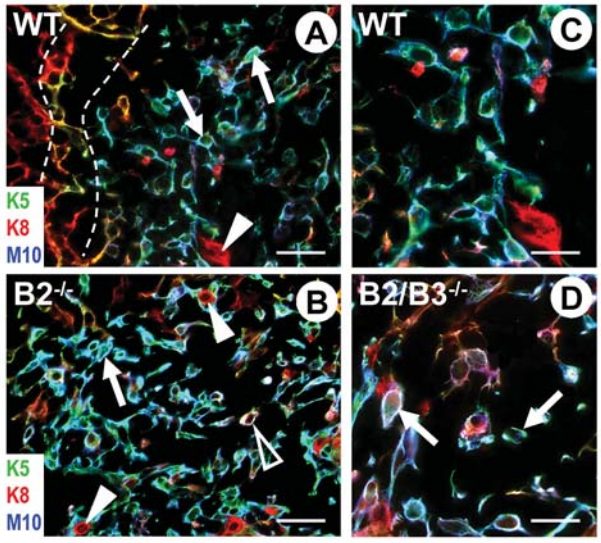


Fig. 3 (Top). Immunofluorescence study of the thymic medulla of adult WT and EphB-deficient mice. *The immunofluorescence analysis was performed by using specific reagents to identify keratin 5 (K5), keratin 8 (K8) and MTS10 (M10) as specific markers for TECs.* **(A)** *WT medulla mainly contains K5$^+$K8$^-$MTS10$^+$ cells (arrow), and a minor K5$^-$K8$^+$MTS10$^-$ cell population (arrowhead); K5$^+$K8$^+$MTS10$^-$ cells appear largely in the cortico-medullary region (limited yellow cells).* **(B)** *Like the WT thymus, EphB2$^{-/-}$ medulla showed both K5$^+$K8$^-$MTS10$^+$ cells (arrow) and K5$^-$K8$^+$MTS10$^-$ cells (arrowhead) but also a K5$^+$K8$^+$MTS10$^+$ cells (open arrowhead) population.* **(C,D)** *Detail of the morphology of medullary TEC either from WT (C) or EphB2/B3$^{-/-}$ thymi (D). Note that medullary TECs from mutant thymi exhibit shorter cell processes than those of WT ones (see 3D) (arrow). Scale bars: (A,B) 35 μm; (C,D) 15 μm.*

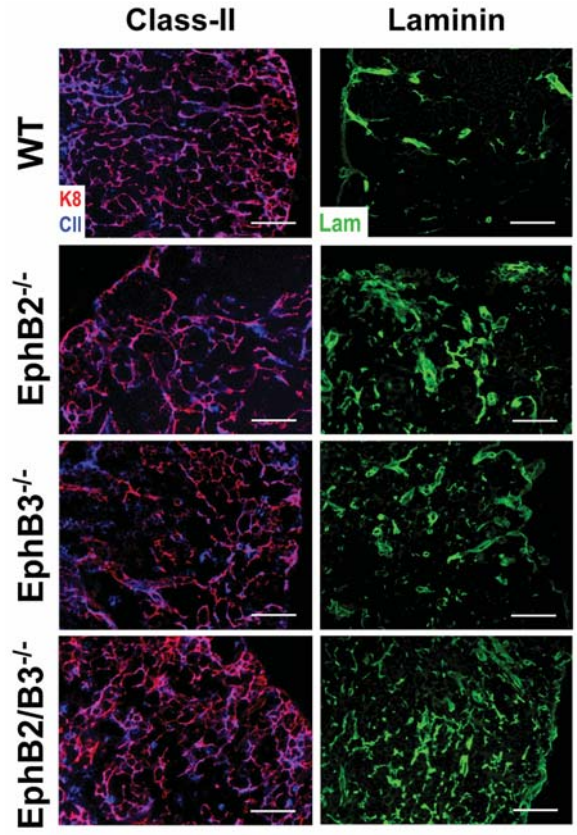


Fig. 4 (Right). MHC Class II and laminin expression in adult WT and Eph deficient thymi. *EphB-deficient mice did not show significant modifications of the MHC class II (CII) expression as compared to the WT pattern. On the other hand, increased expression of laminin (Lam) occurred in the EphB-deficient thymi. Scale bars, 50 μm.*

epithelial network appeared less disorganized than in the EphB2-deficient thymi consisting of parallel columns of TEC which showed long cell processes arranged perpendicularly to the connective tissue capsule (Fig. 2G). Furthermore, numerous K5$^+$K8$^+$ cells occurred in the cortex of these mice (Fig. 2G). The thymi of double EphB2/B3 deficient mice showed features observed in each one of the two single mutants. In some areas, these mice showed an extremely reduced (Fig. 2D) and disorganized cortex as observed in EphB2-deficient mice (Fig. 2H) whereas in the other ones the cortical TEC exhibited long cell processes as found in EphB3-deficient thymi (Fig. 2I). In the cortex of these double EphB2/B3 mutant mice as well as in the other deficient mice studied, typical medullary TEC, including K5$^+$K8$^-$MTS10$^+$ cells and K5$^+$K8$^+$MTS10$^+$ occurred (Fig. 2J).

In the three deficient mice, regions devoid of K5 and K8 expression were scattered at random throughout the thymic parenchyma in the EphB2 mutants (Fig. 2B, asterisks) and largely confined to the medulla and inner cortex in the EphB3-deficient mice (Fig. 2C). In the EphB2/B3 mutants huge K5$^-$K8$^-$ areas invaded the thymic cortex that in these areas, as mentioned above, was reduced to a few discontinuous cell layers (Fig. 2D). These were not, however, "empty regions" devoid of any kind of cells. On the contrary, they contained numerous cells, as shown by using Hoechst 33342 staining (Fig. 2K) but were totally devoid of keratins as demonstrated by their negative reaction (Data not shown) with pan-keratin reagents (ICN, GPCK) that recognize all keratins or specific stainings for other keratin-expressing cells previously described to be expressed in the thymus, such as K14 (Klug *et al.*, 1998). We could conclude, therefore, that these areas did not contain epithelial marker-expressing cells. Their existence could be related to the occurrence of numerous degenerated TEC in both thymic cortex and medulla observed by electron microscopy in the EphB-deficient thymi (Fig. 1J).

The medullary TEC population of EphB-deficient mice included principally K5$^+$K8$^-$MTS10$^+$ cells and K5$^-$K8$^+$MTS10$^-$ (Fig. 3B) cells as found in the WT thymi (Fig. 3A), but there were also K5$^+$K8$^+$MTS10$^+$ cells not described in the WT mice (Fig. 3D). Furthermore, numerous medullary TECs showed shortened and broader cell processes (Fig. 3D), compared to the WT condition (Fig. 3C), suggesting that the lack of EphB2 and/or EphB3 receptors importantly affected the organization of cell processes of both cortical and medullary epithelial cells.

The presence of numerous K5$^+$K8$^+$ cells does not correlate with an increase in MTS20$^+$ TEC progenitors

Another remarkable feature of the EphB-deficient thymi was the frequent occurrence of K5$^+$K8$^+$ cells. When the expression of MTS20, a marker for TEC progenitors (Bennett *et al.*, 2002; Gill *et al.*, 2002), was evaluated within that cell population we

found high numbers of $K5^-K8^-MTS20^+$ cells but not $K5^+K8^+MTS20^+$ cells (Fig. 2M) in the EphB-deficient thymi.

Changes in the laminin but not in the MHC class II expression occur in the EphB-deficient thymi

Finally, described alterations in the thymic epithelial network of adult EphB-deficient mice correlated well with changes in the pattern of laminin expression, an extracellular matrix protein intimately associated with TEC processes, but not with that of MHC Class II molecules that remarkably remained largely unchanged compared to their expression in WT thymi (Fig. 4).

Changes observed in EphB2 and/or EphB3-deficient thymi appear early in ontogeny

For a better understanding of the observed alterations in the adult thymus of EphB-deficient mice we analyzed the maturation of the epithelial cell component during thymus ontogeny. Our results support that the observed phenotype in EphB-deficient adult thymus is due to an early alteration of the thymic epithelium that gradually increases, rather than to a restricted condition of the adult thymus.

The first differences between WT and mutant embryonic thymi became evident at E13.5 days. The thymic primordium of E11.5 EphB-deficient mice, with the used markers, did not show significant differences in size, histological organization or topological distribution of TEC types compared to WT primordium (Data not shown). At 13.5 embryonic days, the thymic primordium was totally separated from the pharynx and the central cavity had disappeared. In both WT and EphB-deficient mice the thymus had grown but in the mutant mice less (Fig. 5 B-D) than in control animals (Fig. 5A). A non-uniform $K8^+$ network (Fig. 5E, red cells) constituted the WT parenchyma with stronger staining in the inner zone, where many cells were $K5^+K8^+MTS10^+$ (Fig. 5G), than in the outer one (Fig. 5E). In the periphery of the lobe, there were also a few $K5^+K8^-$ cells and $K5^+K8^+$ cells (Fig. 5F). In EphB2- and/or EphB3-deficient mice the thymic parenchyma was also formed by a $K8^+$ epithelial network but the centrally located groups of $K5^+K8^+$ cells were smaller, more numerous (Fig. 5 B-D) and contained few $MTS10^+$ cells (Fig. 5 J,M,P), an immature medullary TEC type (Fig. 5G). In addition, the peripheral epithelial network already appeared disorganized containing $K5^-K8^-$ areas (Fig. 5 H,K,N) and high numbers of $K5^+K8^+$ cells (Fig. 5 I,L,O). This apparent slowness of the thymic epithelium development observed in the single mutants was more severe in the thymic primordium of EphB2/B3 double mutants. In these animals, the medullary $K5^+K8^+$ cell groups were very small and contained very few $MTS10^+$ cells (Fig. 5P); although some mutants showed a single medullary $K5^+K8^-$ area containing a few $K5^+K8^+MTS10^+$ cells (Data not shown).

At 15.5 days of gestation, the phenotype of WT thymus remained similar (Fig. 6A, E) but the organ had grown notably making the differences with the EphB-deficient thymi more evident (Fig. 6 B-D). An important disorganization of the $K5^-K8^+$ epithelial network occurred largely in both $EphB2^{-/-}$ and $EphB3^{-/-}$ thymi (Fig. 6 F,G), the size of which was significantly reduced compared to WT ones (Fig. 6 B,C). In the $EphB2^{-/-}$ thymi, a loose epithelial network appeared showing numerous $K5^-K8^-$ areas (Fig. 6F). In both single mutants, numerous $K5^+K8^+$ cells and $K5^+K8^-$ cells were scattered throughout the thymic parenchyma, frequently in the subcapsulary region (Fig. 6G; yellow and green cells, respectively). A similar phenotype was found in the EphB2/B3 double mutants that showed a significant reduction in thymus size (Fig. 6D), important epithelial disorganization and presence of numerous $K5^+K8^+MTS10^-$ cells (Fig. 6H).

Thymic alterations become more severe at the end of fetal life

Both 17.5 day old fetal and newborn WT thymi (Fig. 7 A,E) already showed the main features above described for control adult thymi: a $K5^-K8^+$ cortical epithelial network perpendicularly

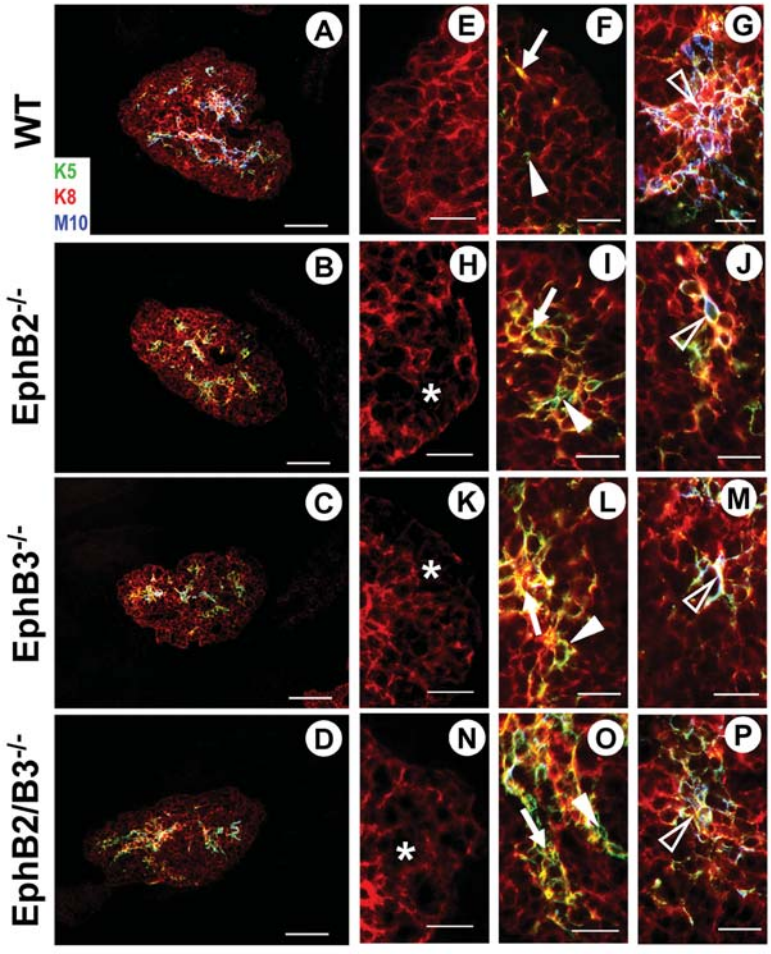
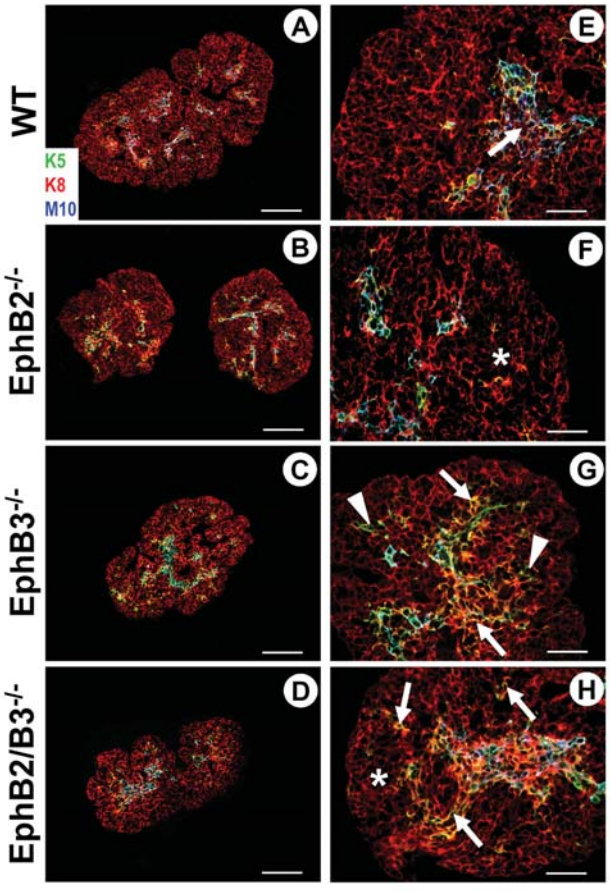


Fig. 5. Morphological and phenotypical differences between WT and EphB-deficient thymi appear at 13.5 days post-coitum. (A-D) *Notice the reduced size of E13.5 mutant thymi (B-D), as compared to that of WT one (A).* **(E-G)**. *WT thymus (E) exhibits a well-defined $K5^-K8^+MTS10^-$ cortical epithelial network (red cells) and inner $K5^+K8^+MTS10^+$ (open arrowhead) (G) medullary regions. A few $K5^+K8^+$ cells (arrow, F) and $K5^+K8^-$ cells (arrowhead, F) occur also in the cortex of WT thymi. This histological organization was severely altered in EphB-deficient thymi* **(H-P)** *that showed: small groups of $K5^+K8^+MTS10^+$ cells (open arrowhead)* **(J,M,P)**, *numerous $K5^+K8^+$ cells (arrow) and $K5^+K8^-$ cells (arrowhead, I,L,O) as well as $K5^-K8^-$ areas (asterisks)* **(H,K,N)** *which appeared frequently in the outer cortex. Scale bars: (A-D) 100 μm; (E-P) 25 μm.*

arranged to the connective tissue capsule (Data not shown), several, small, well defined, centrally located medullary $K5^+K8^-$ $MTS10^+$ cell groups and $K5^+K8^+MTS10^-$ cells in the cortico-medullary limit (Fig 7E, insert). Phenotypical differences between WT and EphB-deficient thymi began to be particularly important in these developmental stages. EphB-deficient mice showed smaller thymi than the control ones and their medullary groups, consisting of a few cells, remained scattered throughout the parenchyma (Fig. 7 B-D,F-H). In addition, some specific features were observed in the thymi of EphB2-, but not EphB3-deficient mice and vice versa. Shortened cell processes occurred in the cortical $K5^-K8^+$ TEC of EphB2-deficient mice but not in those of EphB3 mutants, although these contained numerous $K5^+K8^+$ cells in the thymic cortex (Data not shown). In the thymi of EphB2/B3 double mutants, typical features found in the adult thymi occurred also at these stages including a severe disorganization of the cortical epithelial network with large $K5^-K8^-$ areas, small cortex, shortening of the epithelial cell processes and frequent $K5^+K8^+$ cells. However, numerous, dispersed, small medullary areas still remain (Fig. 7 D,H). In summary, our results conclusively demonstrate that the phenotypes observed in the adult Eph-deficient thymi are already evident from the early stages of development and notably increased in the last fetal stages and in



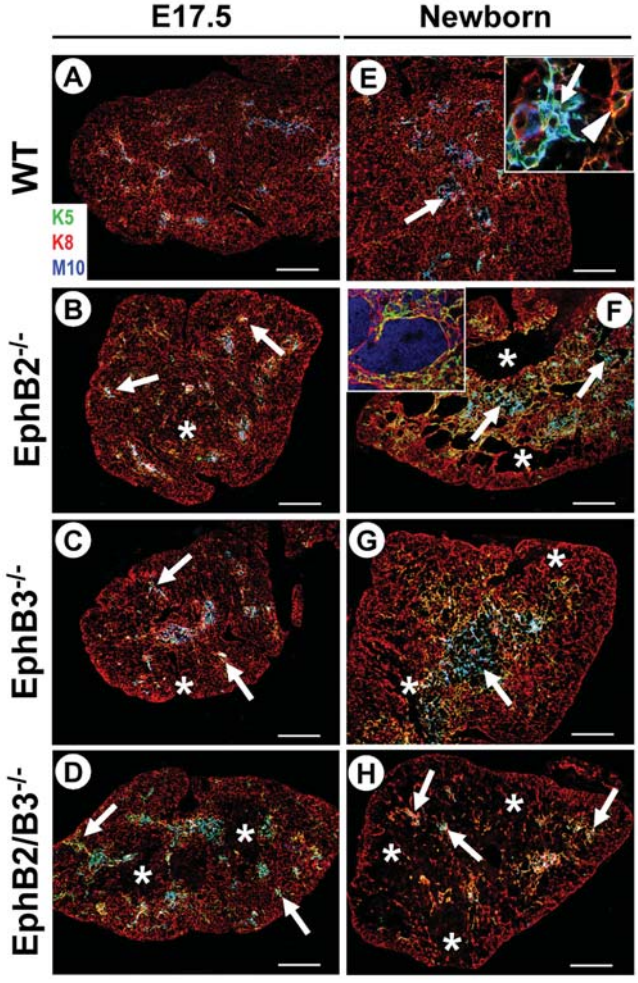


Fig. 6 (Left). Thymi from E15.5 WT and EphB-deficient mice showed important phenotypical and morphological differences. (A-D) *Notice the reduced size of EphB-deficient thymus and the increased disorganization of the thymic parenchyma, as compared to that of the WT mice.* **(E)** *Detail of the $K5^-K8^+$ cortical (red cells) and $K5^+K8^{lo}MTS10^+$ medullary area (arrow) of a WT embryonic thymus.* **(F)** *A well-organized $K5^-K8^+$ cortical epithelial network was not found in EphB2$^{-/-}$ embryonic thymi (asterisk).* **(G)** *Detail of an EphB3$^{-/-}$ embryonic thymus showing increased number of $K5^+K8^+$ cells (arrow) and $K5^+K8^-$ cells (arrowhead).* **(H)** *Increased numbers of both $K5^+K8^+$ cells (arrow) and $K5^-K8^-$ regions (asterisk) appeared in the E15.5 EphB2/B3 double mutant thymi. Scale bars: (A-D) 200 μm; (E-H) 50 μm.*

Fig. 7 (Right). In both E17.5 and newborn EphB-deficient mice the thymus phenotype became more severe. (A-D) *EphB-deficient mice present smaller thymi than the WT ones and contain scattered medullary foci (arrows) and increased numbers of $K5^+K8^+$ cells (yellow cells) and $K5^-K8^-$ regions (asterisks).* **(E)** *Thymic section of WT newborn mice. Well-defined medullary areas (arrow) were scattered throughout the cortical network (red cells). Insert shows the major medullary $K5^+K8^-MTS10^+$ TEC population (arrow) and the occurrence of $K5^+K8^+MTS10^-$ cells in the cortico-medullary limits (arrowhead).* **(F,G)** *Neonatal thymi of both EphB2$^{-/-}$ (F) and EphB3$^{-/-}$ mice (G) exhibited, as compared to the WT thymi, smaller size, numerous $K5^+K8^+$ cells (yellow) and $K5^-K8^-$ areas (asterisks). Note the presence of cells (blue) into the $K5^-K8^-$ areas, revealed by Hoeschst 33342 staining (F, insert). However, EphB2$^{-/-}$ thymi contained small, dispersed medullary foci and EphB3$^{-/-}$ ones a unique, central medulla (arrows).* **(H)** *The thymus of EphB2/B3$^{-/-}$ newborn mice was severely affected showing importantly reduced size, severe disorganization of the cortical epithelial network (red cells) that contained large $K5^-K8^-$ areas (asterisk) and numerous small medullary foci (arrow). Scale bars, 200 μm.*

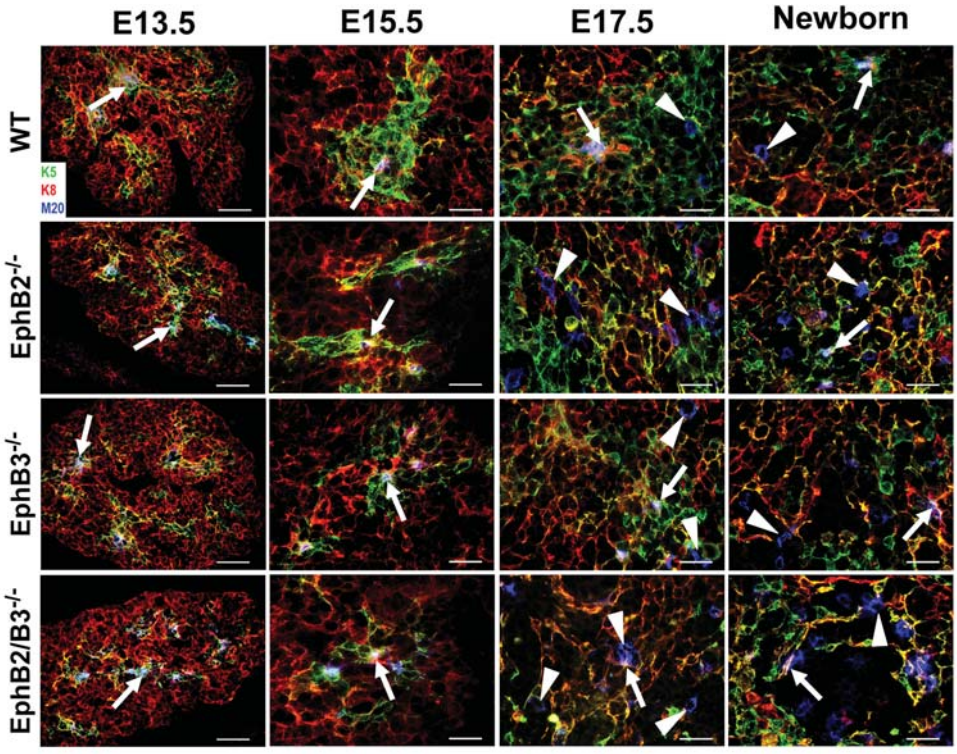


Fig. 8. Appearance and evolution of MTS20-expressing cells during thymus ontogeny. *The immunofluorescence study was carried out by using reagents to identify keratin 5 (K5), keratin 8 (K8) and MTS20 (M20). Presence of $K5^+K8^+MTS20^+$ (arrow, white cells) cells during thymus ontogeny. Note their distribution largely restricted to both medulla and cortico-medullary border. This cell population remained higher in EphB-deficient mice than in WT ones until E15.5 becoming less evident in the next stages. On the other hand, $K5^-K8^-MTS20^+$ cells appeared for the first time at E17.5 (arrowhead, blue cells) increasing in the next stages of EphB-deficient thymus, mainly in the EphB2/B3$^{-/-}$ double mutants. Scale bars: E13.5, 50 μm; E15.5, E17.5 and Newborn, 25 μm.*

the neonatal period.

On the other hand, MTS20-expressing cell precursors gradually disappeared along thymus ontogeny and were topologically restricted to medulla and cortico-medullary border in WT thymi, as previously reported by other authors (Bennett *et al.*, 2002; Gill *et al.*, 2002). A gradual reduction in $MTS20^+$ cell progenitors also occurred in the EphB-deficient mice but their numbers, at each studied stage, remained high, especially in the EphB2/B3 double mutant mice. Furthermore, these were scattered throughout the thymic parenchyma rather than restricted to the medullary limits (Fig. 8). On the other hand, $K5^-K8^-MTS20^+$ cells appeared for the first time in the 17.5 fetal day-old thymi of both WT and EphB-deficient mice. As found in adult mice, the numbers of these cells were significantly high in the mutant thymi, principally in the EphB2/B3 double deficient mice (Fig. 8).

TABLE 1

ANALYSIS OF THE CELL CYCLE AND APOPTOSIS IN THE CD45$^-$ CELL FRACTION OF EPHB-DEFICIENT MICE

	Proliferating Cells			
	WT	**EphB2$^{-/-}$**	**EphB3$^{-/-}$**	**EphB2/B3$^{-/-}$**
E15.5	13.45±1.52	11.57±1.55*	11.82±1.36*	10.80±1.44*
Adult	4.29±1.60	1.60±0.20**	2.40±0.90*	1.75±0.05**
	Apoptotic Cells			
	WT	**EphB2$^{-/-}$**	**EphB3$^{-/-}$**	**EphB2/B3$^{-/-}$**
E15.5	2.23±0.20	3.97±0.90*	4.10±0.88*	3.96±0.71*
Adult	41.5±7.70	63.18±6.69**	65.57±5.78**	58.84±6.76**

Reduction in the percentage of cycling TEC and increased numbers of apoptotic epithelial cells are observed in EphB-deficient mice at E15.5 and adult stages, data represent the mean of SD of, at least, five independent animals. The significance of a Student t test probability is indicated *, p≤ 0.05; **, p≤ 0.01.

TEC proliferation and survival are altered in EphB-deficient mice

In order to evaluate possible factors contributing to the observed small sizes and hypocellularity of EphB-deficient thymi, we analyzed both proliferating rate and the proportions of apoptotic cells within the TEC population. Both fetal (E15.5) and adult EphB-deficient thymi showed significant decreased proliferation rates as well as increased proportions of the CD45$^-$ thymic cells respect to WT values, as evaluated by flow cytometry (Fig. 9, Table 1).

Discussion

The current results demonstrate a role for EphB2 and EphB3 in the organization and maturation of the thymic epithelial network, confirming the involvement of these molecules in the biology of epithelial tissues previously pointed out by other authors (Batlle *et al.*, 2002; Dravis *et al.*, 2004; Holmberg *et al.*, 2006; Merlos-Suarez and Batlle, 2008). Although most Eph and their ligands, ephrins, are expressed in the thymus (Munoz *et al.*, 2002; Vergara-Silva *et al.*, 2002; Wu and Luo, 2005; Alfaro *et al.*, 2008), their functional role in this primary lymphoid organ was controversial. *In vitro* and *in vivo* studies by our group had demonstrated previously the effects of EphA family on T-cell differentiation (Munoz *et al.*, 2002; Munoz *et al.*, 2006) and we recently found that EphB-deficient mice show a slight blockade of T-cell maturation, largely affecting the double negative (DN) ($CD4^-CD8^-$) cell compartment (Alfaro *et al.*, 2008). However, it has also been reported that both EphB6$^{-/-}$ and EphB2$^{-/-}$ mice exhibit normal thymic cellularity (Shimoyama *et al.*, 2002; Coles *et al.*, 2004; Luo *et al.*, 2004) although the first ones have compromised T-cell function (Luo *et al.*, 2004) and EphB6 overexpression under CD2 promoter control results in important hypocellularity, increased numbers of DN cells and altered cortex/medulla ratio (Coles *et al.*, 2004).

This is, however, the first study to report profound and specific alterations in the thymic epithelial network due to the lack of EphB2 and/or EphB3 receptors. Previously, we had correlated the decreased numbers of double positive (DP) ($CD4^+CD8^+$) thymocytes observed in EphA4-deficient mice with an important collapse of the cortical epithelial network (Munoz *et al.*, 2006) and we also found that the treatment of ephrinB1-Fc fusion proteins *in*

vitro disorganize the 3D epithelial network of reaggregates established with fetal TEC and DP thymocytes (Alfaro *et al.*, 2007).

The three mutants studied exhibit some common phenotypical features, including: thymi significantly smaller than the WT ones, the presence of $K5^+K8^+MTS10^+$ immature medullary epithelial cells, high numbers of $K5^-K8^-MTS20^+$ cells and the occurrence of both cortical $K5^+K8^+$ cells and $K5^-K8^-$ areas. However, every mutant also showed specific features: for example, randomly scattered small medulla in the EphB2$^{-/-}$ thymi or long epithelial cell processes arranged in parallel columns in the thymic cortex of EphB3$^{-/-}$ mice. The lack of EphB2 is obviously not compensated for by the presence of EphB3, and vice versa, although there could be a certain degree of redundancy between the two molecules because the phenotype of the EphB2/B3 double mutants is more severe than that of the single EphB-deficient mice. It is important to remark the specificity of observed phenotypes because some authors have justified the lack of a phenotype in mice deficient in some Ephs or ephrins by the presence of other molecules of the same family, which is, obviously, not the case.

The phenotypes observed in the adult mutant thymi appear early in ontogeny, presumably from the organization of thymic anlage (E11.5), although, with the used markers, at this stage they are not evident. At E13.5, the deficient thymi already show, compared to WT ones, a smaller size and phenotypical variations, including changes in the location of medullary $K5^+K8^+$ areas and the appearance of incipient $K5^-K8^-$ regions. These early changes remain and gradually increase throughout ontogeny but the thymus phenotype of EphB-deficient mice becomes more severe at the end of fetal life and in the neonatal thymus. Thus, changes in the extension and arrangement of epithelial cell processes in both cortical and medullary TEC become more evident from 15.5 embryonic days onward. In addition, the intermediate $K5^+K8^+MTS10^+$ medullary cell population, presumably related to the lack of thymocyte-TEC interactions, appears at 13.5 days of gestation in the WT thymi, but it is scarcely represented in the EphB-deficient ones, to disappear two days later except in the thymus of EphB2/B3 double mutant mice.

The thymus size is determined, at least in part, by the numbers of TEC it contains. Flow cytometry analysis of the CD45$^-$ thymic cell fraction demonstrated a decreased proliferation rate of both fetal and adult EphB-deficient mice, suggesting that EphB receptors could be directly or indirectly regulating the proliferation of thymic epithelium. In this regard, in the intestinal crypts, EphB2 and EphB3 regulate the proliferation of epithelial cell progenitors (Holmberg *et al.*, 2006). On the other hand, thymus size has also been related with the production of different factors by the mesenchyme surrounding the organ, including IGF-1, IGF-2, FGF-7 and FGF-10 (Jenkinson *et al.*, 2003; Jenkinson *et al.*, 2006). Eph/ephrins are involved in governing the activity of receptors for these growth factors (Yokote *et al.*, 2005).

In addition, fetal and adult EphB-deficient thymi showed increased proportions of apoptotic CD45$^-$ cells. On this regard, there is conclusive evidence relating both Eph and ephrins with survival, death and proliferation of thymocytes and peripheral T lymphocytes (Luo *et al.*, 2001; Munoz *et al.*, 2002; Freywald *et al.*, 2003; Yu *et al.*, 2003a; Yu *et al.*, 2003b; Freywald *et al.*, 2006; Yu *et al.*, 2006; Alfaro *et al.*, 2007). We recently demonstrated that the thymocyte hypocellularity observed in EphB2 and/or EphB3-deficient mice correlated with increased proportions of apoptotic cells, largely both DN and DP cells, and decreased proportions of cycling DN cells (Alfaro *et al.*, 2008). Accordingly, changes in the proportions of apoptotic cells and proliferating cells affecting both thymocytes and TEC account for the reduced size observed in the adult and fetal thymus of EphB-deficient mice.

Apart from their small size, profound alterations in the cell content, histological organization and topological distribution of epithelial cell types characterize the thymus gland of embryonic and adult EphB-deficient mice. Other reports have described similar phenotypes in mice with defects in different molecules, known to be involved in the maturation of TEC. Adult Foxn1$^{\Delta/\Delta}$ mice that express Foxn1, a transcription factor required for TEC development (Blackburn *et al.*, 1996; Anderson *et al.*, 2006), deprived of its N-terminal domain, show a similar, even more severe phenotype (Su *et al.*, 2003). Importantly, several studies relate EphB2 and EphB3 with the Forkhead-box (Fox) family of transcription factors. In the intestine, a direct relationship has been demonstrated between the expression of EphB2 and EphB3 and regulation of the β-catenin/TCF-4 complex, a key component of the Wnt signalling pathway, by Foxl1,

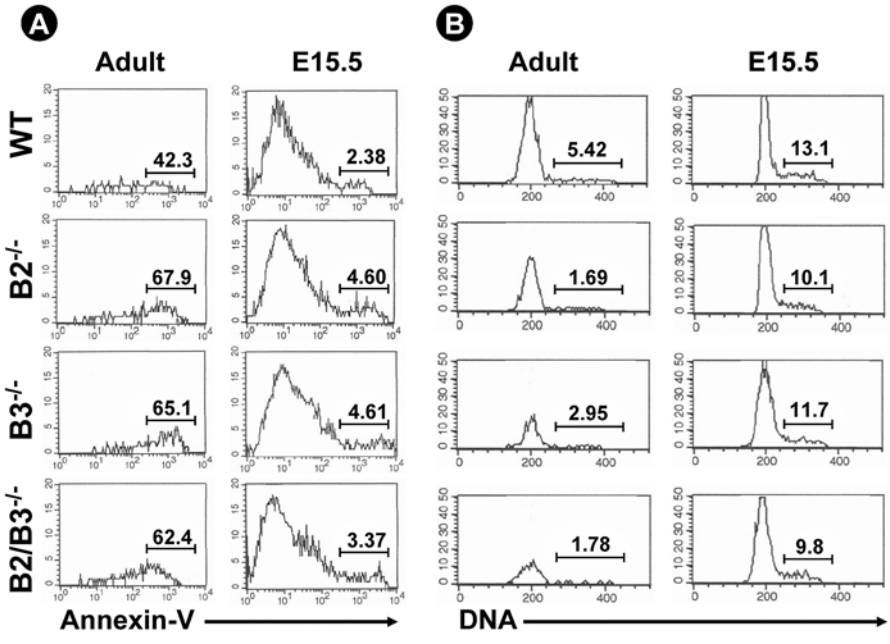


Fig. 9. Adult and E15.5 EphB-deficient mice showed increased proportions of apoptotic TEC (CD45$^-$ cell fraction) and reduced numbers of cycling TEC. (A) *Annexin-V staining demonstrated increased proportions of apoptotic cells in both adult and E15.5 mutant TEC. Dead cells were excluded from the analysis by PI staining and gating on PI-negative cells.* **(B)** *The cell cycle of the CD45$^-$ thymic fraction was analyzed by DNA staining. The percentage of cells in S-G2-M phases is indicated. A reduction of cycling cells in both adult and E15.5 mutant TEC is observed. The example corresponds to one representative result.*

another member of the Fox family (Takano-Maruyama *et al.*, 2006). On the other hand, the lack of Kremen 1 (Krm1), which negatively regulates the canonical pathway of Wnt signalling, results in profound alterations in the thymic epithelium, including cortical areas that do not express epithelial cell markers, increased number of $K5^+K8^+$ cells, decreased numbers of mature TEC, etc.. (Osada *et al.*, 2006). Nevertheless, $Krm1^{-/-}$ mice did not show a decreased size or cellularity and are capable of a normal T-cell maturation (Osada *et al.*, 2006). Furthermore, as found in EphB-deficient mice, the early appearance of a phenotype affecting the thymic epithelium has also been reported in both $Foxn1^{\Delta/\Delta}$ mice (Su *et al.,* 2003) and $Krm1^{-/-}$ mice (Osada *et al.*, 2006). Together, these results suggest some coordination in the roles of Wnt, Foxn1 and EphB in the regulation of both development and organization of thymic epithelium. Another transcription factor, Stat3, involved in control of the TEC network, is also related to Eph molecules. EphA4, EphB2 and EphB3 are able to phosphorylate and activate Stat3 (Lai *et al.*, 2004; Yuan *et al.*, 2004) and conditioned mice that do not express Stat3 in cells expressing the keratin 5 gene promoter, exhibit hypoplastic thymi with important alterations in the epithelial phenotype (Sano *et al.*, 2001).

On the other hand, EphB-deficient mice apparently show a delayed or slow maturation of $K5^+K8^+MTS20^+$ epithelial progenitor cells that results in the occurrence of a high number of these cells at E13.5, and even, E15.5 mutant thymi, as well as in the late appearance of $K5^+K8^-MTS10^+$ mature medullary TEC. Both findings have also been observed in $Foxn1^{\Delta/\Delta}$ mice (Su *et al.*, 2003). Altered location and organization of medullary areas also appear early in the ontogeny of EphB-deficient thymus and, although other factors could be implied, it is obvious that altered migration of TEC progenitors to the adequate thymic areas, a process governed by EphB2 and EphB3 in other tissues (Zou *et al.*, 1999; Miao *et al.*, 2005), is involved. Presumably, these early stages of TEC maturation are independent of the occurrence of thymocyte-TEC interactions, established beyond the E12.5 stage, when lymphoid progenitors colonize the thymus primordium. Thus, mice with severe early blockade of thymocyte differentiation (Anderson *et al.*, 2006) are able to organize a thymic primordium. The subsequent development of thymic cell populations, thymocytes and TEC is, however, closely linked.

Thymic epithelial cells from EphB-deficient mice also show changes that contribute to the histological disorganization of a three-dimensional thymic epithelial network. These alterations affect both medullary and cortical epithelial cells that showed two different patterns: shortened cell processes in the $EphB2^{-/-}$ thymi and disappearance of the lateral cell processes that joined the cortical epithelial columns in the EphB3-deficient thymi. These results support a specific and distinct regulation of the emission and disposition of thymic epithelial cell processes by EphB2 and EphB3. Both receptors have been suggested to regulate the morphology of neuronal dendrite spines (Henkemeyer *et al.*, 2003) as well as the integrin-mediated cell interactions with the extracellular matrix components (Zou *et al.*, 1999; Miao *et al.*, 2005). Furthermore, thymocyte-TEC interactions, known to be important for normal maturation of the thymic epithelial network are also affected by Eph/ephrins. We demonstrated that the supply of ephrinB1-Fc fusion proteins to cell reaggregates formed by WT TEC and DP thymocytes resulted in the retraction of epithelial cell processes (Alfaro *et al.*, 2007) and bone marrow lymphoid progenitors from EphB2 and EphB3 deficient mice *in vivo* injected to SCID mice induced altered maturation of the TEC network (D. Alfaro, unpublished). On the other hand, increased numbers of $K5^+K8^+MTS10^+$ cells observed in EphB-deficient mice could reflect altered thymocyte-TEC interactions. $K5^+K8^+MTS10^+$ cells are generated during thymus organogenesis before lymphoid colonization, maturing once the thymocyte-TEC interactions have been established (Klug *et al.*, 2002). In the mutant thymi, the alterations of these interactions as a consequence of the lack of Eph/ephrin signalling could result in delayed maturation and high numbers of that TEC subpopulation.

The occurrence of areas devoid of TEC markers, another remarkable feature of EphB-deficient thymi, has been observed in the thymus of $Krm1^{-/-}$ mice (Osada *et al.*, 2006). Although these areas correlate well with both the ultrastructural evidence of degenerated cells and the increased numbers of apoptotic TEC found in the EphB-deficient mice, we can not rule out the possibility that they are a consequence of downregulated K8 expression in the cortical $K5^-K8^+$ cells, rather than the result of a massive epithelial degeneration. On the other hand, Eph and ephrins are key molecules for determining the cell positioning that establishes tissue limits or domains (Durbin *et al.*, 1998; Mellitzer *et al.*, 1999) in which the intermingling of different cell types is rigorously impeded. Accordingly, the altered Eph/ephrin signalling that occurs in EphB-deficient thymi could result in the mutual exclusion of thymocytes and TEC in some thymic areas.

Increased numbers of $K5^+K8^+$ cells did not correlate with high numbers of $MTS20^+$ progenitor cells, but instead was more related to $K5^+K8^+MTS20^-$ cells, the capacity of which to produce a mature thymic epithelial stroma is a matter of discussion (Bennett *et al.*, 2002; Gill *et al.*, 2002). These findings support a relationship between high numbers of $K5^+K8^+$ cells in EphB-deficient mice and altered regulation of K5 expression in TEC rather than with changes in the biology of epithelial progenitor cells. In this respect, the increased numbers of $K5^-K8^-MTS20^+$ cells, a thymic cell population never reported before, found in the mutant thymi could be a consequence of downregulated expression of both K5 and K8 in the $MTS20^+$ epithelial progenitor population.

On the other hand, high numbers of $K5^+K8^+$ cells have also been reported in other mice with defective maturation of thymic epithelium, such as $Foxn1^{\Delta/\Delta}$ (Su *et al.*, 2003), conditioned Stat3 deficient mice (Sano *et al.*, 2001) and $Krm1^{-/-}$ mice (Osada *et al.*, 2006). As mentioned above, both Foxn1 and Wnt pathways modulate the expression of both EphB2 and EphB3, and these Ephs and others are concerned with Stat3 activation (Lai *et al.*, 2004), which indirectly affects activity of the transcription factor AP-1 (Galang *et al.*, 1996), a regulator of the keratin 5 gene promoter (Rossi *et al.*, 1998). A similar situation could be occurring in the EphB-deficient TEC, resulting in altered regulation of keratin genes. It is also possible that altered cell-to-cell communication between thymocytes and TEC, which occurs in EphB-deficient mice could affect the thymic epithelial phenotype. We have demonstrated that *in vitro* Eph/ephrinB-Fc treatment decreases the formation of cell conjugates by DP ($CD4^+CD8^+$) thymocytes and TEC (Alfaro *et al.*, 2007). Moreover, in the adult thymus of both hCD3εtg26 and $Rag2^{-/-}\gamma c^{-/-}$ mice, that shows an important blockade of T-cell differentiation, there are also increased numbers of $K5^+K8^+$ TEC (Klug *et al.*, 2002). Thus, apart

from the gradual accumulation of early defects, a correct cell-to-cell communication between thymocytes and TEC could be the key to establishing the definitive phenotype in the adult thymus of EphB-deficient mice.

In conclusion, our results emphasize the importance of Eph/ephrinB in establishing the relationships that link the histological organization, topological distribution and phenotypical differentiation of thymic epithelial cell progenitors, an area that is still largely unknown.

Materials and Methods

Mice

EphB2 and/or EphB3 deficient mice (*Mus musculus,* L) in a CD1 background were provided by Dr. Mark Henkemeyer (University of Texas, Southwestern Medical Center at Dallas, Dallas, Texas). All animals were bred and maintained under pathogen-free conditions in the facilities of the Complutense University of Madrid. The day of vaginal plug detection was designated as day 0.5. Wild type (WT) and mutant descendents from heterozygous parents were used for analysis in all cases. At least five animals of each experimental group (WT and mutants) were used in each analyzed developmental stage. In the earliest developmental stages the thymic primordia were totally sectionated and all sections stained and analyzed. At least 20 sections covering all thymic area were studied in the E17.5, neonatal and adult WT and deficient mice.

Immunofluorescence studies

For immunofluorescence, thymic cryosections from either WT, EphB2$^{-/-}$, EphB3$^{-/-}$ and EphB2/B3$^{-/-}$ mice sacrificed at different developmental stages: E11.5 (12 μm thickness), E13.5, E15.5, E17.5, newborn and adult (8 weeks) (6 μm thickness), were fixed in acetone for 10 minutes and air dried. Cryosections were stained with either anti-keratin 5 (Covance, Berkeley, CA, USA), anti-keratin 8 (Troma-1, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-mouse thymic stroma 10 (MTS10) and anti-mouse thymic stroma 20 (MTS20) (Kindly gifted by Richard Boyd, Monash University, Melbourne, Australia), anti-laminin (Sigma Aldrich, Saint Louis, Missouri, USA) and biotin-conjugated anti-Class II (BD Biosciences, Erembodegem Belgium) monoclonal antibodies. Primary antibodies were incubated during 1 hour at room temperature. After washing, they were detected using anti-rabbit IgG Alexa-fluor 488, anti-rat IgG AMCA and anti-rat IgM Texas Red antibodies (Molecular Probes, Invitrogen, Eugene, Oregon, USA) incubated during 45 minutes at room temperature. Sections were, then, washed in cold PBS 1x three times during 5 minutes. Cell nuclei were detected by staining with Hoechst 33342 (0.2 μg/ml) (Molecular Probes, Invitrogen, Eugene, Oregon, USA) during 5 minutes. Finally, sections were mounted with antifade Prolong Gold (Molecular Probes, Invitrogen, Eugene, Oregon, USA). All sections were analyzed using a Zeiss Axioplan microscope, photographed with a Spot 2 digital camera and analyzed using Metamorph software (MDS Inc., Toronto, Canada) at the Microscopy and Cytometry Centre (Complutense University, Madrid, Spain).

Light and electron microscopy

For electron microscopy, thymi isolated from WT and EphB-deficient mice of the mentioned developmental stages were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate at 4°C for 3–5 hours and embedded in Durcopan resine (Fluka AG, Buchs, Switzerland) as previously described (Willett *et al.*, 1999). For light microscopy analysis, semithin sections were stained with toluidine blue during 5 minutes, dehydrated in 70%-100% ethanol (5 minutes) (Panreac, Barcelona, Spain) and in xylene baths (5 minutes). Sections were mounted in DPEX (Panreac, Barcelona, Spain). Same sections were also used to identify the most interesting areas for further ultrastructural analysis. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined in a JEOL 10.10 electron microscope at the Microscopy and Cytometry Centre of Complutense University (Madrid, Spain).

Cell cycle and cell death analysis

Adult and E15.5 TEC were obtained as previously described (Gray *et al.*, 2002). For cell cycle analysis, after staining with anti-CD45 (BD Biosciences, Erembodegem, Belgium), cells were fixed in Cellfix (BD Biosciences, Erembodegem, Belgium) overnight and stained with Hoechst 33342 (Molecular Probes, Invitrogen, Eugene, Oregon, USA) in EtOH 30% in PBS 1% BSA for 30 minutes at room temperature. Cells were analyzed in a BD LSR (BD Biosciences, San Jose, CA, USA) and CellQuest software at the Microscopy and Cytometry Center (Complutense University, Madrid, Spain). For cell death analysis, CD45^{-} cell suspensions were stained with Annexin-V-FITC (Roche Diagnostics, Penzberg, Germany) in HEPES buffer 1% FCS for 20 mininutes at 4°C. After stained cell suspension were washed in HEPES buffer 1% FCS and resuspended for analysis. To discard complete cell death to apoptotic cells, a couple minutes before analyze cell suspension were stained with propidium iodide (PI). Considering apoptotic cells Annexin-V^{+}/PI^{-}. Cells were analyzed in a FACSCalibur (BD Biosciences, San Jose, CA, USA) and CellQuest software at the Centro de Microscopia y Citometria (Complutense University, Madrid, Spain). At least 20,000 cells/sample were analyzed and non- viable cells were excluded by forward-side scatter in all cases.

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