

# Thymic Medullary Epithelial Cell Differentiation, Thymocyte Emigration, and the Control of Autoimmunity Require Lympho–Epithelial Cross Talk via LT $\beta$ R

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## Abstract

Thymocytes depend on the interaction with thymic epithelial cells for the generation of a diverse, nonautoreactive T cell repertoire. In turn, thymic epithelial cells acquire their three-dimensional cellular organization via instructive signals from developing thymocytes. The nature of these signals has been elusive so far. We show that thymocytes and medullary epithelial cells (MECs) communicate via the lymphotoxin  $\beta$  receptor (LT $\beta$ R) signaling axis. Normal differentiation of thymic MECs requires LT $\beta$ R ligand on thymocytes and LT $\beta$ R together with nuclear factor- $\kappa$ B-inducing kinase (Nik) in thymic epithelial cells. Impaired lympho–epithelial cross talk in the absence of the LT $\beta$ R causes aberrant differentiation and reduced numbers of thymic MECs, leads to the retention of mature T lymphocytes, and is associated with autoimmune phenomena, suggesting an unexpected role for LT $\beta$ R signaling in central tolerance induction.

Key words: thymus • thymopoiesis • development • epithelial cells • selection

## Introduction

The thymus is the primary lymphoid organ that generates a diverse, nonautoreactive T cell repertoire. Maturation of T lymphocytes is accompanied by a highly ordered migration pattern in the thymus (1, 2). During this movement, developing T lymphocytes constantly interact with a dense network of epithelial reticular cells. Thymic epithelial cells are organized in a three-dimensional network rather than as a sheet of cells on a basement membrane (3). The interaction between T cell receptors on immature thymocytes and peptide–MHC complexes expressed by stromal cells ultimately determines the fate of developing T cells. Specifically, epithelial cells in the cortex mediate positive selection whereas bone marrow–derived dendritic cells and medullary epithelial cells (MECs) mediate negative selection of developing thymocytes (4). Recent work has demonstrated an important role for MECs in the prevention of autoimmunity (5–8). MECs express tissue-specific antigens such as thyroglobulin, insulin, renin, proteolipid protein, and myelin oligodendrocyte glycoprotein in a promiscuous fashion presumably to present the entire immunologi-

cal self in the thymus for repertoire selection. Mutations in the *aire* gene, so far the only known regulator of promiscuous gene expression in MECs, lead to the autoimmune polyendocrine syndrome type I in man and to the lack of a subset of promiscuous transcripts with organ-specific autoimmunity in mice (8). Thus, MECs may play a crucial role in the control of autoimmunity by the promiscuous expression of tissue-specific antigens that are thought to negatively select autoreactive T cell clones and possibly to promote the generation of regulatory T cells. How MECs develop and acquire their specific differentiation in three-dimensional space to achieve this control of autoimmunity is poorly understood. The aforementioned interaction between lymphocytes and epithelial cells is reciprocal and, thus, also determines the fate of thymic epithelial cells. In the absence of mature thymocytes (e.g., in SCID or RAG-deficient mice), the thymus has a markedly underdeveloped medulla, which is restored to wild-type configuration after transfer of wild-type bone marrow (9, 10). However, the molecular basis for lympho–epithelial cross talk that governs the differentiation of epithelial cells in the thymus is unknown.

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*Abbreviations used in this paper:* BrdU, 5-bromo-2'-deoxyuridine; LT $\beta$ R, lymphotoxin  $\beta$  receptor; MEC, medullary epithelial cell; Nik, nuclear factor- $\kappa$ B-inducing kinase; RTE, recent thymic emigrant; UEA-1, ulex europaeus agglutinin 1.

The large family of TNF receptors is involved in such diverse biological processes as inflammation, apoptosis, autoimmunity, and lymphoid organogenesis (11). During organogenesis of peripheral lymphoid organs, the lymphotoxin  $\beta$  receptor (LT $\beta$ R) is absolutely required for the development of Peyer's patches, peripheral lymph nodes, and gut-associated lymphoid tissues (i.e., all peripheral lymphoid organs apart from the spleen; reference 12). In addition, LT $\beta$ R, which is expressed in stromal cells but not T and B cells, is required for the organization of microenvironments within the spleen as well as the germinal center reaction (13). Two ligands have been described previously for LT $\beta$ R, namely LT $\alpha$ 1 $\beta$ 2 and LIGHT (14, 15). LT $\alpha$ 1 $\beta$ 2 consists of one LT $\alpha$  and two LT $\beta$  subunits and is crucial for lymphoid organogenesis. The absence of either subunit, LT $\alpha$  or LT $\beta$ , leads to the lack of most peripheral lymph nodes and a disturbed splenic architecture in mutant mice (16–18). Lack of the recently identified homotrimeric LT $\beta$ R ligand LIGHT does not impair lymphoid organogenesis (19). However, on an LT $\beta$ -deficient background (i.e., in LT $\beta$ <sup>-/-</sup> LIGHT<sup>-/-</sup> mice), the absence of LIGHT enhances the phenotype in that most LT $\beta$ <sup>-/-</sup> LIGHT<sup>-/-</sup> mice now lack mesenteric lymph nodes, which are present in all LT $\beta$ <sup>-/-</sup> LIGHT<sup>+/-</sup> mice (19). Concerning the signal transduction of LT $\beta$ R, a spontaneously occurring point mutation in the nuclear factor- $\kappa$ B-inducing kinase (Nik), a MAP3K-related kinase, has been shown to specifically affect LT $\beta$ R but not TNF receptor-mediated NF- $\kappa$ B activation (20). This point mutation in the *Nik* gene is the defect in *aly/aly* mice that lacks Peyer's patches and lymph nodes and shows a disturbed splenic and thymic microarchitecture (20, 21). Although the exact role of the *Nik* point mutation in *aly/aly* mice is unclear, complete removal of *Nik* leads to a strikingly similar phenotype (22). Despite the obvious similarities between LT $\beta$ R<sup>-/-</sup> and *aly/aly* mice, the *aly/aly* phenotype appears to be more complex than can be explained by disturbed LT $\beta$ R signaling alone. It has been shown for example that *aly/aly* lymphocytes, i.e., cells which do not express LT $\beta$ R, are unable to home to the gut-associated lymphoid tissues (23).

In this paper, we characterize the role of the LT $\beta$ R signaling pathway for lympho-epithelial cross talk in the thymic medulla. Mice deficient for LT $\beta$ R ligand, LT $\beta$ R itself, and mice carrying a point mutation in the signaling component *Nik* all show abnormally differentiated MECs. LT $\beta$ R signaling is critically required for proper differentiation and normal steady-state numbers of MECs. Functionally, the absence of LT $\beta$ R signaling leads to the retention of mature T cells in the thymic medulla and is associated with autoantibody production, which may be a result of incomplete central tolerance induction by MECs.

## Materials and Methods

**Mice.** C.B-17 scid/scid (SCID), RAG2<sup>-/-</sup>, Balb/c, C57BL/6, C57BL/6-Ly5.1, LT $\beta$ R<sup>-/-</sup>, and *aly/aly* mice were kept under specific pathogen-free conditions in the mouse facility of the Max

Planck Institute for Immunobiology. LT $\beta$ R<sup>-/-</sup> mice had been backcrossed to C57/BL6 at least six times and were genotyped as described previously (12). *aly/aly* mice had been backcrossed to Balb/c and were genotyped by PCR plus sequencing, using the primers 5'-TCGAAGGCCTCTCAGAGCTCACG-3' and 5'-CTCTGTTCTGGGAACCCTTGG-3'. LT $\beta$ <sup>-/-</sup>, LIGHT<sup>+/-</sup>, and LT $\beta$ <sup>-/-</sup> LIGHT<sup>-/-</sup> mice were generated and genotyped as described previously (19). Unless stated otherwise, age-matched 4–8-wk-old mice were used for experiments.

**Immunohistochemistry.** 5- $\mu$ m frozen sections were fixed in a mixture of 75% acetone and 25% methanol for 15 min at 4°C, incubated with PBS/1% H<sub>2</sub>O<sub>2</sub> at room temperature for 15 min, washed, blocked with PBS/0.5% BSA, and incubated with the respective antibody solution overnight. The following antibodies and reagents were used: ulex europaeus agglutinin 1 (UEA-1; Vector Laboratories), Troma-1 (anti-cytokeratin 8, a gift by R. Kemler [Max Planck Institute for Immunobiology, Freiburg, Germany]), MTS10 (BD Biosciences), polyclonal rabbit antiserum to I-O (K535, a gift by C. Surh [Scripps Research Institute, La Jolla, CA]), F4/80 (Serotec), MIDC-8 (Serotec), and CD45.1 (BD Biosciences). Staining was developed directly or after incubation with an appropriate biotinylated second step antibody using the StreptABCComplex/HRP according to the manufacturer's recommendations (DakoCytomation). For the detection of autoantibodies, frozen sections from RAG2<sup>-/-</sup> mice were processed as described earlier in this paragraph, sera were diluted 1:40, and an anti-mouse Ig-FITC conjugate (Southern Biotechnology Associates, Inc.) was used for visualization on a TCS SP2 confocal microscope system (Leica). Sera from MRL.lpr/lpr mice were used as a positive control.

**Bone Marrow Chimeras.** Mice before and after transfer of bone marrow cells were maintained in individually ventilated cages containing sterilized food and water. SCID and RAG2<sup>-/-</sup> mice were irradiated sublethally with 350 rad, LT $\beta$ R<sup>-/-</sup> and *aly/aly* mice were irradiated sublethally with 450 rad, and irradiated mice were injected i.v. 24 h later with 2 × 10<sup>6</sup> erythrocyte-lysed bone marrow cells of the appropriate genetic background. Thymi of bone marrow chimeras were analyzed 6–8 wk after bone marrow transfer, and engraftment was confirmed either by the detection of mature CD4 and CD8 single-positive thymocytes by FACS<sup>®</sup> or by the detection of thymocytes positive for CD45.1 (Ly5.1) by FACS<sup>®</sup> or immunohistochemistry.

**LT $\beta$ R-Fc Treatment of Mice.** C57BL/6 mice, two in each group, received weekly intraperitoneal injections of 100  $\mu$ g LT $\beta$ R-Fc or 100  $\mu$ g human polyclonal IgG preparation as control (Sandoglobulin; Novartis), both a gift by J. Browning (Biogen, Cambridge, MA). Thymi of injected mice were harvested after 3 and 6 wk, respectively.

**Quantification of UEA-1-Positive Medullary Thymic Epithelial Cells.** To measure the surface area of thymic epithelial cells, UEA-1-stained frozen sections were prepared from littermates with different genotypes on one and the same slide. High resolution pictures were obtained with a CCD camera mounted on an Axioplan2 microscope (original magnification 20; Carl Zeiss MicroImaging, Inc.). From these pictures, medullary areas of 6.25 × 10<sup>-8</sup> m<sup>2</sup> were extracted, and the UEA-1-stained brown pixels were counted using the public domain National Institutes of Health (NIH) Image program (written by W. Rasband at NIH, Bethesda, MD). To quantify the number of nuclei of medullary thymic epithelial cells in wild-type versus LT $\beta$ R<sup>-/-</sup> mice, high magnification images were obtained using the described setup, and nuclei were counted by eye. Nuclei of medullary thymic epithelial cells were defined as completely surrounded by UEA-1

staining, large, and weakly hematoxylin-positive when compared with nuclei of thymocytes. Quantification of UEA-1-positive epithelial cells by FACS<sup>®</sup> was done by digesting thymic stroma after mechanically removing free thymocytes in 5 ml RPMI 1640 containing 2% FCS, 20 mM Hepes, 0.4 mg/ml collagenase/dispase (Roche), and 25  $\mu$ g/ml DNase I (Roche) at 37°C for 15 min. Cells in the supernatant were collected, and digestion was repeated two more times. A representative sample from each thymus of four  $LT\beta R^{-/-}$  and four wild-type mice was analyzed, and the total number of CD45-negative CDR1-negative cells expressing high levels of UEA-1 per thymus was calculated. This staining protocol was adopted because cortical, CDR1-positive epithelial cells, and other nonepithelial cells have been shown to express low levels of UEA-1 (24).

**FACS<sup>®</sup> Analysis and Sorting.** FACS<sup>®</sup> analyses were performed on freshly prepared, erythrocyte-lysed single cell suspensions of thymocytes in PBS/0.3% BSA/5 mM EDTA. Stainings were analyzed on a FACSCalibur<sup>™</sup> machine (BD Biosciences) using the following: FITC-, PE-, allophycocyanin- or biotin-conjugated monoclonal antibodies purchased from BD Biosciences (clone name given in parenthesis): CD4 (GK1.5), CD8 (53-6.7), CD3 $\epsilon$  (145-2C11), TCR- $\beta$  (H57-597), CD62L (MEL-14), CD69 (H1.2F3), CD45 (30-F11), Ly5.1 (A20), B7.1 (16-10A1), and CD45 (30F11). The monoclonal antibody G8.8 (a gift by B. Kyewski [German Cancer Research Center, Heidelberg, Germany]) was conjugated to Alexa Fluor 647 according to the manufacturer's recommendations (Molecular Probes). The unlabeled monoclonal antibodies CDR1 (a gift by B. Kyewski) and M293 (anti-integrin  $\beta$ 7; BD Biosciences) were visualized with a FITC-labeled goat anti-rat antiserum. The biotin label was visualized with streptavidin-Red670 (GIBCO BRL). For stainings with  $LT\beta R$ -Fc, a modified protocol was used to avoid background staining; stainings were performed in PBS/2% normal mouse serum. Thymocytes were preincubated with unlabeled monoclonal antibody to CD16/CD32 (2.4G2; provided by M. Lamers [Max Planck Institute for Immunobiology, Freiburg, Germany]) at 1  $\mu$ g/10<sup>6</sup> cells for 10 min on ice, washed, and incubated with  $LT\beta R$ -Fc at 1  $\mu$ g/10<sup>6</sup> cells for at least 45 min on ice.  $LT\beta R$ -Fc staining was visualized using a PE-conjugated donkey anti-human IgG F(ab')<sub>2</sub> fragment (Jackson ImmunoResearch Laboratories). Sorting of MECs for RT-PCR was done largely as described previously (5). In brief, thymi of five mice (4-8-wk-old) were digested by three rounds of collagenase/dispase followed by two rounds of trypsin to free epithelial cells. Cells in suspension were labeled and sorted on a high-speed FACS<sup>®</sup> (MoFlo; Dako-Cytomation) to a purity >95%. RT-PCR was performed on RNA equivalent to 30,000 MECs as described previously (25) using primers reported previously (6).

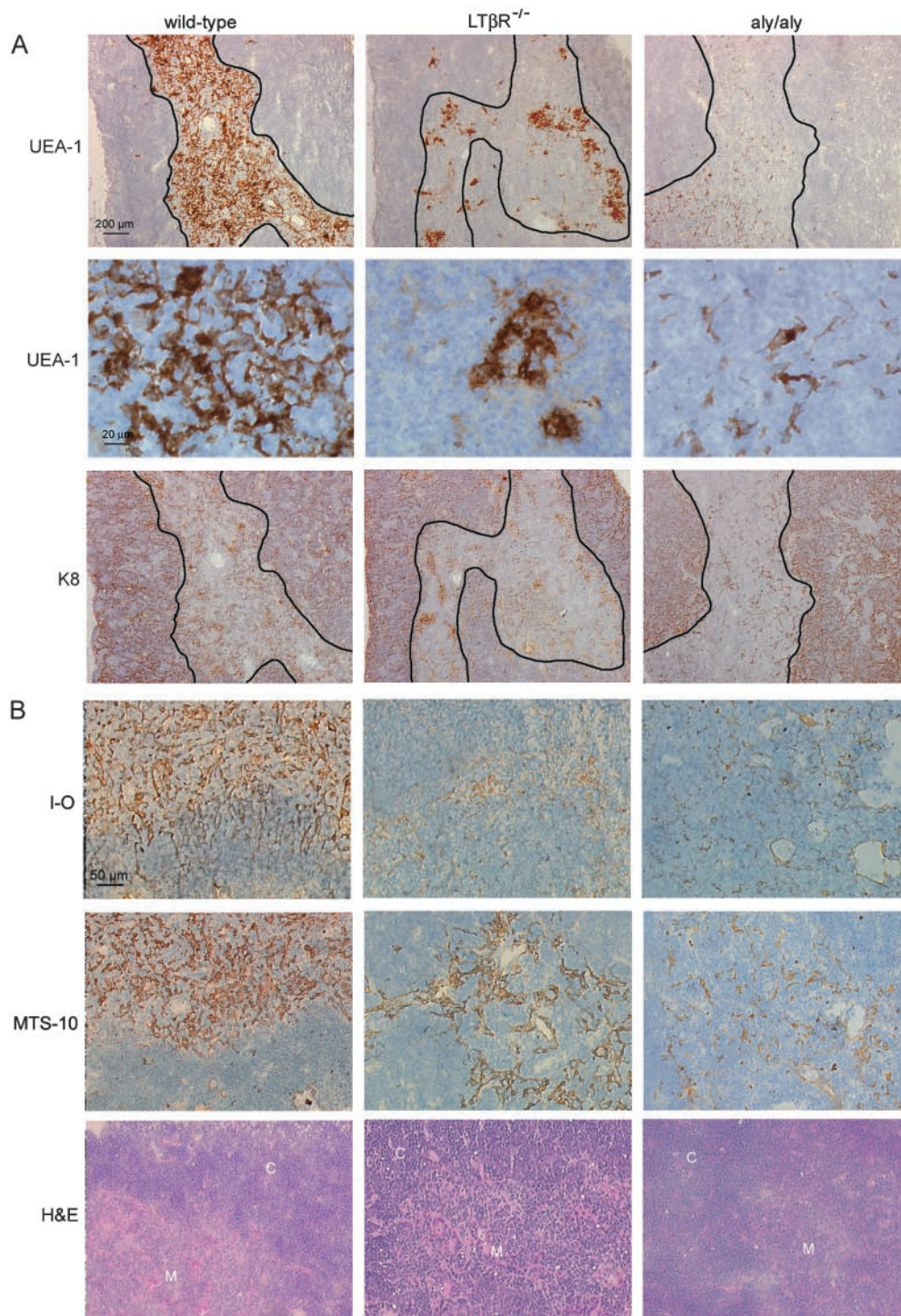
**In Vivo BrdU Labeling and Analysis of Thymic Reentry.** For 5-bromo-2'-deoxyuridine (BrdU) labeling, 12-wk-old  $LT\beta R^{-/-}$  and wild-type control mice were injected with 0.6 mg BrdU in PBS i.p. on day 0 and kept on drinking water containing 1 mg/ml BrdU. Thymocytes were sorted by FACS<sup>®</sup> on day 3, and each population was stained separately as described previously (26). For the detection of reentry of peripheral lymphocytes to the thymus,  $3 \times 10^7$  lymphocytes isolated from lymph nodes and spleen of C57BL/6-Ly5.1 mice were i.v. injected into  $LT\beta R^{-/-}$  and wild-type control mice. Thymocytes of these mice were analyzed 16 h later for Ly5.1 expression.

**Online Supplemental Material.** Immunohistochemistry reveals the presence of dendritic cells and macrophages in the thymic medulla of  $LT\beta R^{-/-}$  and aly/aly mice as shown on Fig. S1. The de-

fect causing abnormal differentiation of MECs in aly/aly mice lies in the stroma compartment. Fig. S2 shows low magnification images of UEA-1-stained thymus sections of the indicated aly/aly bone marrow chimeras. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20030794/DC1>.

## Results

**The Characteristic Arrangement of Thymic MECs in Three-dimensional Space Depends on  $LT\beta R$  Signaling.** To investigate a possible role for  $LT\beta R$  signaling in thymic development, we stained sections from  $LT\beta R^{-/-}$  and aly/aly mice with markers for medullary and cortical epithelial cells (Fig. 1 A). Cortical and medullary regions can be found in the thymi of these mutant mice with the same ratio observed in wild-type mice. However, although cortical epithelial cells marked by anticytokeratin 8 staining appeared unaffected, the normal configuration of MECs is lost in  $LT\beta R^{-/-}$  and aly/aly mice. In wild-type mice, UEA-1-positive epithelial cells form a homogeneous, three-dimensional network of stellate cells that builds the thymic medulla (Fig. 1 A, middle). Although UEA-1-positive cells can still be detected in  $LT\beta R^{-/-}$  and aly/aly mice, these cells form clumps rather than a network (Fig. 1 A). FACS<sup>®</sup> analyses confirmed the epithelial nature of UEA-1-positive cells from  $LT\beta R^{-/-}$  thymi as they are large G8.8 positive cells that lack CD45 and CDR1 expression (unpublished data). MECs staining positive for UEA-1 have been shown previously to represent one of two subsets that make up the medullary epithelial stroma (27). The other subset is characterized by the expression of the nonpolymorphic MHC class II antigen I-O, and this subset is similarly affected in  $LT\beta R^{-/-}$  and aly/aly mice when compared with wild-type mice (Fig. 1 B). Apart from epithelial cells, the stroma of the thymic medulla consists of dendritic cells, macrophages, and poorly defined cells of nonepithelial origin that are cytokeratin-negative but recognized by the monoclonal antibody MTS-10, which also stains epithelial cells (28). Dendritic cells and macrophages are readily detectable in  $LT\beta R^{-/-}$  and aly/aly mice by MIDC-8 and F4/80 staining, respectively (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20030794/DC1>). MTS-10 staining is strongly reduced in aly/aly mice but only mildly affected in  $LT\beta R^{-/-}$  mice (Fig. 1 B). Although  $LT\beta R$  is expressed in stromal but not lymphoid cells (29-31), Nik has been shown to be ubiquitously expressed (32), and there is evidence that the aly/aly mutation affects cells which do not express  $LT\beta R$  (e.g., lymphocytes; reference 23). Thus, MTS10-positive cells of nonepithelial origin may be unaffected by the absence of  $LT\beta R$  but depend on the presence of undisturbed signaling through Nik. In hematoxylin and eosin stainings of thymus sections, the demarcation between cortex and medulla is blurred in  $LT\beta R^{-/-}$  and aly/aly mice due to a significant increase in the density of strongly hematoxylin-positive nuclei (i.e., lymphocytes) in the medulla (Fig. 1 B). Collectively, the data reveal an aberrant development of thymic MECs in

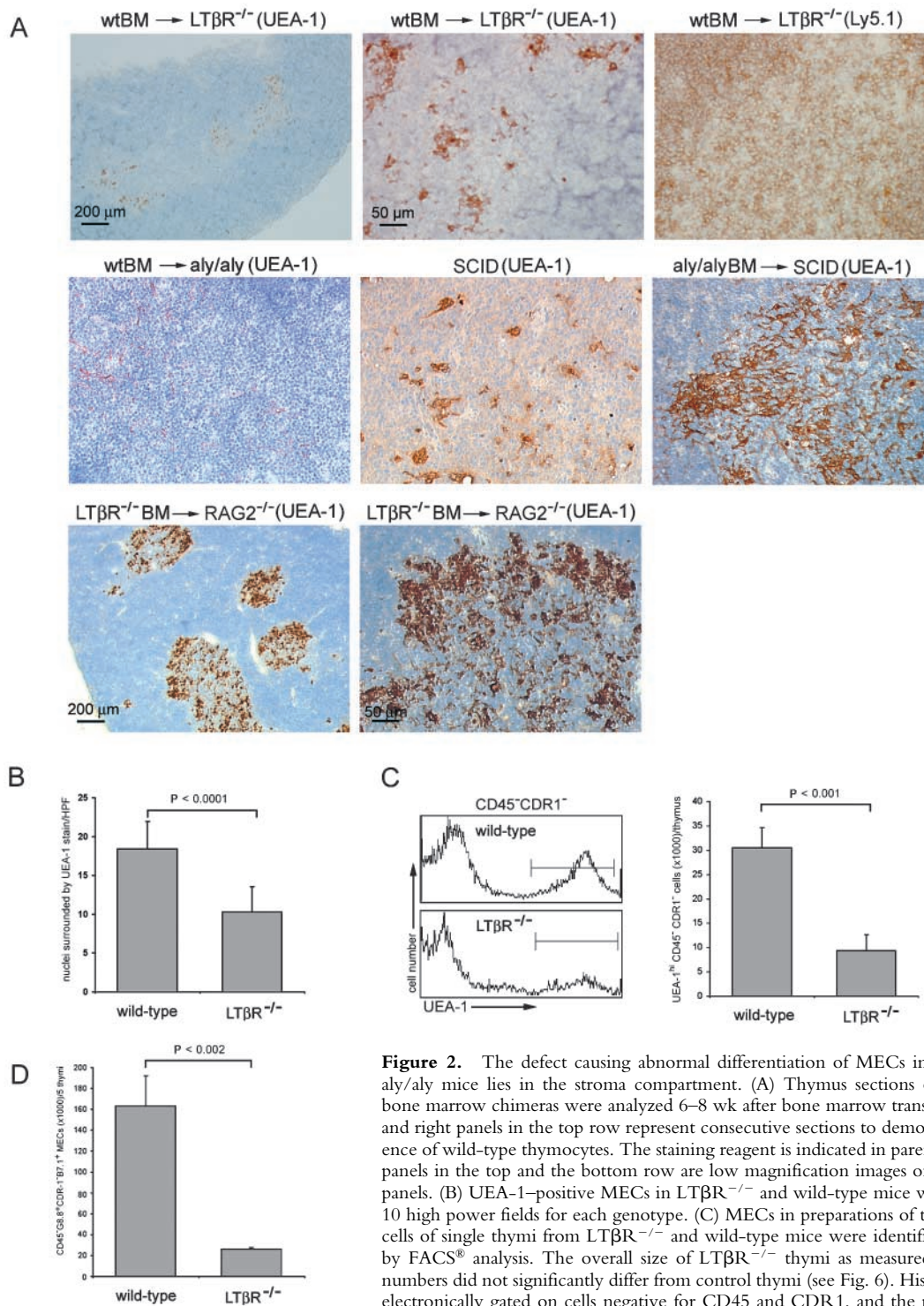


**Figure 1.** Normal development of thymic MECs requires  $LT\beta R$  signaling. (A) UEA-1 staining reveals the disturbed differentiation of MECs in  $LT\beta R^{-/-}$  and *aly/aly* mice. Consecutive sections of thymic lobes stained with the lectin UEA-1 for MECs and a monoclonal antibody specific for cytokeratin 8 (K8) expressed by cortical epithelial cells are shown (top and bottom, respectively). The dark line marks the border between cortex and medulla. Panels in the middle represent higher magnification images of the top panels. (B) Stainings of medullary regions of thymi of the respective mice were performed with a polyclonal rabbit antiserum directed against the nonpolymorphic MHC class II antigen I-O and the monoclonal antibody MTS10. Hematoxylin and eosin stainings (H&E) show an increased density of lymphocytes in the medulla compared with wild-type mice. Cortical areas (C) and medullary areas (M) are indicated.

mice defective for  $LT\beta R$  signaling that affects UEA-1 as well as I-O-positive MECs.

To locate the defect of the aberrant development of the thymic MECs, we generated bone marrow chimeras. The transfer of Ly5.1-marked, wild-type bone marrow did not restore the normal three-dimensional epithelial network in  $LT\beta R^{-/-}$  mice, even though Ly5.1-marked thymocytes readily repopulated the thymic medulla (Fig. 2 A). Likewise, transfer of wild-type bone marrow into *aly/aly* mice

could not rescue defective MEC development (Fig. 2 A; and Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20030794/DC1>). However, the latter experiment was done without the congenic Ly5.1 marker so that we could not formally demonstrate the presence of wild-type cells in the thymus of these bone marrow chimeras. Therefore, we transferred *aly/aly* bone marrow into SCID mice to test if *aly/aly* thymocytes induced MEC differentiation in these mice. The transfer of *aly/aly* bone marrow into

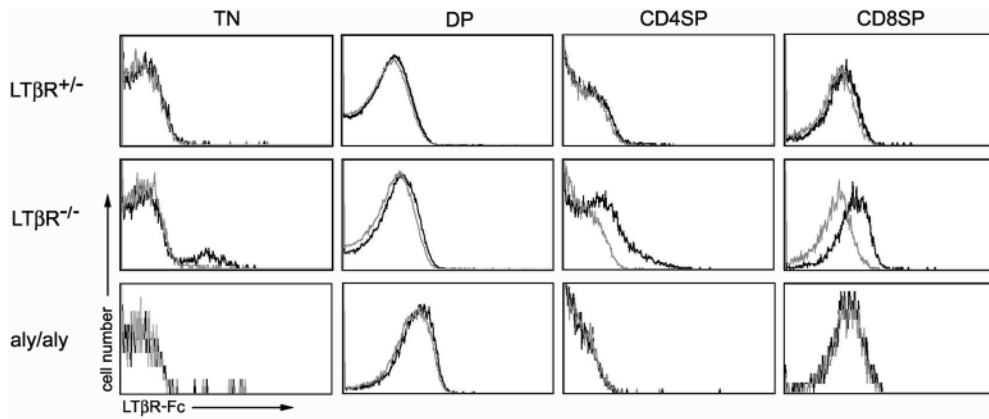


**Figure 2.** The defect causing abnormal differentiation of MECs in LTβR<sup>-/-</sup> and aly/aly mice lies in the stroma compartment. (A) Thymus sections of the indicated bone marrow chimeras were analyzed 6–8 wk after bone marrow transfer. The middle and right panels in the top row represent consecutive sections to demonstrate the presence of wild-type thymocytes. The staining reagent is indicated in parentheses. The left panels in the top and the bottom row are low magnification images of the subsequent panels. (B) UEA-1-positive MECs in LTβR<sup>-/-</sup> and wild-type mice were counted on 10 high power fields for each genotype. (C) MECs in preparations of thymic epithelial cells of single thymi from LTβR<sup>-/-</sup> and wild-type mice were identified and counted by FACS<sup>®</sup> analysis. The overall size of LTβR<sup>-/-</sup> thymi as measured by thymocyte numbers did not significantly differ from control thymi (see Fig. 6). Histogram plots are electronically gated on cells negative for CD45 and CDR1, and the marker indicates the fluorescence intensity scored as high level UEA-1 expression. MEC numbers of

four individual thymi per genotype were determined. (D) The absolute number of CD45<sup>+</sup>G8.8<sup>+</sup>CDR1<sup>-</sup>B7.1<sup>+</sup> MECs from preparations of epithelial cells of pools from five mice were determined. The gate that identifies these MECs among CD45<sup>+</sup>G8.8<sup>+</sup> thymic epithelial cells is shown in Fig. 7. The results of three independent experiments for each genotype are shown. Error bars represent standard deviations from the mean and statistical analysis was done using Student's *t* test.

SCID mice completely restored the configuration of MECs in three-dimensional space (Fig. 2 A; and Fig. S2). The same is true for the transfer of LTβR<sup>-/-</sup> bone marrow into RAG2-deficient mice. We conclude from these experi-

ments that the defect in the development of UEA-1 as well as I-O-positive MECs in LTβR<sup>-/-</sup> and aly/aly mice lies in the stromal compartment. To investigate if LTβR signaling influenced cell proliferation, which affected the steady-state

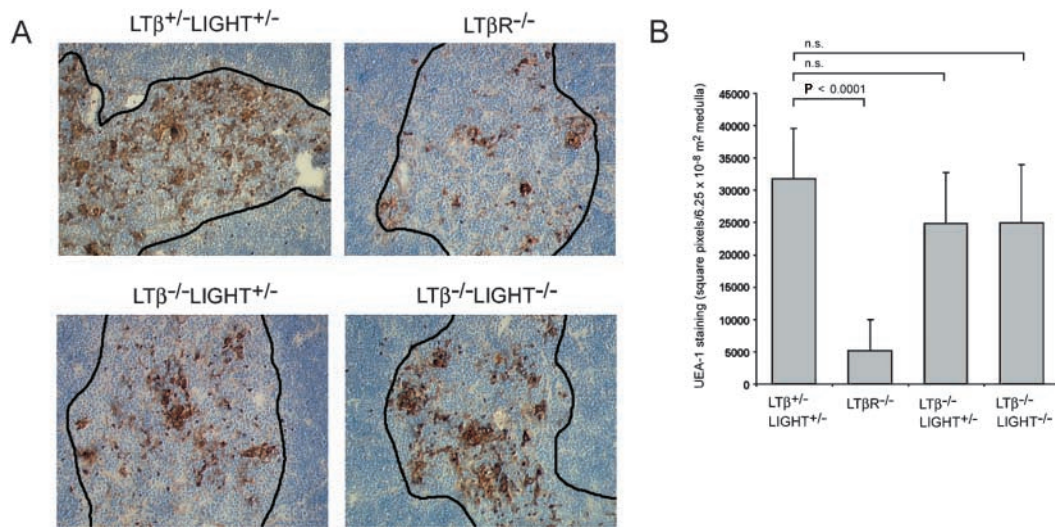


**Figure 3.** FACS<sup>®</sup> analysis detects LTβR ligands on thymocytes in LTβR<sup>-/-</sup> but not control mice. Histogram plots for LTβR-Fc stainings of electronically gated triple-negative (TN), coreceptor double-positive (DP), and CD4 and CD8 single-positive (CD4SP and CD8SP) thymocyte subsets for mice of the indicated genotypes are shown. TN cells are defined as thymocytes negative for CD4, CD8, CD3ε, and TCRβ. The gray lines show background staining levels without the fusion protein. One representative staining of at least four for each genotype is shown.

number of MECs in addition to their differentiation in three-dimensional space, we determined the number of MECs in LTβR<sup>-/-</sup> versus wild-type thymi in three different ways. First, MECs were counted as large cells with lightly hematoxylin-positive nuclei completely surrounded by UEA-1 staining on high-power magnifications. They are significantly reduced in LTβR<sup>-/-</sup> mice (Fig. 2 B). Second, we counted cells expressing high levels of UEA-1 by FACS<sup>®</sup> analysis, excluding hematopoietic cells (as identified by CD45) and cortical epithelial cells (as identified by CDR1) in preparations of individual thymi (Fig. 2 C). By this method, thymi of LTβR<sup>-/-</sup> mice contained three times fewer cells than wild-type control thymi. Third, another staining protocol for MECs was used. The absolute cell number of CD45<sup>-</sup>G8.8<sup>+</sup>CDR1<sup>-</sup>B7.1<sup>+</sup> MECs that could be isolated from pools of five LTβR<sup>-/-</sup> versus five

wild-type thymi was determined by FACS<sup>®</sup> sorting. MECs defined by these markers were reduced in LTβR<sup>-/-</sup> mice sixfold compared with controls (Fig. 2 D). In contrast, total thymocyte numbers did not differ significantly between mutant mice and appropriate controls. Collectively, our data demonstrate that in LTβR<sup>-/-</sup> mice MECs identified by the independent markers UEA-1 and I-O show aberrant differentiation. At the same time, UEA-1<sup>hi</sup> CD45<sup>-</sup>CDR1<sup>-</sup> and even more pronounced CD45<sup>-</sup>G8.8<sup>+</sup> CDR1<sup>-</sup>B7.1<sup>+</sup> MECs are significantly reduced in absolute numbers per thymus in LTβR<sup>-/-</sup> mice compared with controls.

*Mature Single-positive Thymocytes Are the Main Source of LTβR Ligands.* To identify the cells that express LTβR ligands, we performed FACS<sup>®</sup> analyses on thymocytes using a soluble LTβR-Fc fusion protein to stain ligand-pos-



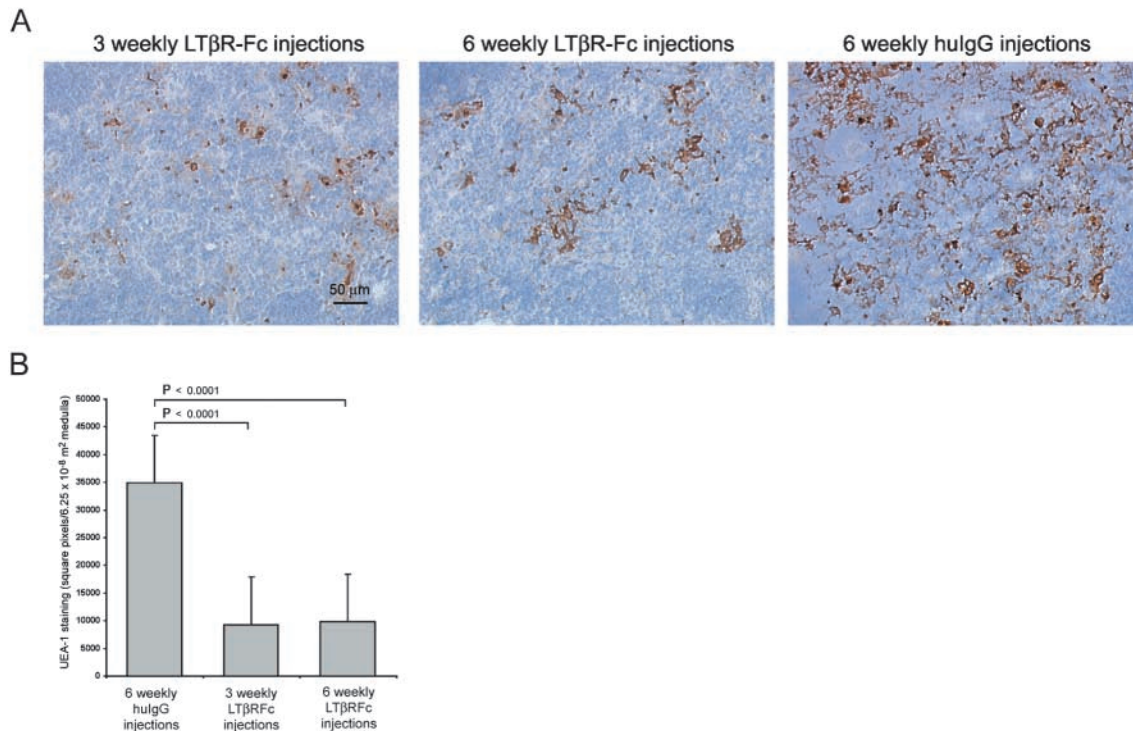
**Figure 4.** Mice deficient for the LTβR ligand LTα1β2 show disturbed differentiation of MECs. (A) UEA-1 stainings of thymic medullary regions of LTβR<sup>-/-</sup>, LTβ<sup>-/-</sup>LIGHT<sup>-/-</sup>, LTβ<sup>-/-</sup>LIGHT<sup>+/+</sup> and LTβ<sup>+/+</sup>LIGHT<sup>+/+</sup> mice. The panels show representative medullary areas from 8-wk-old LTβR ligand-deficient mice of the same litter processed on the same slide. The dark line marks the border between cortex and medulla. (B) Quantification of the surface area of UEA-1-positive MECs in LTβR<sup>-/-</sup>, LTβ<sup>-/-</sup>LIGHT<sup>-/-</sup>, LTβ<sup>-/-</sup>LIGHT<sup>+/+</sup>, and LTβ<sup>+/+</sup>LIGHT<sup>+/+</sup> mice. Brown pixels (i.e., UEA-1 staining) were counted from 10 digital images of each genotype using the public domain software NIH image. Results are given as square pixels per 6.25 × 10<sup>-8</sup> m<sup>2</sup> thymic medulla.

itive cells. We found that neither wild-type nor  $LT\beta R^{+/-}$  thymocytes bound  $LT\beta R$ -Fc (Fig. 3 and not depicted). On the other hand, lymph node B lymphocytes from these mice showed low but significant levels of  $LT\beta R$  ligands as described previously (29, 33). To our surprise, mature CD4 or CD8 single-positive thymocytes of  $LT\beta R^{-/-}$  mice showed easily detectable levels of  $LT\beta R$  ligand expression, whereas CD4 and CD8 double-positive cells and the majority of coreceptor- and TCR-negative thymocytes showed no staining (Fig. 3). Thus, in the absence of  $LT\beta R$  on stromal cells, mature thymocytes appear to up-regulate  $LT\beta R$  ligands to supraphysiological levels. This overexpression of  $LT\beta R$  ligands in  $LT\beta R^{-/-}$  mice suggests the presence of a regulatory loop that modulates  $LT\beta R$  ligand expression. In contrast,  $LT\beta R$  ligands could not be detected on the surface of  $aly/aly$  thymocytes (Fig. 3). Although the situation is complicated by the fact that Nik is ubiquitously expressed, there is the possibility that the direct interaction between ligand(s) and receptor induces the down-regulation of ligand(s) on thymocytes.

*Proper Development of UEA-1-positive MECs Depends on the Presence of the  $LT\beta R$  Ligand  $LT\alpha 1\beta 2$ .* The results presented thus far identify the  $LT\beta R$  signaling pathway as a crucial determinant in MEC development. Next, we investigated thymi of mice rendered deficient for  $LT\alpha 1\beta 2$ , a  $LT\beta R$  ligand that is crucial for lymphoid organogenesis

(16–18). Thymi of  $LT\beta^{-/-}$  mice consistently show a loss of the homogeneous distribution of UEA-1-positive cellular extensions covering the entire thymic medulla (Fig. 4 A). Clumps of strongly UEA-1-staining cells are found next to medullary areas entirely devoid of UEA-1 staining. Despite this severe morphological impairment, the overall surface area, as a measurement for MEC functionality, provided by UEA-1-positive cells in the thymic medulla of  $LT\beta^{-/-}$  mice was not significantly reduced (Fig. 4 B). In contrast, the surface area of UEA-1-positive MECs in  $LT\beta R^{-/-}$  mice was significantly smaller than in control mice. This difference between receptor and ligand knock-outs could be explained if another  $LT\beta R$  ligand, such as LIGHT (15), that is dispensable by itself for lymphoid organogenesis compensated for the absence of  $LT\alpha 1\beta 2$  in  $LT\beta^{-/-}$  mice. However, this is not the case as  $LT\beta^{-/-}$  LIGHT $^{-/-}$  thymi are indistinguishable from  $LT\beta^{-/-}$  LIGHT $^{+/-}$  thymi (Fig. 4, A and B). These results demonstrate that proper differentiation of UEA-1-positive MECs depends on the presence of  $LT\beta$  and suggest the existence of (an) additional  $LT\beta R$  ligand(s) apart from  $LT\alpha 1\beta 2$  and LIGHT that signal through  $LT\beta R$  in the absence of the two known ligands.

*Lympho-epithelial Interaction in the Thymic Medulla Is a Dynamic Process.* To investigate lympho-epithelial cross talk in a normal adult mouse, we injected wild-type mice once a week with 100  $\mu$ g  $LT\beta R$ -Fc, a fusion protein of  $LT\beta R$

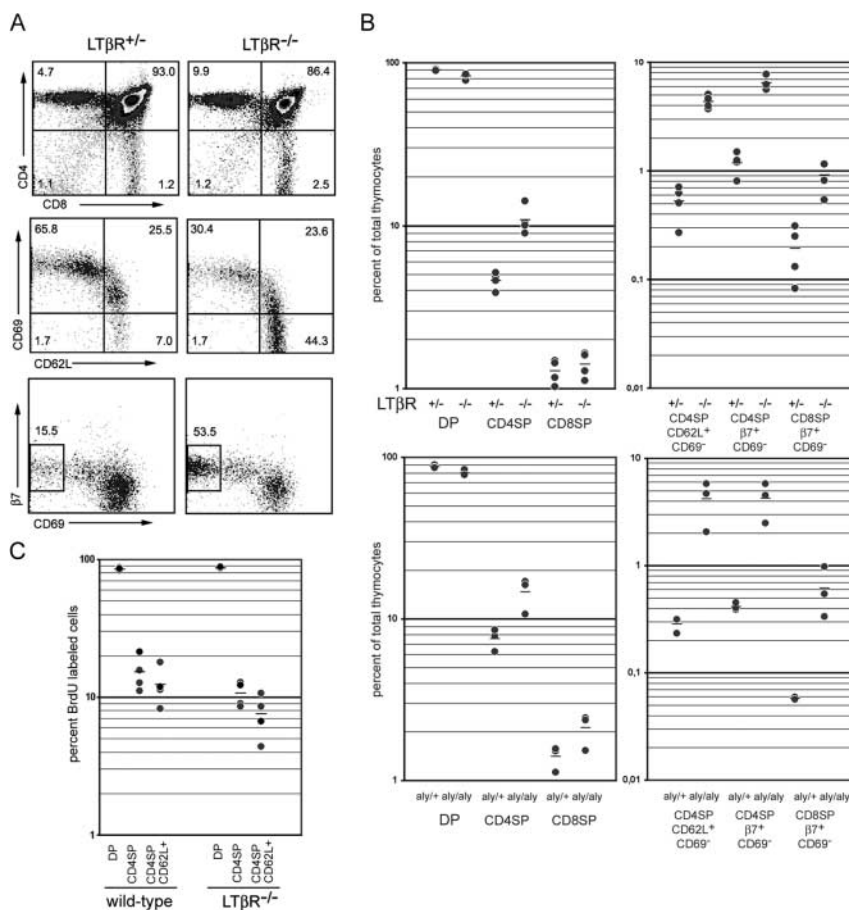


**Figure 5.** The three-dimensional organization of thymic MECs depends on continuous signaling through  $LT\beta R$ . C57BL/6 wild-type mice received intraperitoneal injections of  $LT\beta R$ -Fc or human polyclonal IgG as control once a week for 3 and 6 wk, respectively. (A) Thymi were stained with UEA-1 and medullary regions are shown. (B) Quantification of the surface area of UEA-1-positive MECs in thymi from  $LT\beta R$ -Fc-treated mice. Brown pixels (i.e., UEA-1 staining) were counted from 10 digital images derived from multiple sections of each experimental condition using the public domain software NIH image.

and human IgG that blocks interaction of ligands with the LT $\beta$ R in vivo (34). Three injections were sufficient to completely abolish the physiological configuration of UEA-1–positive thymic epithelial cells (Fig. 5 A) and to significantly reduce the detectable surface area of these cells (Fig. 5 B). Six injections did not have an additional effect. UEA-1–positive cells were still detectable and showed the same aberrant phenotype found in LT $\beta$ R<sup>-/-</sup> mice (Fig. 1 A). Control injections with human immunoglobulin did not affect UEA-1–positive MEC organization. From this experiment, we conclude that thymic MECs crucially depend on the continuous presence of signals mediated by LT $\beta$ R.

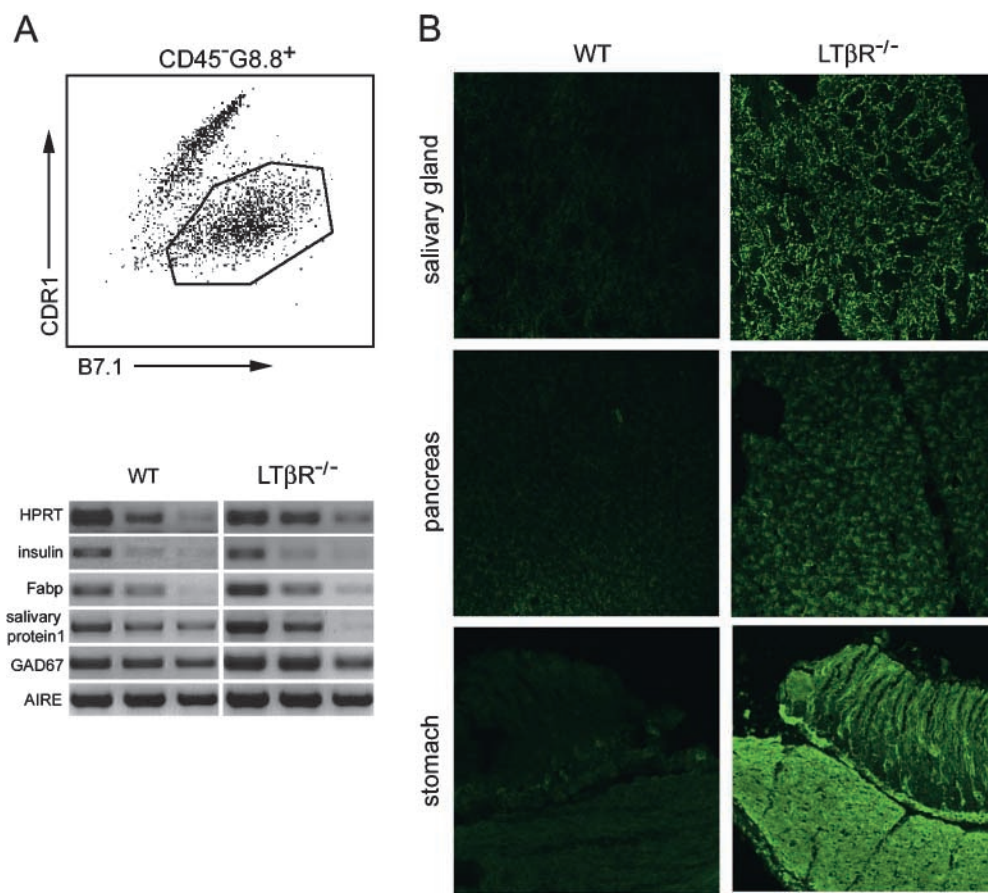
**LT $\beta$ R Signaling Controls Export of Mature Thymocytes.** We investigated the basis for the increased density of medullary thymocytes found on hematoxylin and eosin stains in LT $\beta$ R<sup>-/-</sup> and aly/aly mice. By fluorescence immunohistochemistry, we found that double–positive and single–positive thymocytes were correctly located to cortical and medullary regions, respectively, which ruled out the possibility that aberrant thymocyte homing within the thymus leads to increased medullary cellularity (unpublished data). Therefore, we analyzed mature thymocytes by FACS<sup>®</sup> analysis, which revealed a mild increase in single–positive T lymphocytes in LT $\beta$ R<sup>-/-</sup> and aly/aly mice compared with heterozygous controls (Fig. 6, A and B). This led us

to investigate in more detail the population of thymocytes in the process of leaving the thymus. These cells carry the characteristic CD69–negative CD62L–positive integrin  $\beta$ 7–positive phenotype of recent thymic emigrants (RTEs; reference 35). In FACS<sup>®</sup> analyses, CD4 single–positive thymocytes from 12–wk–old LT $\beta$ R<sup>-/-</sup> mice showed a marked shift toward the CD69–negative CD62L–positive integrin  $\beta$ 7–positive RTE phenotype compared with heterozygous controls (Fig. 6 A), which translated into an eightfold increase in the percentage of CD4 single–positive CD69–negative CD62L–positive RTEs in LT $\beta$ R<sup>-/-</sup> mice compared with heterozygous control thymocytes ( $4.33 \pm 0.60\%$  vs.  $0.52 \pm 0.19\%$  of total thymocytes;  $P < 0.0001$ , Student's *t* test). Consistently, CD8 single–positive LT $\beta$ R<sup>-/-</sup> thymocytes showed a 4.7–fold increase in the percentage of CD8 single–positive CD69–negative integrin  $\beta$ 7–positive RTEs ( $0.91 \pm 0.29\%$  vs.  $0.19 \pm 0.10\%$ ;  $P < 0.005$ ; Fig. 6 B). Similar results were obtained by comparison of aly/aly mutant and aly/+ heterozygous controls; the observed increase for aly/aly mice is 14.9–fold for CD4 single–positive CD69–negative CD62L–positive RTEs and 10.7–fold for CD8 single–positive CD69–negative integrin  $\beta$ 7–positive RTEs. The marked increase in RTEs together with only a mild increase in less mature stages of single–positive thymocytes suggested a disturbed export of thymocytes from the thymus. Consistently, the



**Figure 6.** The absence of LT $\beta$ R signaling leads to the accumulation of thymocytes with the phenotype of recent thymic emigrants. (A) FACS<sup>®</sup> analysis of thymocytes in LT $\beta$ R<sup>-/-</sup> mice and heterozygous controls. Thymocytes of 12–wk–old mice were stained with the indicated antibodies and gated on lymphocytes for the top panels and on CD4 single–positive cells (CD4SP) for the middle and bottom panels. Total thymocyte numbers did not differ significantly between mutant mice and appropriate controls ( $1.19 \pm 0.27 \times 10^8$  per LT $\beta$ R<sup>-/-</sup> thymus and  $1.68 \pm 0.37 \times 10^8$  per LT $\beta$ R<sup>+/-</sup> control thymus;  $1.43 \pm 0.58 \times 10^8$  per aly/aly thymus and  $1.77 \pm 0.46 \times 10^8$  per aly/+ control thymus). Percent of cells in each quadrant are indicated. For the bottom panel, the percent of integrin  $\beta$ 7<sup>+</sup> CD69<sup>-</sup> cells among CD4SP in the indicated box is given. (B) Quantification of thymocyte subsets in LT $\beta$ R<sup>-/-</sup>, aly/aly, and heterozygous control mice. Thymocytes from 12–wk–old mice were stained as shown in A. Results are shown as percent of total thymocytes. Each dot indicates one mouse and the mean percentage is indicated by a horizontal bar. (C) 12–wk–old LT $\beta$ R<sup>-/-</sup> mice and appropriate wild–type controls were injected with BrdU once and kept on BrdU containing drinking water for 3 d, after which the indicated thymocyte subpopulations were sorted by FACS<sup>®</sup> and stained with a FITC–labeled anti–BrdU monoclonal antibody. The percentage of cells that incorporated BrdU was determined. Each dot indicates one mouse and the mean percentage is indicated by a horizontal bar.





**Figure 7.** LTβR<sup>-/-</sup> mice show signs of autoimmunity. (A) The FACS<sup>®</sup> plot shows thymic epithelial cells electronically gated on CD45<sup>-</sup>G8.8<sup>+</sup> cells. CD45<sup>-</sup>G8.8<sup>+</sup>CDR1<sup>-</sup>B7.1<sup>+</sup> MECs were isolated by FACS<sup>®</sup> from wild-type and LTβR<sup>-/-</sup> mice using the indicated gate. Semi-quantitative RT-PCR results of undiluted, 5-fold, and 25-fold diluted cDNAs for several tissue-specific transcripts, HPRT, and AIRE are shown. (B) The presence of autoantibodies directed against salivary gland, pancreas, and stomach was detected in sera from LTβR<sup>-/-</sup> but not control mice. Immunohistochemistry was performed on sections of tissues isolated from RAG2<sup>-/-</sup> mice using 1:40 dilutions of the indicated sera. Images were obtained by confocal microscopy with identical settings for all samples shown.

transfer of  $3 \times 10^7$  Ly5.1-marked lymphocytes into LTβR<sup>-/-</sup> mice did not reveal an increased reentry of peripheral lymphocytes into the thymus when compared with wild-type mice (<0.01% Ly5.1<sup>+</sup> cells among both LTβR<sup>-/-</sup> and wild-type thymocytes in transferred mice). To rule out the possibility that enhanced proliferation rates of RTEs rather than their impaired export caused the observed increase in RTEs, we measured proliferation of double-positive, CD4 single-positive, and CD4 single-positive CD62L-positive thymocytes in LTβR<sup>-/-</sup> mice and controls by *in vivo* BrdU labeling studies (Fig. 6 C). Mature thymocytes of mutant mice showed the same if not a lower propensity for BrdU uptake compared with control cells, ruling out abnormally high proliferation of RTEs in LTβR-deficient mice.

**LTβR-deficient Mice Show Signs of Autoimmunity.** Finally, we asked if the disorganization and absolute reduction of MECs in LTβR<sup>-/-</sup> mice was associated with autoimmune processes. There is accumulating evidence that MECs play a crucial role in the control of autoimmunity by the promiscuous expression of tissue-specific antigens that are thought to negatively select autoreactive T cell clones and possibly to promote the generation of regulatory T cells (6, 7). To investigate promiscuous gene expression in LTβR<sup>-/-</sup> mice, we performed RT-PCR on RNA isolated from MECs of LTβR<sup>-/-</sup> and wild-type mice identified as large CD45<sup>-</sup>G8.8<sup>+</sup>CDR1<sup>-</sup>B7.1<sup>+</sup> cells by FACS<sup>®</sup>

analysis. This approach revealed that promiscuous gene expression and the expression of AIRE, so far the only known regulator of promiscuous gene expression (8), is unaffected in LTβR<sup>-/-</sup> MECs (Fig. 7 A). Because only 1–5% of MECs express a given tissue-specific antigen (6), central tolerance induction to these promiscuously expressed antigens has been suggested to require extensive interaction of mature lymphocytes with a large number of MECs (7). Given the disturbed three-dimensional organization and the absolute reduction of MECs in LTβR<sup>-/-</sup> mice, we hypothesized that interaction between lymphocytes and MECs should be grossly disturbed in these mice and, therefore, we sought signs of autoimmunity. Although LTβR<sup>-/-</sup> mice develop perivascular lymphocytic infiltrates in lungs, liver, pancreas, salivary gland, adrenal gland, and kidney (12), these infiltrates could be due to homeostatic rather than autoimmune processes considering that these mice lack lymph nodes. More conclusively, we detect autoreactive antibodies in sera of LTβR<sup>-/-</sup> but not wild-type mice directed against salivary gland, stomach, and pancreas (Fig. 7 B). Autoreactive antibodies to liver, adrenal gland, and kidney where lymphocytic infiltrations can also be found were not observed. Older mice appear to be frequently affected: all sera obtained from four 6-mo-old LTβR<sup>-/-</sup> mice contained autoantibodies recognizing determinants of at least two of these organs whereas control sera remained negative (Table I). Antibodies directed

**Table I.** Staining Intensities of Autoantibodies Detected in the Sera of 6-mo-old  $LT\beta R^{-/-}$  and Wild-type Controls by Immunohistochemistry

	Stomach	Pancreas	Salivary gland
$LT\beta R^{-/-}$ no. 7	+++	0	+++
$LT\beta R^{-/-}$ no. 8	++	0	+
$LT\beta R^{-/-}$ no. 9	+	+++	+++
$LT\beta R^{-/-}$ no. 10	+	+	+
WT no. 1	0	0	0
WT no. 2	0	0	0
WT no. 3	0	0	0
WT no. 4	0	0	0

against dsDNA or immunoglobulin in the sera of  $LT\beta R^{-/-}$  mice were not detected. These findings suggest that promiscuous expression of tissue-specific genes may not be sufficient to prevent autoimmune processes and that proper three-dimensional organization in conjunction with a critical number of MECs may also be required.

## Discussion

Thymic epithelial cells have long been recognized for their ability to generate a diverse, nonautoreactive T cell repertoire by the interaction with developing thymocytes. This reciprocal lympho-epithelial interaction is a unique feature of thymopoiesis. Interaction of T cell receptors and peptide-MHC complexes represents the classical example of an interaction that determines the developmental fate of thymocytes. Here, we provide to our knowledge the first example of how lymphocytes determine the development of thymic MECs that are thought to control autoimmunity. Although transcription factors (e.g., Foxn1 [36] and Hoxa3 [37]), signaling molecules (e.g., RelB; reference 38, 39), and the Wnt proteins (40) have been implicated in thymus development, none of these have been shown to depend on activation by developing lymphocytes *in vivo*. Indeed, Wnt proteins, which are produced by thymocytes and even more abundantly by thymic epithelial cells, have been so far the only soluble factors influencing epithelial cell differentiation. Our paper identifies the  $LT\beta R$  signaling axis as an important means of communication between developing lymphocytes and epithelial cells in the thymic medulla. The absence of any of its components, namely ligand (i.e.,  $LT\alpha 1\beta 2$ ), receptor, or intracellular signaling molecule (i.e., Nik), leads to the disorganization of MECs. Functionally, malformed MECs result in the retention of mature thymocytes and are associated with autoantibody production, suggesting key roles for  $LT\beta R$  signaling in thymic lymphocyte homeostasis and central tolerance induction.

The requirement of instructive signals for thymic epithelial cell differentiation from developing lymphocytes was

suggested by the phenotype of mice with defective T cell development (3, 9, 41), but the nature of these signals has remained elusive. Our results now show that defective lympho-epithelial cross talk in  $LT\beta R^{-/-}$  mice specifically affects medullary but not cortical epithelial cells. Thus, other signals must exist which organize the three-dimensional network in the cortex. This observation is in good agreement with the fact that supra-physiological levels of  $LT\beta R$  ligands were found in  $LT\beta R^{-/-}$  mice on mature single-positive (situated in the medulla) but not on double-positive thymocytes (situated in the cortex). More work is required to determine the molecular basis for increased ligand expression on  $LT\beta R^{-/-}$  thymocytes.

The medullary stroma of the thymus consists of a complex mixture of epithelial cells, interdigitating dendritic cells, macrophages, and fibroblastoid cells. Although a series of markers exist that are MEC-specific, one has to consider that any one may only stain a certain MEC subset. UEA-1 and I-O expressed on mutually exclusive MEC subsets in the adult mouse thymus have been shown convincingly to mark the majority of epithelial cells in the thymic medulla when used in combination (27). Although we cannot rule out the possibility that a minor subset of MECs remains unaffected by the lack of  $LT\beta R$  signaling, our data unequivocally demonstrate disturbed differentiation of UEA-1 as well as I-O-positive MECs. Both of these markers are also detectable on thymic epithelial cells during embryogenesis, which should have allowed the identification of MECs in our experiments even if they remained in an embryonic differentiation state. Studies are under way to determine if MECs deficient for  $LT\beta R$  signaling are locked in their embryonic form.

Our paper shows that the marked reduction in UEA-1 staining for MECs in  $LT\beta R^{-/-}$  mice is caused by both the loss of the characteristic three-dimensional organization and the reduction in absolute numbers of epithelial cells. The maintenance of this three-dimensional structure in the thymic medulla requires the continuous signal transduction through  $LT\beta R$  as demonstrated by the loss of the medullary epithelial network after three  $LT\beta R$ -Fc injections. This rapid regression from a three-dimensional organization to isolated clusters of cells is an unexpected finding. The view of thymic stroma as a static, radio-resistant scaffold for developing thymocytes that, once it has reached its ultimate state of differentiation, remains unchanged may have to be revised. Additional support for the proliferative effect of  $LT\beta R$  signaling comes from analyses of mice that are transgenic for LIGHT under the control of the proximal *lck* promoter (42). These mice show a significant enlargement of the thymic medulla with only small cortical areas. Although the situation is complicated by the fact that LIGHT binds the herpes virus entry mediator expressed on lymphocytes (15) in addition to  $LT\beta R$ , this may suggest that overexpression of  $LT\beta R$  ligands leads to increased numbers of MECs and an enlarged medulla.

Concerning the  $LT\beta R$  ligands involved in organizing the thymic medulla, our results show that the phenotype of the  $LT\beta R^{-/-}$  mouse is more severe than that of the

LT $\beta$ <sup>-/-</sup>LIGHT<sup>-/-</sup> mouse that lacks both known LT $\beta$ R ligands. This would indicate that additional, so far unknown ligand(s) partially compensate for the absence of LT $\beta$  and LIGHT in the thymic medulla. A recent analysis of the development of Peyer's patches, peripheral, and mesenteric lymph nodes in LT $\beta$ <sup>-/-</sup>LIGHT<sup>-/-</sup> mice also hinted at additional ligand(s) of the LT $\beta$ R (19). Because not all functional LT $\beta$ R ligands are known, the transfer of LT $\beta$ <sup>-/-</sup>LIGHT<sup>-/-</sup> bone marrow into SCID mice (to demonstrate the inability of LT $\beta$ R ligand-deficient thymocytes to induce normal medullary architecture in SCID mice) will yield inconclusive results; LT $\beta$ <sup>-/-</sup>LIGHT<sup>-/-</sup> bone marrow would still be able to induce differentiation of MECs in SCID mice as can be observed in LT $\beta$ <sup>-/-</sup>LIGHT<sup>-/-</sup> mice.

In the absence of LT $\beta$ R signaling, malformation of MECs leads to increased numbers of the most mature stages of thymopoiesis, suggesting that these cells are retained in the medulla rather than efficiently exported. This view is supported by the fact that abnormal proliferation of mature T cells or reentry of peripheral lymphocytes to the thymus was not observed in LT $\beta$ R<sup>-/-</sup> mice. This may mean that mature T cells ready to leave the thymus require signals from MECs for emigration that are only inefficiently supplied by malformed MECs in LT $\beta$ R<sup>-/-</sup> mice. Indeed, a checkpoint may exist that allows thymocyte emigration only when extensive interaction with MECs has been completed to prevent autoreactive clones from escaping negative selection. The need for extensive lympho-epithelial interaction in the thymic medulla appears to be even more important keeping in mind that only 1–5% of MECs express a given tissue-specific antigen, presumably necessary to prevent organ specific autoimmunity in a mosaic pattern (6); if each T cell had to interact with at least one MEC expressing a particular tissue-specific antigen to guarantee central tolerance, many cell-cell contacts would have to be made. From our data, it is obvious that such lympho-epithelial interaction in the medulla cannot take place normally in LT $\beta$ R<sup>-/-</sup> mice, which may result in autoimmunity, specifically in lymphocytic infiltrations and autoantibody production. However, our data by no means prove that MEC malformation leads to autoimmunity. They simply reveal a correlation between disturbed lympho-epithelial cross talk and autoimmune processes, and further work will be necessary to investigate this issue in more detail. Preliminary results from Western blot analyses using sera of LT $\beta$ R<sup>-/-</sup> mice on whole tissue lysates show reactivity to multiple proteins, suggesting a polyclonal set of autoantibodies. It is interesting to note that we detect autoantibodies in LT $\beta$ R<sup>-/-</sup> mice that lack peripheral lymph nodes, whereas priming of autoreactive T cells to autoantigens is thought to occur primarily in local lymphoid tissues (43).

Intriguingly, promiscuous expression of tissue-specific antigens in MECs appears to be unaffected in LT $\beta$ R<sup>-/-</sup> mice and does not depend on lympho-epithelial interaction. In situ hybridization studies suggested initially that AIRE, so far the only known regulator of promiscuous gene expression, was undetectable in MECs of LT $\beta$ R<sup>-/-</sup>

mice (unpublished data); because AIRE detection via in situ hybridization is not very sensitive and may have been complicated by the aberrant phenotype of MECs in LT $\beta$ R<sup>-/-</sup> mice, we investigated this issue by a different, more sensitive procedure. RT-PCR analyses using sorted CD45<sup>-</sup>G8.8<sup>+</sup>CDR1<sup>-</sup>B7.1<sup>+</sup> MECs demonstrate unequivocally that on a per cell basis, LT $\beta$ R<sup>-/-</sup> MECs express normal levels of AIRE. Thus, autoimmunity occurs in LT $\beta$ R<sup>-/-</sup> mice, whereas promiscuous gene expression is unaffected. This suggests an important role for the three-dimensional network and the absolute number of MECs and all the factors necessary to maintain these parameters, such as LT $\beta$ R<sup>-/-</sup> signaling, to prevent autoimmunity.

In summary, our data identify the LT $\beta$ R signaling axis as an important pathway that governs the three-dimensional configuration of MECs via lympho-epithelial cross talk. We describe the ligand, the receptor, and a signaling mediator that are crucial components in this signaling axis and provide evidence for the importance of the proper differentiation of MECs for thymocyte export and possibly the prevention of autoimmunity.

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