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Lymphotoxin β Receptor Is Required for the Migration and Selection of Autoreactive T Cells in Thymic Medulla

Mingzhao Zhu, Robert K. Chin, Alexei V. Tumanov, Xiaojuan Liu, and Yang-Xin Fu

How organ-specific central tolerance is established and regulated has been an intriguing question. Lymphotoxin β receptor (LTβR) deficiency is associated with autoimmune phenotypes characterized by humoral and cellular autoreactivity to peripheral organs. Whether this results from defective negative selection of T cells directed at tissue-restricted Ags has not been well understood. By tracing the development of OT-I thymocytes in rat insulin 2 promoter-mOVA transgenic mice on either Ltbr+/+ or Ltbr−/− background, we demonstrate that LTβR is necessary for thymic negative selection. LTβR deficiency resulted in a dramatic escape of “neo-self” specific OT-I cells that persist in circulation and lead to development of peri-insulitis. When the underlying mechanism was further explored, we found interestingly that LTβR deficiency did not result in reduced thymic expression of mOVA. Instead, LTβR was revealed to control the expression of thymic medullary chemokines (secondary lymphoid tissue chemokine (SLC) and EBV-induced molecule 1 ligand chemokine (ELC)) which are required for thymocytes migration and selection in medulla. Furthermore, RIP-mOVA transgenic mice on SLC/ELC deficient background (plt) demonstrated significant impaired negative selection of OT-I cells, suggesting that the dysregulation of SLC/ELC expression alone in Ltbr−/− thymi can be sufficient to impair thymic negative selection. Thus, LTβR has been revealed to play an important role in thymic negative selection of organ-specific thymocytes through thymic medullary chemokines regulation. The Journal of Immunology, 2007, 179: 8069–8075.

The thymus plays an essential role in facilitating T cell self-tolerance by organizing negative selection and generating regulatory T cells (Treg). Although it is easy to understand how central tolerance to ubiquitous self-Ags is established in the thymus, how these same mechanisms might forestall autoimmunity against peripheral tissue-specific Ags remains a mystery, until the appearance of recent clues (1, 2). A lengthening list of tissue-restricted self-Ags (TRAs), including insulin, have been found ectopically expressed in medullary thymic epithelial cells (mTECs). The appropriate expression of TRAs and interaction between developing thymocytes and thymic APCs in thymic medulla are regarded important for the efficient negative selection of autoreactive T cells and the generation of regulatory T cells. Autoimmune regulator (Aire) was found clearly involved in the regulation of thymic expression of TRAs (2–5). A few other molecules have also been suggested to take part in the regulation of central tolerance, including lymphotoxin β receptor (LTβR), NF-κB-inducing kinase, TNFR-associated factor 6 (TRAF6), v-rel reticulonendotheliosis viral oncoprotein homolog B (Rel B), NF-κB2, and chemokine (c-c motif) receptor 7 (6–11), although their roles have not been directly demonstrated. Clear definition of their functions in the organ-specific central tolerance will not only help to uncover the complex regulation of organ-specific autoimmune diseases, but also provide new targets for future diagnostic and therapeutic intervention of organ-specific autoimmunity diseases.

LTβR-deficient mice harbor an autoimmune phenotype characterized by increased lymphocytic infiltration of peripheral organs and humoral autoreactivity (8, 12, 13). LTβR was recently proposed to be involved in the control of negative selection of organ-specific autoreactive thymocytes in both Aire-dependent and independent pathways (8, 12, 13). However, this hypothesis has not been formally tested and little is known about the underlying mechanisms. This issue becomes even more intriguing given the controversial data available at present. Although one study showed that LTβR signaling may be directly involved in the regulation of Aire expression (12), another study suggested an indirect mechanism via the development and differentiation of thymic medulla epithelial cells (13). A third study demonstrated apparently normal Aire expression in Lta−/− mice, in which the ligand for LTβR is deficient, raising the possibility that increased peripheral tissue infiltration is not related to the defect in Aire-dependent negative selection (11). Whether and how LTβR is involved in organ-specific thymic negative selection remains to be determined. In this study, we clarify and confirm the essential role of LTβR in the negative selection of organ-specific autoreactive T cells. Our data further suggests that LTβR controls this process by regulating medullary chemokine expression for cortex-to-medulla thymocyte migration.

Materials and Methods

Mice

Ltbr−/− mice have been previously described (8). Aire−/− mice were generated with a mixed genetic background as described (14) and back-crossed to C57BL/6 for at least six generations in our laboratory. OT-I TCR transgenic mice on C57BL/6 background were purchased from The Jackson Laboratory.
Laboratory. RIP-mOVA transgenic mice on C57BL/6 background were obtained from Dr. W. R. Heath, Division of Immunology (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). The plt mice were obtained from J. G. Cyster (University of California, San Francisco, CA). Animal care and experiments were performed in accordance with the institution and National Institutes of Health guidelines and approved by the animal use committee at the University of Chicago.

**Bone marrow reconstitution and splenocytes adoptive transfer**

Mice were lethally irradiated with 1000 rads and adoptively transferred i.v. with 3 x 10^6 bone marrow cells the next day. Bactrim was added to the drinking water for 3 wk following irradiation. Thymocytes and splenocytes were analyzed 8–9 wk after transfer and in some cases 12 wk after transfer. For splenocyte adoptive transfer experiments, splenocytes from bone marrow chimeric mice were recovered when mice were sacrificed and 20 x 10^6 splenocytes were transferred to sublethally (400 rad) irradiated rats inulin-2 promoter (RIP-mOVA) mice. Bactrim was applied for 3 wk following irradiation. Mice were sacrificed for pancreas histopathology analysis 4 wk after splenocytes adoptive transfer.

**Immunofluorescence staining**

Six-micron frozen sections were fixed in acetone at 4°C for 10 min, rehydrated in PBS/saponin (0.1%), and blocked with goat serum (5%) or BSA (1%) in PBS at room temperature for 1 h. Primary Abs include anti-EpCAM (G8.8; BD Biosciences), UEA-I-bio (Sigma-Aldrich), anti-Cd8 (53-6.7; BD Biosciences), anti-CD11c (N418; BD Biosciences), anti-CD19 (1D3; BD Biosciences), anti-F4/80 (BM8; eBiosciences), and anti-insulin (abCAM). Tissue sections were incubated with primary Abs at 4°C overnight and visualized with appropriate fluorescence reagents.

**Thymy morphometrics analysis**

Mice were sublethally irradiated with 400 rad. Thymic sectioning and immunofluorescence staining were performed as described above. ImageJ 1.34 (National Institutes of Health) software was used to calculate the cortex and medulla areas. The medulla was defined as a continuous area with UEA-I-positive staining. Because only rare CD4CD8 DP thymocytes were present in sublethally irradiated mice, CD8 SP thymocytes distribution and quantity were visualized by FITC-conjugated anti-CD8 Ab (53-6.7; BD Biosciences). CD8 SP cells in medulla/cortex were counted and their distribution density was calculated.

**Flow cytometry analysis**

Single cell suspensions from the thymus and spleen were stained with anti-Cd4 (GK1.5), anti-Cd8 (53-6.7), anti-Cd24 (30-F11), anti-Cd69 (H1.2F3) mAbs (BD Biosciences), and OVA Tetramer H-2 Kb SIINFEKL-PE (Beckman Coulter) in PBS containing 0.2% BSA and 0.09% sodium azide. Before staining, the cells were preincubated with anti-FcγRII receptor (2.4G2; BD Biosciences). Foxp3 intracellular staining was conducted using Mouse Regulatory T Cell Staining Kit following the manufacturer’s instructions (eBioscience). Stained cells were analyzed on FACS Canto (BD Biosciences). mTECs sorting was performed as previously described (6). The purity was routinely higher than 90%.

**Real-time PCR**

Real-time PCR was conducted on cDNA prepared from DNase I-treated RNA extracted from whole thymus or purified mTECs from 4- to 6-week-old mice. The primers and probes used are as follows. For Insulin2: Forward, 5’-CTTTACAGGCATGCAGGTGGCA-3’, Reverse, 5’-ATGCT GGTCGCACTGCATC-3’; Probe, 5’-FAM-CGCAGGGACGAGCTGGGC-3’; For mOVA: Forward, 5’-ATTCGCAAGGAGATGGTTTGGAG-3’, Reverse, 5’-TGCCATGGTGTGCTATACAGA-3’, Probe, 5’-FAM-CGCTTACAGCGATCATCCTCA-TAMRA-3’. For secondary lymphoid tissue chemokine (SLC): Forward, 5’-GGCCAGGCCCACGACGACG-3’, Reverse, 5’-GTGAAGGCAGGACGAGCTTGG-3’, Probe, 5’-FAM-CCA CCTACTGCGCCTCGCTAT-CAMRA-3’. For CD80: Forward, 5’-AGACTCAGGCAGCAGCAAGACG-3’, Reverse, 5’-GTGGAAGGCAGGACGAGCTTGG-3’, Probe, 5’-FAM-CCA CCTACTGCGCCTCGCTAT-CAMRA-3’. For CD86: Forward, 5’-AGGATGGGCCAGTGTGTTTGG-3’, Reverse, 5’-GGCCAGGCCCACGACGACG-3’, Forward, 5’-GGCCAGGCCCACGACGACG-3’, Reverse, 5’-GGCCAGGCCCACGACGACG-3’, Forward, 5’-GGCCAGGCCCACGACGACG-3’, Reverse, 5’-GGCCAGGCCCACGACGACG-3’. For CD4: Forward, 5’-CTCTCACTAGACGAAAGTGC-3’, Reverse, 5’-GGCCAGGCCCACGACGACG-3’, Forward, 5’-GGCCAGGCCCACGACGACG-3’, Reverse, 5’-GGCCAGGCCCACGACGACG-3’. For CD69: Forward, 5’-CTTCATGCGCCTCGCTAT-CAMRA-3’, Reverse, 5’-CTTTACAGGCATGCAGGTGGCA-3’, Forward, 5’-CTTTACAGGCATGCAGGTGGCA-3’, Reverse, 5’-CTTTACAGGCATGCAGGTGGCA-3’, Forward, 5’-CTTTACAGGCATGCAGGTGGCA-3’, Reverse, 5’-CTTTACAGGCATGCAGGTGGCA-3’, Forward, 5’-CTTTACAGGCATGCAGGTGGCA-3’, Reverse, 5’-CTTTACAGGCATGCAGGTGGCA-3’, Forward, 5’-CTTTACAGGCATGCAGGTGGCA-3’. Reactions were run on the ABI/Prism 7300 (Applied Biosystems), in a final volume of 25 μl containing 100 nM of the forward and reverse primers and 200 μM of the probe using 2x Taqman Master Mix (Applied Biosystems) containing AmpliTaq Gold polymerase. Cycling conditions were a single denaturing step at 95°C for 15 min followed by 45 cycles of 94°C for 15 s and 60°C for 1 min. Analysis of In52, mOVA, SLC, ELC, and Gapdh gene expression were performed with a standard curve, and then normalized to sample Gapdh. The standard curves had R² values >0.99.

**Histopathology**

Tissues for histological examination were fixed in 10% buffered formalin and embedded in paraffin. Four- to five-micron sections were obtained from the paraffin blocks and stained using H&E stain methods. All sections were then examined by a pathologist in a blinded fashion.

**Results**

**LTβR deficiency rescues autoreactive T cells from thymic negative selection**

In mice with a native polyclonal TCR repertoire, the fate of autoreactive thymocytes can be difficult to trace. We thus took advantage of the OT-I-RIP-mOVA transgenic system to uncover the role of LTβR in regulating negative selection. In this system, mOVA expression is driven by RIP, mimicking an organ-specific self-Ag. OT-I thymocytes are routinely negatively selected in the thymus of RIP-mOVA transgenic mice (4, 15, 16). To determine whether LTβR controls the negative selection of organ-specific autoreactive thymocytes, we made bone marrow chimeras with OT-I−/− donors bone marrow transplanted into either RIP-mOVA +/− or RIP-mOVA −/− hosts. The development of OT-I + T cells was analyzed 8–9 wks later by flow cytometric staining with OT-I clonotype-specific tetramer and thymocyte maturation markers.

Following OT-I bone marrow transplantation, thymocytes in recipients wild type (WT) (Ltbr+/−) and Ltbr−/− developed normally and CD8+ OT-I T cells were predominantly positively selected (Fig. 1A). In RIP-mOVA+/− recipients of OT-I bone marrow transplantation, the CD8+ single positive (SP) population in the thymus was significantly reduced by 3-fold. Within this reduced CD8+ SP population, the percentage of OT-I+ clonotypic cells was even more severely decreased from around 77% in WT to 21% in RIP-mOVA−/− mice (Fig. 1, A and B). This led to, in absolute terms, about a 10-fold reduction of CD8+ OT-I clonotypic T cells in the presence of thymic mOVA expression in the RIP-mOVA recipients, suggesting an efficient thymic negative selection process (Fig. 1, A and B and Refs. 4, 16). The further addition of LTβR deficiency (RIP-mOVA/Ltbr−/−), however, rescued 2.5-fold of OT-I+ clonotypic cells (Fig. 1, A and B). When CD24 was added as a marker to further define the postselection, fully mature OT-I cells, few CD24+ OT-I cells were seen in the RIP-mOVA−/− recipients. In contrast, analysis of RIP-mOVA−/− thymi revealed a 40-fold increase in postselection mature OT-I+ population (CD24 negative), apparently rescued by LTβR deficiency (Fig. 1, A and B). Dramatic rescue of mature OT-I cells by LTβR deficiency was also seen when CD69 was used as a maturation marker (Fig. 1, A and B). These data thus strongly suggested an important role of LTβR in the thymic negative selection of OT-I+ thymocytes.

**Normal CD4+Foxp3+ Treg development in Ltbr−/− thymus**

Development of CD4+ Foxp3+ Treg is another important mechanism in the maintenance of central tolerance. To determine whether Treg development was impaired in the absence of LTβR, thymocytes from WT and Ltbr−/− mice were first intracellularly stained with Foxp3 and analyzed by flow cytometry. No differences in the percentage or the absolute number of CD4+ Foxp3+ cells were found between WT and Ltbr−/− mice (Fig. 2, A and B). To confirm this finding, Foxp3 expression level was also analyzed by quantitative real-time PCR and found unchanged in the thymus of Ltbr−/− mice (Fig. 2C). The expression level of Foxp3 also found no difference by comparing the mean fluorescence intensity between WT and Ltbr−/− Treg cells (data not shown), indicating...
a normal Treg function (17). Therefore, it appears that LTβR deficiency does not significantly influence the development of conventional CD4⁺Foxp3⁺ Treg cells. Whether other regulatory T cell populations, e.g., Th1, Th1, or even CD8⁺ regulatory T cells (18), are generated during thymic development and influenced by LTβR remains to be determined.

**LTβR deficiency leads to preserved ectopic mOVA expression but reduced medullary chemokine expression and defective migration**

To further dissect how LTβR deficiency impinges on thymic negative selection, we first analyzed the ectopic thymic expression level of the neo-self Ag, as this is a crucial factor for thymocytes selection (4, 19, 20). Thymic expression levels of the neo-Ag mOVA were determined by quantitative real-time PCR. LTβR was shown to be necessary for thymic Aire expression, and Aire shown necessary for thymic insulin 2 expression. Given that the RIP-mOVA gene is driven by insulin 2 promoter, we expected reduced thymic mOVA expression in the absence of LTβR signaling. We found rather that expression levels of mOVA in the thymi of RIP-mOVA⁻/⁻ and RIP-mOVA⁻/⁻ were equivalent (Fig. 3A). This is consistent with a recent study demonstrating that mOVA thymic expression is not reduced on Aire⁻/⁻ background (4, 19, 20). However, the native insulin 2 expression was indeed reduced in RIP-mOVA⁻/⁻ thymi (Fig. 3A). The RIP promoter’s independence from Aire control may be due to its deficiency in necessary cis-elements, either due to species specific differences or loss during cloning. Chromosomal integration-site effect on the RIP-driven expression of transgenes is a further possible explanation (4, 21).

Given that OT-I thymocytes in RIP-mOVA⁻/⁻ mice are prominently rescued without a significant decrease in thymic expression of mOVA, we wondered whether LTβR acts via other mechanisms in organizing thymic negative selection. Thymic medulla is the major site for negative selection of TSA-specific autoreactive T cells. Appropriate cortex-to-medulla migration of thymocytes is critical for their differentiation development and maturation (22, 23). Mice with severely diminished thymic medulla, as in Relb⁻/⁻ mice, demonstrate significant autoimmune phenotype (24–26). Because LTβR has been known to regulate the expression of chemokines in secondary lymphoid organs (27, 28), we hypothesized that chemokine expression might be defective in the Ltbr⁻/⁻ thymus and the defective negative selection in Ltbr⁻/⁻ mice might result from impaired thymocytes cortex-to-medulla migration. We therefore examined the thymic expression of CCL2 (SLC) and CCL19 (ELC) in WT and Ltbr⁻/⁻ mice. These two chemokines are produced predominantly in the medulla of thymus. Their receptor, CCR7, is expressed on semimature thymocytes, and has been recently shown to be important for the thymocytes migration from cortex to medulla (29, 30). By quantitative real-time PCR, we found both SLC and ELC were significantly reduced in the Ltbr⁻/⁻ thymus compared with those in WT mice (Fig. 3B). Because the mTECs population is slightly reduced in Ltbr⁻/⁻ thymi, to more specifically determine the role of LTβR in intrinsic regulation of SLC/ELC expression within the mTECs population, we purified mTECs (CD45⁺G8.8.7⁺B7.1⁺) by FACS.
FIGURE 3. Cellular mechanisms of LTβR control thymic negative selection. A. Real-time PCR analysis of relative mOVA and Ins2 gene expression in RIP-mOVA/Ltbr−/− thymus. Expression values are shown in arbitrary units and normalized relative to Gapdh. Mean ± SD (n = 3). **, p < 0.01; *, p < 0.05; NS, no significance. B. Real-time PCR analysis of relative thymic SLC and ELC expression in Ltbr−/− mice. Expression values are shown in arbitrary units and normalized relative to Gapdh. Data shown are mean ± SD (n = 3). *, p < 0.05. C. Real-time PCR analysis of SLC and ELC expression in purified mTECs from WT and Ltbr−/− mice. Expression values are shown in arbitrary units and normalized relative to Gapdh. Data shown are mean ± SD (n = 3). *, p < 0.05. D. CD8 SP thymocytes localization in 400 rad irradiated mice. Red, UEA-1; Green, CD8. M, medulla; C, cortex. E. Mean ± SD (n = 3) of the numbers of CD8 SP cells preunit area (0.1 mm²) in indicated thymic regions from different mice. *, p < 0.05, compared with WT.

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The data shown are mean ± SD (n = 3). **, p < 0.01; *, p < 0.05; NS, no significance. Thus, LTβR deficiency not only results in down-regulation of thymic SLC/ELC expression, but also impairs the cortex-to-medulla migration of CD8SP thymocytes.

Impaired thymic negative selection leads to peri-insulitis

To characterize the impact of this defect in negative selection on the peripheral T cell repertoire, splenocytes from bone marrow chimeras were first analyzed by flow cytometric staining with OT-I specific tetramer. We found a significantly increased peripheral population of OT-I+ T cells in RIP-mOVA/Ltbr−/− recipients relative to RIP-mOVA+/+ recipients (Fig. 4, A and B), suggesting that this thymic defect may have real peripheral consequences.

To determine the functional impact of these rescued OT-I+ T cells, we first checked whether this selection defect will culminate in insulitis or hyperglycemia in RIP-mOVA+/+ mice. However, we found neither insulitis nor hyperglycemia in RIP-mOVA+/+ mice, even at 12 wks after bone marrow transfer (data not shown). With the consideration that this may represent a falsely negative result, given the absence of draining lymph nodes in Ltbr−/− mice for priming, and congenitally disturbed spleen microarchitecture, we designed adoptive transfer experiments. Splenocytes from OT-I−/−/RIP-mOVA+/+ and OT-I−/−/RIP-mOVA+/+ bone marrow chimeric mice were secondarily adoptively transferred to sublethally irradiated RIP-mOVA−/− mice. To determine the functional impact of these rescued OT-I+ T cells, we first checked whether this selection defect will culminate in insulitis or hyperglycemia in RIP-mOVA−/− mice. However, we found neither insulitis nor hyperglycemia in RIP-mOVA−/− mice, even at 12 wks after bone marrow transfer (data not shown). With the consideration that this may represent a falsely negative result, given the absence of draining lymph nodes in Ltbr−/− mice for priming, and congenitally disturbed spleen microarchitecture, we designed adoptive transfer experiments. Splenocytes from OT-I−/−/RIP-mOVA+/+ and OT-I−/−/RIP-mOVA+/+ bone marrow chimeric mice were secondarily adoptively transferred to sublethally irradiated RIP-mOVA−/− mice to test whether they lead to autoimmune response targeting to the pancreas. Four weeks after secondary transfer, mice were sacrificed and pancreata were checked for lymphocyte infiltration by histostaining. Although there was only occasional perilislet infiltration noted in the RIP-mOVA+ recipients of OT-I−/−/RIP-mOVA+/+ splenocytes, significant infiltration was seen in >75% of the islets analyzed in RIP-mOVA+ recipients.
earlier shown to have impaired medullary migration of SP thymocytes. Similarly to RIP-mOVA mice and RIP-mOVA transgenic system, we present direct evidence that their contribution to organ-specific central tolerance has not been well defined. Aire is considered a major transcriptional factor for the deletion of organ-specific T cells in thymus, but how Aire itself is regulated is also poorly defined. LTβR-deficient mice have peripheral organ lymphocytic infiltration that may be associated with defects in negative selection. Some studies have proposed that the LTβR pathway controls negative selection in an Aire-dependent fashion (12, 13), while others have shown no change in Aire expression in Ltbr−/− mice, challenging this hypothesis (11). In this study, we present direct evidence for impaired negative selection in Ltbr−/− mice. Furthermore, the underlying mechanisms of LTβR signaling that regulate organ-specific central tolerance remained unclear. Over many studies, molecules other than Aire have been noted to be important for negative selection, including LTβR, NF-κB-inducing kinase, TRAF6, RelB, NF-κB2, and CCR7 (6–12). In mice genetically deficient in these factors, there is clear lymphocytic infiltration targeting multiple peripheral organs. However, the mechanism of their contribution to organ-specific central tolerance has not been directly demonstrated. In this study, by taking advantage of OT-I and RIP-mOVA transgenic system, we present direct evidence that LTβR is critical in the thymic negative selection of organ-specific T cells. We further revealed that LTβR controls thymic expression of SLC and ELC, key chemokines controlling the migration of developing thymocytes from the thymic cortex to the medulla (9, 29, 30, 35). Dysregulation of the CCR7-CCR7L (SLC/ELC) axis has been proposed to be associated with impaired central tolerance (9), but it is not clear whether negative selection, Treg generation,

![Image](http://www.jimmunol.org/)

**FIGURE 4.** Impaired thymic negative selection results in peri-insulitis. A, Islet-specific T cells in the peripheral repertoire of Ltbr−/− mice. Splenocytes of OT-I Tg mice in various backgrounds were stained with CD4, CD8, and OT-I tetramer. Representative profiles are shown. Numbers shown are mean ± SD (n = 4–8) for each group. B, Total splenocytes and proportion of indicated clonotypic splenocytes were analyzed. Statistic analysis was performed using Student t test. Mean ± SD (n = 4–8). ***, p < 0.001; NS, no significance. C, Impaired thymic negative selection leads to peri-insulitis. Total splenocytes from bone marrow chimeric mice were secondarily adoptively transferred to sublethally irradiated RIP-mOVA+ mice and pancreatic infiltration was checked 4 wks later by H&E staining. Representative results from two experiments (n = 2 in each group) are shown.

OT-I → RIP-mOVA+Ltbr−/− chimera mice (Fig. 4C). Immunofluorescence staining revealed that the infiltrates were composed of CD8+ T cells, B cells, macrophages, and dendritic cells (data not shown), a similar composition to previously reported insulitis (34). Thus, not only do more OT-I+ cells escape thymic negative selection in the absence of LTβR, but also they carry a functional impact, leading to infiltration of the islets and development of peri-insulitis.

**Thymic chemotactic defect alone is sufficient to impair the negative selection**

To evaluate whether the dysregulation of chemokines in the Ltbr−/− thymus are in themselves sufficient to produce the witnessed defects in thymic negative selection, we used RIP-mOVA+plt mice as OT-I BM transfer recipients. plt thymi harbor an isolated defect in the expression of thymic SLC/ELC and were earlier shown to have impaired medullary migration of SP thymocytes. Similarly to RIP-mOVA+Ltbr−/− recipients, we found significantly increased populations of clonotypic OT-I+ T cells in RIP-mOVA+plt recipients (Fig. 5, A and B). This result confirms regulation of thymocyte chemotaxis as a significant component in the schema of LTβR control of negative selection. However, our current data do not exclude other mechanisms used by LTβR for control of thymic negative selection (see Discussion).

**Discussion**

Thymic medullary epithelial cells are known to play an important role for organ-specific central tolerance, but how these cells are regulated and which ligand or receptors are involved in this process has not been well defined. Aire is considered a major transcriptional factor for the deletion of organ-specific T cells in thymus, but how Aire itself is regulated is also poorly defined. LTβR-deficient mice have peripheral organ lymphocytic infiltration that may be associated with defects in negative selection. Some studies have proposed that the LTβR pathway controls negative selection in an Aire-dependent fashion (12, 13), while others have shown no change in Aire expression in Ltbr−/−, challenging this hypothesis (11). In this study, we present direct evidence for impaired negative selection in Ltbr−/− mice. Furthermore, the underlying mechanisms of LTβR signaling that regulate organ-specific central tolerance remained unclear. Over many studies, molecules other than Aire have been noted to be important for negative selection, including LTβR, NF-κB-inducing kinase, TRAF6, RelB, NF-κB2, and CCR7 (6–12). In mice genetically deficient in these factors, there is clear lymphocytic infiltration targeting multiple peripheral organs. However, the mechanism of their contribution to organ-specific central tolerance has not been directly demonstrated. In this study, by taking advantage of OT-I and RIP-mOVA transgenic system, we present direct evidence that LTβR is critical in the thymic negative selection of organ-specific T cells. We further revealed that LTβR controls thymic expression of SLC and ELC, key chemokines controlling the migration of developing thymocytes from the thymic cortex to the medulla (9, 29, 30, 35). Dysregulation of the CCR7-CCR7L (SLC/ELC) axis has been proposed to be associated with impaired central tolerance (9), but it is not clear whether negative selection, Treg generation,
or anergy is affected. We have now provided the first direct evidence that SLC/ELEC, dependent on LTβR signaling, is required for proper thymic negative selection.

Because it is difficult to trace the fate of organ-specific autoreactive T cells in a native polyclonal repertoire, the OT-I/RIP-mOVA transgenic system was used in this study. Although the mOVA gene expression is under control of RIP in these mice and is supposed to mimic the regulation of insulin 2 expression, a recent study has shown clear evidence that significant differences remain (4). In our system, mice deficient in LTβR fail to demonstrate reductions in thymic RIP-mOVA expression, even while native thymic Ins2 expression is reduced (4). Although RIP-mOVA is an imperfect mimic of native insulin expression, the OT-I—RIP-mOVA system was valuable in dissecting other mechanisms essential for negative selection. As presented earlier, LTβR also controls several key medullary chemokines required for proper thymocyte migration, in a manner similar to what has been found in Aire−/− thymi (4). Given the reduced Aire expression in Libr−/− thymi, it therefore becomes an interesting question of whether these defects in Libr−/− thymi are dependent on Aire function. To address this question, we compared several genes regulated by Aire and/or LTβR. SLC and ELC are two key chemokines known to be involved in the regulation of thymocyte migration into the thymic medulla. Although both are significantly reduced in Libr−/− thymi, neither of them was reduced in Aire−/− thymi (data not shown). This difference suggests that, although both LTβR and Aire are involved in the negative selection process, they may have both overlapping and distinct mechanisms.

Although our data suggest that reduced expression of medullary chemokine (SLC and ELC) expression in Libr−/− thymi is in itself sufficient to disturb thymic negative selection, other mechanisms may also be involved. This is indicated by the partial rescue of OT-I T cells in RIP-mOVAπt mice relative to RIP-mOVA Libr−/− mice. Boehm et al. (13), for example, reported disorganized thymus medulla in Libr−/− mice. Other potential mechanisms of LTβR control of thymic negative selection might reside in function of mTECs or other thymic APCs. Their relative contributions need to be determined in further studies.

Dendritc cells (DC), upon activation, also express CCR7 and become responsive to SLC/ELEC (36). Their migration and localization were also found defective in the secondary lymphoid organs of DLI mice (37). Because DCs have been shown to mediate thymic negative selection in the OT-I/RIP-mOVA system (16), the impaired negative selection in our Libr−/− mice could be also due to improper DCs localization in the thymus. However, tissue staining failed to show any differences in thymic localization of DCs among WT, Libr−/−, and DLI mice. Most DCs were properly located into the medulla region (data not shown and Ref. 9). Thus, thymic migration and localization of DCs may not rely on the CCR7-CCR7L chemotaxis and is unlikely to be a major contributor to impaired thymic negative selection in our Libr−/− mice. Whether our Libr−/− DCs have an intrinsic functional defect that contributes to the Libr−/− phenotype, as was suggested recently (38), remains an interesting question for future study.

Although significantly more OT-I T cells escaped thymic negative in the RIP-mOVA Libr−/− than in RIP-mOVA Libr+/+ hosts, these autoreactive T cells were held in check by the absence of lymph nodes necessary for priming in their Libr−/− hosts. Flora autoimmune developed upon secondary transfer, when this restriction was relieved. The absence of hyperglycemia in these mice, which could be due to the limited number of autoreactive T cells transferred. This is consistent with previous findings that transfer of small numbers of OT-I T cells (< 1 × 108), which is the case in our experiments) into RIP-mOVA+ mice fails to induce diabetes (34).

In conclusion, we have now clearly demonstrated that LTβR is required for the thymic negative selection of organ-specific T cells. Medullary chemokine regulation has been revealed to be an important pathway used by LTβR to control organ-specific negative selection. Our study defines the novel role of LTβR in establishing organ-specific central tolerance, helps to unravel the complex regulation of organ-specific autoimmunity, and provides new targets for future diagnostic and therapeutic intervention of organ-specific autoimmune diseases.

Note added in proof. During the proofreading of this manuscript, the Mathis group had just published their study about the indirect role of LTβR in thymic Aire expression via regulation of mTECs (39). It appears that LTβR controls the development/structural organization of mTECs rather than direct control of Aire expression cell-intrinsically. But the blocking LTβR signaling could reduce the number of mTECs, suggesting LTβR is required for constantly maintenance of organization. Furthermore, the administration of agonistic Ab to LTβR can enhance the expression of Aire within a few hours. While LTβR signaling is certainly required for development/organization of mTECs, the authors revealed insignificant influence of LTβR deficiency on clonal deletion using OT-II/RIP-mOVAgt system. But our present study has revealed a significant influence of LTβR on thymic clonal deletion of OT-I thymocytes.

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Disclosures

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References