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Lymphotixin Signal Promotes Thymic Organogenesis by Eliciting RANK Expression in the Embryonic Thymic Stroma

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It has recently become clear that signals mediated by members of the TNFR superfamily, including lymphotixin-β receptor (LTβR), receptor activator for NF-κB (RANK), and CD40, play essential roles in organizing the integrity of medullary thymic epithelial cells (mTECs) required for the establishment of self-tolerance. However, details of the mechanism responsible for the unique and cooperative action of individual and multiple TNFR superfamily members during mTEC differentiation still remain enigmatic. In this study, we show that the LTβR signal upregulates expression of RANK in the thymic stroma, thereby promoting accessibility to the RANK ligand necessary for mTEC differentiation. Cooperation between the LTβR and RANK signals for optimal mTEC differentiation was underscored by the exaggerated defect of thymic organogenesis observed in mice doubly deficient for these signals. In contrast, we observed little cooperation between the LTβR and CD40 signals. Thus, the LTβR signal exhibits a novel and unique function in promoting RANK activity for mTEC organization, indicating a link between thymic organogenesis mediated by multiple cytokine signals and the control of autoimmunity. The Journal of Immunology, 2011, 186: 5047–5057.

The thymus provides a microenvironment in which T cells gain the ability to discriminate between self and nonself (1, 2). Developing thymocytes recognizing self-Ags in the thymic stroma either develop into immunoregulatory T cells or are deleted by apoptosis, depending on the strength and/or nature of the reactivity with self-Ags (3). Medullary thymic epithelial cells (mTECs) seem to play pivotal roles in this cross talk with thymocytes by expressing a set of self-Ags (4). This scenario has been supported by gene expression studies showing that mTECs are a specialized cell type in which promiscuous expression of a broad range of tissue-restricted Ag (TRA) genes is an autonomous property (1). Remarkably, mice deficient for autoimmune regulator (AIRE), a mouse homolog of the gene responsible for the development of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy in humans, show reduced expression of many, though not all, TRA genes from mTECs, which has been implicated in the development of autoimmune pathogenesis caused by AIRE deficiency (5–8). The significance of TRA gene expression in the thymic stroma for the establishment of central tolerance has been further supported by the fact that mice deficient in several signal-transducing molecules and NF-κB components downstream of TNFR superfamily (TNFRsf) members have a similar or, in many cases, more profound reduction of TRA gene expression (9–15), and it has been shown that embryonic thymi taken from these mice induce autoimmune disease phenotypes when grafted into recipient mice. In this regard, it is important to emphasize that, in contrast to AIRE-deficient mice, the reduction of TRA gene expression in these mice is strongly associated with a defect in the mTEC differentiation program; the thymi show easily discernable structural abnormalities such as a small medulla, a paucity of mTECs including AIRE-expressing cells, and loss of architectural integrity of mTECs, as assessed by morphological observation and flow cytometric analysis. Subsequently, one of the upstream TNFRsf members responsible for thymic organogenesis was iden-

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Abbreviations used in this article: Aire, autoimmune regulator; 2-DG, 2-deoxyglucose; DKO, double knockout; E, embryonic day; EpCAM, epithelial cell adhesion molecule; FTOC, fetal thymus organ culture; IKKx, IkB kinase; RANK, receptor activator for NF-κB; RANKL, lymphotixin-β receptor; MHC II, MHC class II; mLT, membrane-bound form of lymphotixin; mTEC, medullary thymic epithelial cell; NIK, NF-κB–inducing kinase; RANK, receptor activator for NF-κB; RANKL, RANK ligand; Sap1, salivary protein 1; sLT, secreted form of lymphotixin; TNFRsf, TNFR superfamily; TRA, tissue-restricted Ag; TRAF, TNFR-associated factor; UEA-1, Ulex europaeus agglutinin 1.

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tified as receptor activator for NF-κB (RANK) (16). These studies clearly suggested a link between cytokine-mediated thymic organogenesis and the establishment of central tolerance (1, 17). Besides the individual role of each TNFRsf signal in the production and maintenance of mTECs, combined actions of multiple TNFRsf members, RANK and CD40, have recently been demonstrated (18) (see below).

Among the TNFRsf members studied so far, RANK seems to have the strongest impact on the organization of mTECs, at least on the basis of examination of the individual thymic phenotypes of mice deficient for each of the TNFRsf members (19). It has been suggested that RANK is required for the initial differentiation phase of mTECs during embryogenesis and that lymphoid tissue inducer cells with a CD4+CD3− cell signature are the main source of the RANK ligand (RANKL) in this period (16). In the postnatal phase, the CD40 signal also becomes indispensable (20), and cooperation between RANK and CD40 signals is required for the optimal organization and homeostasis of mTECs (18). Furthermore, mature single-positive thymocytes play essential roles in providing the ligands for RANK and CD40 in postnatal mice (21, 22).

Compared with the essential roles of the RANK and CD40 signals in thymic organogenesis, the functional significance of the lymphotixin-β receptor (LTβR) signal for thymic organogenesis is still poorly defined (23). This is rather unexpected when considering the dominant role of the LTβR signal over the RANK signal in secondary lymphoid organogenesis; mice deficient in LTβR (Ltbr-knockout [KO]) lack both lymph nodes and Peyer’s patches and show a disorganized splenic structure, whereas mice deficient in RANK (Rank-KO) lack only lymph nodes, but possess Peyer’s patches and have an apparently normal splenic structure (24–28). Although thymic alterations such as a reduction in the number of Ulex europaeus agglutinin 1 (UEA-1)+ mature mTECs and defective three-dimensional organization of mTECs have been noticed in postnatal Ltbr-KO (29), production of Aire-expressing mTECs remains unchanged in the absence of the LTβR signal (9, 16, 29–32). This relatively mild phenotype in Ltbr-KO is more remarkable when one considers the severe thymic disorganization characterized by lack of Aire+ mTECs in a natural strain of mice with NF-kB–inducing kinase (NIK) mutation [aly mice (33, 34)] (9, 29) or in mice deficient in IκB kinase α (IκKα), downstream of NIK (10, 11). In marked contrast to the different phenotypes of thymic organogenesis, Ltbr-KO and aly mice (and Ikkα-KO) exhibit common abnormalities of secondary lymphoid organogenesis (35), suggesting that NIK-IκKα constitutes a component downstream of LTβR that is essential for secondary lymphoid organogenesis, whereas for thymic organogenesis, NIK-IκKα additionally acts downstream of other receptor(s) beyond LTβR. In this context, it would be important to look for any cooperative action of LTβR together with other TNFRsf members, especially RANK and CD40. Accordingly, an exact picture of the role of LTβR in organization of mTEC integrity is still lacking (23, 30–32).

We have approached these issues by generating both Ltbr/Cd40 double-deficient mice (Ltbr/Cd40-double KO [DKO]) and Rank ligand/Ltbr double-deficient mice (Rankl/Ltbr-DKO), using fetal thymus organ culture (FTOC) to further explore the mechanisms underlying the cooperation by TNFRsf members. The results suggested that the LTβR signal regulates the development of mTECs cooperatively with the RANK signal, but not with the CD40 signal. Through this newly identified cooperation between LTβR and RANK, the LTβR signal was found to upregulate the expression of RANK in immature mTECs (most likely presumptive mTEC progenitors), thereby promoting subsequent differentiation of the mTECs after induction by the RANK signal. Thus, cytokine signals mediated by multiple TNFRsf members exhibit unique cooperation to achieve the mTEC organization required for establishment of self-tolerance in the thymus.

**Materials and Methods**

**Mice**

Ltbr-KO (accession number CBDO531K at the Center for Developmental Biology, RIKEN Kobe) were generated by gene targeting. Briefly, the targeting vector was constructed by replacing a 3′ portion of Ltb exon 1 together with the whole of exon 2–5 with a LacZ and neomycin resistance gene (LacZ-neo) (Supplemental Fig. I.4). The targeting vector was introduced into T72 embryonic stem cells, and the homologous recombination clones were first identified by PCR and confirmed by Southern blot analysis (37). After the targeted embryonic stem cells had been injected into morula-stage embryos, the resulting chimeric male mice were mated with C57BL/6 females (CLEA Japan) to establish germine transmission (Supplemental Fig. I.B–D). Lta-KO (38), Light-KO (39), Rankl-KO (40), and Aire/GFP-knockin mice (K1 (41) were generated as described previously, aly mice (33, 34) and BALB/cem8 mice were purchased from CLEA Japan, and CD40-KO (42) were from The Jackson Laboratory. The mice were maintained under pathogen-free conditions, and the protocols used in this study were in accordance with the Guidelines for Animal Experimentation of Tokushima University School of Medicine and conducted with the approval of the RIKEN Kobe Animal Experiment Committee.

**Immunohistochemistry**

Immunohistochemical analysis of the thymus with rat ER-TR5 mAb (43), rat anti-epithelial cell adhesion molecule (EpCAM) mAb (BD Biosciences), and rabbit polyclonal anti-keratin 5 (K5) Ab (Covance) was performed as described previously (41). Rat anti-Aire mAb (clone RF33-1; IgG1) recognizing the COOH-terminal portion of mouse Aire was produced in our laboratory. UEA-1 was from Vector Laboratories. For the detection of autoantibodies, serum from untreated mice or thymic chimeras was incubated with various organs obtained from Rag2-deficient mice. Alexa 488-conjugated anti-mouse IgG Ab (Invitrogen) was used for the detection.

**TEC preparation and flow cytometric analysis**

Preparation of TECs and flow cytometric analysis with an FACScalibur (BD Biosciences) were performed as described previously (18, 41). The mAbs used were anti-CD4, anti-CD8, anti-CD45, anti–TER-119, anti-EpCAM, anti-CD80, anti-B220, anti-I–A/E, and anti-Ly-51, all purchased from ebioscience except for anti-EpCAM mAb.

**Thymus grafting and FTOC**

Thymus grafting was performed as described previously (9). Briefly, thymic lobes were isolated from embryos at 14.5 d postcoitus and then cultured for 4 d on top of Nucleopore filters (Whatman) placed on RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), 2 mM l-glutamine. 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 mM 2-ME, hereafter referred to as R10, and supplemented with 1.35 mM 2-deoxyglucose (Sigma-Aldrich). Five thymic lobes were grafted under the renal capsule of BALB/c mice. After 6–8 wk, reconstitution of peripheral T cells was determined by flow cytometric analysis, and then thymic chimeras were used for analyses. For the assessment of mTEC differentiation using FTOC, 2-DG–treated fetal thymic organ culture was cultured in R10 supplemented with rRANKL (1 μg/ml; Wako), CD40L (5 μg/ml; R&D Systems), agonistic anti-LTβR mAb AC.H6 (2 μg/ml (44)), and their combinations, as described previously (18). mTEC differentiation and induction of gene expression from thymic stroma were assessed by flow cytometric analysis and real-time PCR, respectively.

**Real-time PCR and semiquantitative RT-PCR**

RNA was extracted from thymic stroma with SuperScript III RT Kits (Invitrogen) in accordance with the manufacturer’s instructions. Real-time PCR (RT-PCR) and semiquantitative RT-PCR (9) were performed as described previously. The primers and the probes for the real-time PCR are as follows. Rank primers: 5′-TCTTGGGCTTTCTCTGACAT-3′ and 5′-CACATCTGGATCCCAAGAT-3′; Rank probe: 5′-FAM-TCAAGATCTGCAACTCCTCTG-3′; salivary gland primers: 5′-ACCTGGTGTGCTCTGCTTTTT-3′ and 5′-TGGACACTGAATCCAGGAAATC-3′; Salp1 primers: 5′-ACTCTGTGGTGTCTGCTTTTT-3′ and 5′-TGGACACTGAATCCAGGAAATC-3′; Salp1 probe: 5′-FAM-TTCAACGACAGAATCAGGACTTCAAGA-3′; Hprt primers: 5′-TGAAGAGCTACGTGTAATGATCGTACAC-3′ and 5′-AGCAAGCTTCACACCTTAA-3′.
Roles of LTβR and its ligands in thymic organogenesis

To gain an insight into the precise roles of the LTβR signal in thymic organogenesis, we first performed immunohistochemical analysis using mice deficient in LTβR and mice deficient in two known ligands of LTβR (i.e., the membrane-bound form of lymphotixin [mLT] and LIGHT) (27) (Supplemental Fig. 2A). Immunohistochemical analyses demonstrated that wild-type mouse thymus contained medullary areas that varied in size, as recognized by reactivity with the ER-TR5 mAb (43), some of the larger areas probably containing a few medullary islets (45) (Fig. 1A).

Areas binding with UEA-1 showed considerable overlap with ER-TR5+ medullary areas. In contrast, the individual ER-TR5+ medullary areas in Lta-KO, which lack both mLT (Lto1β2) and the secreted form of LT (Lto3; sLT), were smaller in size and less frequently connected with each other, as also demonstrated by UEA-1 staining. At higher magnification, disruption of the three-dimensional organization of mTECs, as assessed by UEA-1 staining, was also evident in Lta-KO (Supplemental Fig. 2B). Of note, it has been reported that Ltb-KO showed no significant reduction in the total number of mTECs, although changes in the UEA-1+ cell distribution pattern have been pointed out (29) (Fig. 1B).

Lght-KO showed no major alteration of medullary organization, as assessed by using ER-TR5 mAb and UEA-1 staining, at either low or high magnification (Fig. 1A, Supplemental Fig. 2B). Importantly, however, Lta/Light-DKO showed even smaller medullas than those in Lta-KO at lower magnification (Fig. 1A); the size of each UEA-1+ area was further reduced in comparison with that in Lta-KO. It was also noteworthy that, in Lta/Light-DKO, each UEA-1+ area was significantly smaller than the corresponding ER-TR5+ area, giving the UEA-1+ areas an appearance resembling small nodules, a feature that was not evident in Lta-KO (Fig. 1A). At higher magnification, the Lta/Light-DKO thymus also exhibited a more disorganized three-dimensional organization of UEA-1+ mTECs than was the case in Lta-KO (Supplemental Fig. 2B).

The exaggerated thymic disorganization due to additional lack of LIGHT in Lta-KO is in marked contrast to that seen in Ltb-KO (lacking only mLT and not sLT), in which the introduction of LIGHT in Lta-KO showed no additional defect of medullary organization beyond that evident in single Ltb-KO (compare Fig. 1A and 1C, Supplemental Fig. 2B). Flow cytometric analyses also supported this view, except that there was a small reduction of mTECs expressing MHC IIlow (mTECIIlow), but not MHC IIhigh (mTECIIhigh) [(18), Fig. 2A], in Ltb/Cd40-DKO compared with single Ltb-KO (Supplemental Fig. 3B). This was in marked contrast to that resulting from cooperation between the LTβR and CD40 signals (18), indicating the existence of unique functional cooperation among the TNFRsf members. The apparent lack of cooperation between LTβR and CD40 was also supported by the fact that production of Aire+ mTECs was not impaired in either Ltb-KO or Ltb/Cd40-DKO (Supplemental Fig. 4A); although the total numbers of Aire+ mTECs per medulla were again reduced in both Ltb-KO and Ltb/Cd40-DKO due to the smaller size of each medulla, the relative abundance of mTECs expressing Aire among UEA-1+ mTECs was not affected in these strains (Supplemental Fig. 4B).

The autoimmune pathologies in untreated mice described above were examined histologically together with autoantibody production at between 16 and 20 wk of age. Lta-KO, Lta/Light-DKO, and Ltb-KO showed lymphoid cell aggregates in the liver and lung (Supplemental Fig. 5A, 5B), consisting of B220+ CD45+ CD8+ lymphocytes (data not shown). Interestingly, introduction of CD40 deficiency into Ltb-KO (i.e., Ltb/Cd40-DKO) abolished these pathological changes (Supplemental Fig. 5A). Overall, production of IgG-class autoantibodies was not obvious in untreated mice, including Ltb/Cd40-DKO (Supplemental Fig. 5C) [partly due to the defect of class switching in these strains (24)], and thus the autoimmune pathogenesis seen in Ltb-KO was not further accelerated by additional lack of the CD40 signal.

The lack of an obvious combined effect of the LTβR and CD40 signals for establishing central tolerance was also examined in thymus graft experiments. Lymphoid cell infiltration was observed in the stomach, salivary glands, and pancreas of nude mice grafted with embryonic thymus from Ltb-KO (Supplemental Fig. 6A, 6B), and sera from these recipient mice contained IgG-class autoantibodies against gastric mucosa (Supplemental Fig. 6C, 6D), consistent with a previous report (30). Despite the retention of thymic organization in Cd40-KO, as described above, nude mice grafted with embryonic thymus from Cd40-KO showed patho-
logical changes in the stomach, salivary gland, and pancreas (Supplementary Fig. 6A) together with autoantibody production against these organs (Supplemental Fig. 6C). Importantly, beyond the lack of LTβR or CD40 signal alone, no augmentation of autoimmune pathological changes together with autoantibody production was discernable in nude mice grafted with embryonic thymi from Ltbr/Cd40-DKO (Supplemental Fig. 6A, 6C). Taken together, these results suggest that there is no obvious cooperation between the LTβR and CD40 signals in the thymic organization required for establishment of self-tolerance, in marked contrast to the co-operation between the RANK and CD40 signals we reported previously (18).

Cooperation between LTβR and RANK signals for thymic organization

We next addressed whether the LTβR and RANK signals exert any cooperative action on thymic organogenesis by establishing mice deficient in both signals (i.e., mice deficient for both LTβR and RANKL [Rankl/Ltbr-DKO]) and compared them with those deficient in LTβR (Ltbr-KO) or RANKL (Rankl-KO) alone. Flow cytometric analysis demonstrated that both Ltbr-KO and Rankl-KO showed markedly more severe reduction of mTECs than mice with each deficiency alone. Rankl/Cd40-DKO showed the most severe reduction of mTECs for both mTEChigh and mTEClow among the strains tested. Percentages of cortical thymic epithelial cells remained intact in all of the strains (Fig. 2B).

Immunohistochemical staining of the thymic medulla using anti-K5 Ab, UEA-1 binding, and anti-EpCAM/anti-Aire mAbs gave results consistent with those obtained by flow cytometry (Fig. 2C); disruption of UEA-1 binding together with the defective production of Aire+ mTECs was more obvious in Rankl/Ltbr-DKO than in Ltbr-KO or Rankl-KO, and Rankl/Cd40-DKO again showed the most severe defect. Thus, the role of the LTβR signal in the organization of thymic organogenesis was underscored by its cooperative action with RANK.

LTβR elicits RANK expression in mTECs of fetal thymus

Having established that the LTβR signal cooperates with the RANK signal, but not with the CD40 signal, to ensure the correct development of mTECs, we investigated the mechanism of this phenomenon using FTOC. We first prepared 2-DG–treated embryonic thymic stroma from Aire/GFP-KI (41) at embryonic day (E) 14.5 and stimulated them with rRANKL, rCD40L, ago-
nistic anti-LTβR mAb (44), and their multiple combinations. Stimulation with rRANKL clearly induced the development of mTECs expressing the UEA-1 ligand (Fig. 3A), Aire (as assessed by GFP expression) (Fig. 3B), and the Sap1 gene (Fig. 3C), an Aire-dependent TRA gene (5). In contrast, stimulation with rCD40L or agonistic anti-LTβR mAb had only a modest or no effect on these activities, respectively, which appeared to accord well with the phenotypes of mice that were deficient in each TNFR signal; the thymic phenotypes of Rankl-KO were more obvious than those of Cd40-KO or Ltbr-KO (Figs. 1, 2). We then investigated the combined effect of these stimuli on the induction of mTECs together with TRA gene expression in FTOC. Addition of rCD40L to rRANKL stimulation exerted no additional effect on mTEC development (Fig. 3A, 3B) and Sap1 gene expression (Fig. 3C) beyond that induced by rRANKL alone. In contrast, addition of agonistic anti-LTβR mAb to rRANKL stimulation clearly augmented mTEC development and Sap1 gene expression. This seems remarkable when considering that LTβR stimulation alone did not have any obvious effect on mTEC development and Sap1 gene expression. When agonistic anti-LTβR mAb and rCD40L were added together in FTOC, we observed no obvious effect, perhaps being consistent with the phenotypes of mice doubly deficient in LTβR and CD40. Thus, the cytokine-directed process of mTEC differentiation assessed using FTOC well reflects the in vivo developmental program of mTECs induced by the signals of individual members of the TNFRsf or their combinations.

Because the LTβR signal enhanced the differentiation of mature mTECs induced by RANK in FTOC, despite the fact that the LTβR signal alone did not induce such effects (Fig. 3), we hypothesized that the LTβR signal might facilitate more efficient reception of the RANK signal by mTECs. We therefore examined the effect of LTβR on the induction of RANK expression in FTOC and found that RANK expression in the thymic stroma detected by real-time PCR was enhanced at day 4 after treatment with agonistic anti-LTβR mAb (Fig. 4A). In contrast, addition of rCD40L to FTOC induced no such effect. Interestingly, stimulation with rRANKL also upregulated the expression of RANK, suggesting a possible self-amplification mechanism that might explain, at least in part, why RANK has the strongest impact among TNFRsf members known so far on the induction of mTEC differentiation (19). Currently available mAbs directed against RANK did not allow us to evaluate the expression of RANK in the thymic stroma using flow cytometry (Y. Mouri, T. Akiyama, and M. Matsumoto, unpublished observations).

The expression of RANK in the thymic stroma induced by LTβR was not confined to the experimental setting of FTOC. RANK expression in the embryonic thymus of Ltbr-KO was indeed reduced in comparison with that in control heterozygous littermates.

**FIGURE 2.** Differential effects of multiple combinations of TNFRsf signal loss on mTEC differentiation. A, mTEC differentiation in the thymus of adult mice deficient in TNFRsf signals was assessed by flow-cytometric analysis using anti-MHC II (I-A/I-E) mAb and UEA-1 binding. Thymic cell suspensions were stained with UEA-1, anti-MHC II, anti-CD45, and TER-119 mAbs and analyzed by flow cytometry. A subset of MHC IIhigh in UEA-1+ cells (gated for CD45−TER-119− cells) is labeled as high, and a subset of MHC IIlow in UEA-1+ cells is labeled as low in the figure (rightmost panel). Percentages of the cells in the indicated areas are included. B, Summary of the ratios of mTECs (subsets of UEA-1−MHC IIhigh and UEA-1−MHC IIlow) and cortical thymic epithelial cells (subsets of UEA-1−MHC II) in thymic stromal cells (gated for CD45−TER-119− cells) of the mice shown in A. Note that concomitant loss of RANKL and LTβR exerted an additive effect of each deficiency on mTEC differentiation, although it was less profound than that due to concomitant loss of RANKL and CD40. C, Organization of the thymic medulla was assessed by immunohistochemistry using anti-K5 mAb (top panel), UEA-1 binding (middle panel), and anti-EpCAM (in red)/anti-Aire (in green) mAbs (bottom panel). Scale bar, 200 μm. One representative experiment from a total of more than three repeats is shown. *p < 0.05, **p < 0.01, Student t test. n = 3 for each genotype.
indicating a requirement for the LTβR signal to ensure appropriate RANK expression at the embryonic stage in vivo. The observed upregulation of RANK induced by the LTβR signal can be explained in terms of expansion of the stromal mTEC population expressing RANK. Alternatively, the LTβR signal may enhance the per-cell expression of RANK in pre-existing mTECs. To investigate these possibilities, we performed a kinetic study of RANK expression together with an assessment of mTEC development using FTOC. We harvested RNAs from total embryonic thymi cultured with agonistic anti-LTβR mAb or rRANKL at different time points. We observed upregulation of RANK transcripts as early as 6 h after stimulation with agonistic anti-LTβR mAb, and RANK expression reached a plateau at 24 h, remaining at the same level thereafter until at least 72 h of observation (Fig. 4C, upper panel). mTEC development assessed by flow cytometry in the same kinetic study showed no obvious effect after stimulation with agonistic anti-LTβR mAb during the course of observation (Fig. 4D, middle panel) as analyzed on day 4 (Fig. 3A). In contrast, rRANKL stimulation upregulated RANK transcripts more slowly, and this upregulation continued for 72 h (Fig. 4C, upper panel). Concomitant induction of Sap1 gene expression was observed at 72 h only after rRANKL stimulation (Fig. 4C, lower panel). These results are consistent with the idea that the LTβR signal induces RANK expression in pre-existing mTECs on a per-cell basis, rather by expanding the population of RANK-expressing mTECs. In contrast, upregulation of RANK induced by rRANKL appeared to be associated with expansion of the RANK-expressing mTECs. In contrast, upregulation of RANK induced by rRANKL appeared to be associated with expansion of the RANK-expressing mTECs. In contrast, upregulation of RANK induced by rRANKL appeared to be associated with expansion of the RANK-expressing mTECs. In contrast, upregulation of RANK induced by rRANKL appeared to be associated with expansion of the RANK-expressing mTECs. The LTβR signal conditions mTECs to receive the RANK signal for differentiation As we had clarified that the development of mature mTECs triggered by the RANK signal is enhanced by the LTβR signal through upregulation of RANK in mTECs, we hypothesized that LTβR helps to condition mTECs for receiving the RANK signal to fa-
cilitate further differentiation; the LTβR signal might act on immature mTECs prior to their differentiation mediated by the RANK signal. We therefore examined whether upregulation of RANK by the LTβR signal is prerequisite per se for the combined effect of LTβR and RANK by sequential treatment of embryonic thymi with agonistic anti-LTβR mAb and rRANKL. FTOC with agonistic anti-LTβR mAb for the first 3.5 d followed by switching to rRANKL stimulation for an additional 3 d resulted in the development of more mature mTECs in comparison with those obtained by FTOC using a reverse order of treatment (i.e., FTOC with rRANKL for the first 3.5 d, followed by switching to agonistic anti-LTβR mAb for an additional 3 d) (Fig. 5). Treatment with agonistic anti-LTβR mAb and rRANKL throughout the culture period (i.e., for 6.5 d) exerted a minimal and a strong effect, respectively. These results support the idea that the LTβR signal makes the embryonic thymic stroma more receptive to the RANK signal for mTEC differentiation through upregulation of RANK expression.

The LTβR signal regulates the initial development of mTECs in the fetus

Adult Ltbr-KO showed alteration of the thymic medullary architecture (Fig. 1). However, based on our results demonstrating the ability of the LTβR signal to promote accessibility to the RANK signal in FTOC, and the fact that the RANK signal promotes mTEC differentiation at the embryonic stage (16), we assumed that the process of mTEC development at the embryonic stage might already be affected in the absence of LTβR. We therefore directly examined the development of mTECs from Ltbr-KO at E14.5 and found that this strain had lower numbers of UEA-1+ mTECs expressing MHC II than their control littermates, as assessed by flow cytometry (Fig. 6A, 6B). Thus, although the LTβR signal is not absolutely necessary for mTEC development, it plays an important role for the promotion of thymic organogenesis by optimizing the RANK signal through induction of RANK expression in the thymic stroma. In this context, it is

FIGURE 4. LTβR signal elicits RANK expression in the embryonic thymic stroma. A. Embryonic thymi were stimulated with agonistic anti-LTβR mAb, rRANKL, or rCD40L for 4 d, and expression of the Rank gene was assessed by real-time PCR, as described in the legend for Fig. 3C. One representative experiment from a total of four repeats is shown. B. Rank gene expression from embryonic thymi at E14.5 was assessed by real-time PCR. RNAs were harvested from total thymi of five heterozygous (control; clear circles) and homozygous LTβR-deficient mice (solid circles) for each. One circle corresponds to one mouse analyzed. C. Kinetics of Rank and Sap1 gene expression after stimulation with agonistic anti-LTβR mAb (gray columns) or rRANKL (black columns) at indicated time points. White columns, medium alone. Rank gene expression from thymic stroma induced by the LTβR signal was rapid and preceded that induced by the RANK signal. Relative expression was calculated by determining the values of Rank (upper panel) and Sap1 gene (lower panel) expression assessed by real-time PCR at each time point, using that in the absence of stimuli as 1. One representative experiment from a total of three repeats is shown. D. mTEC development assessed by flow cytometric analysis in the same kinetic study as that demonstrated in C. Cells were analyzed for UEA-1 binding and MHC II expression after gating for CD45 TER-119 EpCAM+ cells. **p < 0.01.

FIGURE 5. LTβR signal conditions mTECs to receive the RANK signal for differentiation. FTOCs were supplemented with rRANKL or with agonistic anti-LTβR mAb for the first 3.5 d, followed by switching to agonistic anti-LTβR mAb and rRANKL for an additional 3 d. FTOC supplemented with agonistic anti-LTβR mAb followed by rRANKL showed more mature mTEC development compared with that from FTOC treated with rRANKL, followed by agonistic anti-LTβR mAb; the latter culture yielded results similar to those for FTOC supplemented with rRANKL for 3.5 d followed by no supplementation for an additional 3 d (upper left panel). Treatment with agonistic anti-LTβR mAb and rRANKL throughout the culture period (i.e., for 6.5 d) exerted a minimal and a strong effect, respectively. Cells were analyzed for UEA-1 binding and MHC II expression after gating for CD45 TER-119 EpCAM+ cells. Percentages of cells in the indicated regions are included. One representative experiment from a total of five repeats is shown.
important to emphasize that lack of the LTβR signal at the embryonic stage results in smaller ER-TR5+ medullas with disruption of the three-dimensional organization of UEA-1+ mTECs at the adult stage (Fig. 1), which is associated with a propensity for development of autoimmunity. Thus, the LTβR signal controls the initial phase of mTEC development at the embryonic stage, thus playing an important role in the control of autoimmunity in later life.

Genes encoding both receptors (i.e., LTβR and RANK) and their corresponding ligands (i.e., LTα/LTβ and RANKL) were expressed in embryonic thymi (Supplemental Fig. 7). Although CD40 was expressed in embryonic thymi, CD40L expression was low, suggesting that the CD40L–CD40 axis may not be involved in the initial phase of thymic organogenesis at the embryonic stage. Instead, the CD40L–CD40 axis may become relevant after the postnatal stage (18, 20, 21).

**Differential requirement of NIK and TNFR-associated factor 6 for upregulation of RANK expression induced by the LTβR and RANK signals**

Given that upregulation of RANK by the LTβR signal is prerequisite for the normal developmental process of mTECs, we investigated the signaling pathways involved in this process. We first examined whether upregulation of RANK induced by the LTβR or RANK signal requires normal NIK activity. In contrast to FTOC using material from control aly/+ mice, FTOC using material from NIK mutant aly mice showed neither mTEC development (Fig. 7A) nor upregulation of RANK expression (Fig. 7B) after any of the tested stimuli, indicating that upregulation of RANK expression by the LTβR or RANK signal is totally NIK dependent.

We then investigated the requirement for TNFR-associated factor (TRAF) 6 in this process. FTOC using material from Traf6-KO demonstrated no mTEC development after rRANKL stimulation (18). Similarly, RANK expression induced by rRANKL was greatly diminished (Fig. 7C). In contrast, upregulation of RANK induced by the LTβR signal was indistinguishable between control mice and Traf6-KO, in agreement with the minimal involvement of TRAF6 downstream of LTβR assessed with embryonic fibroblasts (12). These results suggest that NIK is indispensable for the upregulation of RANK induced by both the LTβR and RANK signals, whereas TRAF6 is required for the activity induced by RANK, but not by LTβR.

**Discussion**

In the current study, we investigated the roles of the LTβR signal in thymic organogenesis, focusing especially on its functional cooperation with two other TNFRsf members, RANK and CD40. Our results demonstrated that the LTβR signal orchestrates thymic organogenesis at the embryonic stage through cooperation with the RANK signal, but not with the CD40 signal. The LTβR signal upregulated RANK expression in stromal cells, allowing immature mTECs, possibly including mTEC progenitors, to become more receptive to the ligand for RANK, thus promoting their differentiation. This supportive role of the LTβR signal in RANK activity may at least partly explain why the thymic phenotype of Libr-KO is relatively mild in comparison with that of mice deficient in the RANK signal, although the LTβR signal in mTECs is no doubt indispensable for preventing autoimmunity.

One of the most intriguing observations in this study was the different effects of various combinations of deficiencies of three TNFRsf members (LTβR, RANK, and CD40) on mTEC development, which were not easily predictable from the phenotypes of each TNFR deficiency alone. The order of the gross severity of the defective thymic organization based on medullary size and the composition and/or structural organization of stromal cells assessed using immunohistochemistry and flow cytometric analysis of mTECs was: Rank-KO (most severe; assuming that the Rankl-KO we analyzed had an equivalent phenotype to Rank-KO), Libr-KO (intermediate), and Cd40-KO (mildest). Nevertheless, Rankl/Cd40-DKO showed a more severe thymic phenotype than that of Rankl/Libr-DKO. Furthermore, Libr/Cd40-DKO showed no additional defect, if any, beyond that seen in Libr-KO. This differential effect of various combinations of TNFRsf deficiencies might be due to the functional characteristics of each TNFR signal in terms of the developmental stage of action (i.e., fetus versus postnatal), the responding cell type(s) (e.g., precursor, immature or mature mTECs), the mode of action (i.e., difference in the target genes controlled by the transcriptional activities), and their combinations. We speculate that the actions of the RANK and CD40 signals have significant redundancy at critical point(s) for mTEC differentiation, because the defect of thymic organization in Rankl/Cd40-DKO was much more profound than that in Rankl-KO, despite the apparently normal thymic architecture of Cd40-KO. Consistent with this view, the combined effect of stimuli exerted by rRANKL and rCD40L on mTEC differentiation in FTOC was rather weak; addition of rCD40L did not induce any further effect other than that of rRANKL. In contrast, the actions of the RANK and LTβR signals seem to be distinct, and the simultaneous stimuli exerted by rRANKL and agonistic anti-LTβR mAb showed an additive effect. Indeed, we demonstrated that the LTβR signal contributes to mTEC differentiation in a stepwise manner with the RANK signal; the LTβR signal elicits RANK expression, thereby permitting RANKL to efficiently control the differentiation program of immature mTECs thereafter. In contrast, crossing Cd40-KO onto Libr-KO resulted in no obvious effect beyond the dominant phenotype attributable to LTβR deficiency, and simultaneous stimulation with agonistic anti-LTβR mAb and rCD40L exerted no effect in FTOC, suggesting that there is little functional association between the LTβR and CD40 signals in thymic organogenesis. These results illustrated many aspects of the unique and cooperative action of multiple TNFRsf members in the cytokine-mediated mTEC differentiation program.

Even after alteration of the thymic structure in postnatal Libr-KO had been reported (29), the precise roles of LTβR in thymic organogenesis remained enigmatic and were investigated only...
We have suggested that a defect in the upregulation of RANK in immature mTECs through loss of the LTβR signal may play a more important role in the initial phase of mTEC development, whereas the RANK signal ensures the differentiation of mTECs together with their maintenance in the later stages of embryo development.

In the search for additional receptor(s) beyond LTβR that could fill the gap between LTβR deficiency and NIK mutation or IKKα deficiency for thymic phenotypes, we hypothesized that such recently. Expression of CCL19/EBL-1 ligand chemokine was reduced in mTECs from Lta-KO, Ltb-KO, or Ltbr-KO (31, 36). Conversely, in vivo agonistic stimulation of LTβR in adult Lta-KO has been demonstrated to upregulate expression of the CCL19 gene from mTEClow (31). Interestingly, in vivo agonistic stimulation of LTβR in Lta-KO also increased the UEA-1 binding to mTEClow together with an increase in the expression of some TRA genes such as Crp. Among the genes upregulated by in vivo agonistic stimulation of LTβR in adult mice (31) or genes differentially expressed in mTECs between postnatal Ltb-KO and control mice (30, 32), the Rank gene, which we identified in the current study as an LTβR-responsive gene relevant to thymic organogenesis in the fetus, was not detected in these previous studies. One obvious reason for this could be that the effect of the LTβR signal in thymic organogenesis is dependent on the developmental stage (i.e., fetal versus postnatal) as suggested by LTβR-mediated secondary lymphoid organogenesis; lymph nodes developed in Lta-KO when agonistic LTβR stimulation was initiated in utero before day 17 of gestation, whereas no such effect was observed when the same treatment was applied beyond this developmental window (44). We speculate that analysis of gene expression in mTECs at the embryonic stage might identify Rank as a gene that is differentially expressed between Ltb-KO and control mice. Thus, factors influencing thymic organogenesis need to be studied in a developmental stage-dependent manner, a precedent of which is the main source of RANKL; in the initial differentiation phase of embryos, Aire+ mTECs develop through interaction with lymphoid tissue inducer cells expressing the RANKL, whereas in the postnatal stage, Aire+ mTECs are maintained by the RANKL (together with the CD40L) provided by mature CD4 single-positive T cells to ensure their turnover and/or survival (16, 18, 20–22).

We have suggested that a defect in the upregulation of RANK in immature mTECs through loss of the LTβR signal at the embryonic stage results in a defect of mTEC organization in the postnatal period; the size of each medulla was smaller, and they were poorly connected to each other in the thymus of adult Ltbr-KO. Given that many aspects of thymic organization are receptive to thymocyte cross talk even in the postnatal period (47), the defective thymic organization in adult Ltbr-KO suggests that the requirement for the LTβR signal to ensure proper organization of the thymic epithelium may not be confined to the embryonic stage. Instead, the continued presence of the LTβR signal provided by thymocytes as a cross talk may also play a role in homeostasis of the thymic microenvironment during the postnatal period. Indeed, a recent study has suggested that continued mTEC development to the stage of expression of involucrin, a marker of terminally differentiated epithelium (48), maps to activation of the LTβR signal pathway by mature thymocytes in adult mice (49).

Although both the LTβR and RANK signals augmented the expression of RANK in the thymic stroma, there was a kinetic and functional difference between the two. RANK expression induced by the LTβR signal was rapid, and this alone was not associated with mTEC differentiation. In contrast, RANK expression induced by the RANK signal itself was more gradual and associated with mTEC differentiation together with the expression of TRA genes such as Sap1. We speculate that this kinetic difference between the two signals through LTβR with RANK enables effective mTEC development in a physiological setting, as we demonstrated by the efficient mTEC differentiation after sequential stimulation with LTβR, followed by RANK stimulation in FTOC. Thus, the LTβR signal may play a more important role in the initial phase of mTEC development, whereas the RANK signal ensures the differentiation of mTECs together with their maintenance in the later stages of embryo development.

In the search for additional receptor(s) beyond LTβR that could fill the gap between LTβR deficiency and NIK mutation or IKKα deficiency for thymic phenotypes, we hypothesized that such
putative receptor(s) should primarily activate the non-canonical NF-κB activation pathway, as is the case for LTβR. This assumption was based on the fact that, for secondary lymphoid organogenesis, Ltb-R-KO show essentially the same phenotype as mice deficient in the NIK–IKKα signal pathway (35) and that the NIK–IKKα signal pathway is a unique component of the non-canonical NF-κB activation pathway (46). We therefore expected that crossing Ltb-R-KO with other mouse strains deficient in TNFRsf members that can activate the non-canonical NF-κB pathway, such as RANK and CD40, would produce thymic phenotypes similar to those of NIK mutant aly mice, but it was not the case. Although it is still possible that there are other combinations of deficiency for non-canonical NF-κB activating receptor(s) beyond LTβR plus RANK or LTβR plus CD40, which we tested in the current study, the fact that the role of NIK in NF-κB activation is a component of not only the non-canonical but also the canonical NF-κB activation pathway, at least downstream of some TNFRsf member such as CD27 (50), merits attention. Although the exact roles of the canonical NF-κB activation pathway for thymic organogenesis have not yet been fully defined, it is possible that the combined defect in the canonical and non-canonical NF-κB activation pathways in NIK mutant aly mice might account for the more severe thymic phenotype in comparison with Ltb-R-KO, where the defect is essentially confined to the non-canonical NF-κB activation pathway (46). Thus, it is possible that mTEC differentiation requires intact NF-κB activities mediated by both the canonical and non-canonical NF-κB activation pathways. In this scenario, redundancy and/or cross talk between the canonical and non-canonical NF-κB activation pathways for thymic organogenesis is likely, although the functional segregation of the two pathways seems to be more blurred than previously thought. In contrast to the complexity of NIK-mediated thymic organogenesis with respect to the upstream receptor(s), TRAF6-dependent thymic organogenesis seems to be better defined; Rankl/Cd40-DKO showed a severe defect of thymic organogenesis similar to that in Traf6-KO, suggesting that the upstream receptor(s) responsible for TRAF6-dependent thymic organogenesis is very likely RANK and CD40 (18). However, the precise mechanisms underlying the cooperation between the RANK and CD40 signals require further study.

Finally, an integrated and detailed phenotypic analysis of mice deficient in the LTβR signaling would help to clarify many aspects of LT biology. Ltb-R-KO showed clear reduction of UEA-1+ mTECs associated with loss of the characteristic three-dimensional organization and also a reduction of absolute numbers (29 and in this study). In contrast, Ltb-R-KO (not Lta-KO) showed no significant reduction in the total mass of mTECs, although changes in the UEA-1+ cell distribution pattern have been pointed out (29). Importantly, introduction of LIGHT deficiency into Ltb-R-KO resulted in no additional deterioration (29). Consequently, the thymic phenotype of Ltb-R-KO was more severe than that of Ltb-R-KO or Lib/Lib-DKO. Based on these phenotypic differences between Lib/Lib-DKO and Ltb-R-KO, the existence of additional ligand(s) for LTβR beyond mLT and LIGHT has been assumed (29). In contrast to Ltb-R-KO, however, reduced numbers of mTECs together with a disorganized thymic structure was evident in Lta-KO (9, 16, 29–32 and this study). More importantly, introduction of LIGHT deficiency into Lta-KO significantly worsened the thymic phenotypes of Lta-KO, resulting in a degree of thymic disorganization similar to that seen in Ltb-R-KO (as summarized in Fig. 1B). Based on these results, we favor the idea that no further additional ligand(s) for LTβR beyond mLT and LIGHT need to be assumed. The extensive search for LTβR ligands for more than a decade without any additional discovery would tend to support this view.

The fact that LTα deficiency (lacking both mLT and sLT) and LTβ deficiency (lacking mLT but not sLT) do not have the same consequences for thymic organogenesis is reminiscent of the fact that Lib-KO showed less profound lymph node genesis phenotypes (i.e., the presence of mesenteric lymph nodes) in contrast to the case of Lta-KO (51, 52). The phenotypic difference in thymic organogenesis between Lta/Light-DKO and Lib/Light-DKO (and most likely already between Lta-KO and Lib-KO) (29 and this study) may suggest a role of sLT in thymic organogenesis (also for lymph node genesis). If this is the case, then interaction between sLT (i.e., LTα3) and TNFR (TNFRsf1a/b) and/or HVEM (TNFRsf14) might play some role in thymic organogenesis. Alternatively, there may be a weak in vivo interaction between sLT and LTβR (53), although an in vitro study did not demonstrate such binding (54). In either case, it would be important to emphasize that deficiency of LTα or LTβR results in a different phenotype of thymic organogenesis and that LIGHT deficiency additional to LTα deficiency fills this gap. This is remarkable when considering that a deficiency of LTα or LTβR produces very similar defects in secondary lymphoid organogenesis. Thus, it is now evident that there is a critical requirement for LIGHT (but only in combination with mLT) through LTβR in thymic organogenesis.

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