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Direct and indirect roles of the LTβR pathway in central tolerance induction

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Medullary thymic epithelial cells (mTECs) play a critical role in thymic negative selection of autoreactive thymocytes, especially for thymocytes specific for peripheral tissue-restricted self-antigens (TRA). Deficiency in lymphotoxin β receptor (LTβR) is associated with peripheral tissue inflammation, but whether this is caused by defective negative selection has been unclear; the significance of the LTβR pathway for negative selection is evident in some models but not others. Here, we revisit the data and clarify the role of LTβR in mTEC development and function and thymic TRA expression. These processes are discussed as potential mechanisms for LTβR-mediated control of negative selection.

Medullary thymic epithelial cells (mTECs), Aire and thymic negative selection

Negative selection of autoreactive thymocytes is a central mechanism for establishing self-tolerance. During this process, self-antigens are presented mainly by medullary thymic epithelial cells (mTECs) and/or thymic dendritic cells (DCs) to developing thymocytes to induce apoptosis of thymocytes with a high-affinity T cell receptor (TCR) against self-antigens [1–6]. Although it is easy to understand how autoreactive T cells against ubiquitous self-antigens are purged, it had been a mystery how the same mechanism might forestall autoimmunity against peripheral tissue-restricted self-antigens (TRAs). The explanation began to emerge by the demonstration that a myriad of genes classified as peripheral tissue-restrictive are also expressed in thymic epithelial cells, especially in mTECs [7,8].

The importance of mTECs and mTEC TRA expression in the establishment of central tolerance is demonstrated mainly by the following two aspects. Firstly, abnormal mTEC development and organization is often associated with autoimmunity. Examples include *Relb*$^{-/-}$ mice [9,10], *aly*/*aly* mice [11], *Ikka*$^{-/-}$ embryonic-thymi-grafted nude mice [12], *Traf6*$^{-/-}$ mice [13], *Nfkb2*$^{-/-}$ mice [14,15], *Ltbr*$^{-/-}$ mice [16] and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice [17]. All these mice have disorganized or reduced cellularity of mTECs to different degrees; they also possess autoantibody and/or peripheral organ lymphocyte infiltration, the prototypical phenotype of autoimmunity. Additional evidence underlying the importance of organized mTECs in preventing autoimmunity is that in several autoimmune models, the disruption of thymic medulla (e.g. reduced mTECs, aberrant mTECs location in cortex) is often associated with, or precedes, the development of autoimmunity [18,19].

Secondly, genetically altered mice with reduced TRA thymic expression develop autoimmunity. A typical case is the autoimmune regulator (Aire) deficient mouse. The *AIRE* gene was first identified and cloned from patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED) [20,21]. A subsequent study in mice revealed that Aire is a master regulator of ectopic expression of a large number of peripherally expressed genes in the thymus, and that Aire deficiency in mice leads to autoimmunity against peripheral organs [22]. This was initially attributed solely to reduced ectopic expression of thymic TRA [22,23]. However, it was later found that Aire might possess roles other than regulation of TRA expression, such as regulation of antigen processing and presentation, mTEC differentiation and thymocyte migration [24–28]. Thus, the relative contribution of each Aire-related mechanism in mediating negative selection needs to be fully unraveled. Even so, a critical role for TRA expression in mTECs was demonstrated recently; investigators found that lack of a single protein, interphotoreceptor retinoid-binding protein (IRBP) in the thymus, even in the presence of Aire, is sufficient to trigger spontaneous eye-specific autoimmunity as found in Aire-deficient mice [29].

Given the critical roles of mTECs and thymic TRA expression in negative selection, their regulation has been actively investigated. In this area of research, the lymphotoxin β receptor (LTβR) has received much attention given its important, yet complicated, role in thymic negative selection. Here, we attempt to revisit the data and clarify the controversial role of LTβR in mTEC development and function and in thymic TRA expression.

Can the LTβR pathway control negative selection of TRA-reactive T cells?

LTβR belongs to the TNFR superfamily and is expressed extensively on stromal cells as well as DCs and macrophages, but not on T or B cells. Two ligands of LTβR have been identified so far: lymphotoxin (LT) and LIGHT. LT is expressed mainly on B, T and NK cells, while LIGHT is expressed on immature DCs, activated T cells and NK cells. The LTβR pathway plays a critical role in secondary lymphoid organ development and function [30,31]. LTβR deficiency is associated with increased numbers of lymphocytes in peripheral organs, which when first described was presumed to be due to the lack of lymph nodes (LNs) in

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  doi:10.1016/j.it.2010.06.005 Trends in Immunology 31 (2010) 325–331

Box 1. TCR and neo-self TRA transgenic systems used in the study of negative selection

OT-I is a $CD8^+$ transgenic TCR that recognizes ovalbumin AA257-264 in the context of $H2K^b$; OT-II is a $CD4^+$ transgenic TCR recognizing ovabumin AA323-339 in the context of $H2A^b$; TAG-I and TGB are both $CD8^+$ transgenic TCRs recognizing different SV40-T antigen epitopes in the context of $H2D^b$ and $H2K^k$, respectively. RIP-mOVA transgenic mice bear membrane-bound ovalbumin under the control of the rat insulin 1 promoter. TRAMP mice bear SV40-T antigen under the control of the probasin promoter. Both insulin 1 and probasin are considered to be TRAs. Thus, mOVA and SV40-Tag driven by these promoters are considered to be expressed in a way that mimics TRA expression.

these mice [32]. However, further careful studies by two groups challenged this view with data showing lymphocyte infiltration in peripheral organs was independent of defective LNs and instead dependent on thymic defects [16,32,33]. This opened a new line of investigation into the control of T cell negative selection by LTβR. So far, four antigen-specific TCR transgenic and neo-self Ag transgenic systems have been employed to address the role of LTβR in thymic negative selection: (1) OT-I/RIP-mOVA, (2) OT-II/RIP-mOVA, (3) TAG-I/TRAMP and (4) TGB/TRAMP (Box 1). Intriguingly, the results obtained from these different studies are somewhat divergent. In a study using the OT-II/RIP-mOVA system, LTβR had little influence on thymic negative selection [34]. However, in other studies using three different $CD8^+$ transgenic TCR systems ((1), (3) and (4) above), a significant role of LTβR on thymic negative selection was revealed [14,35]. These different results, as well as a controversial role for LTβR in the control of TRA and Aire expression, have led to some confusion in the field regarding the role of LTβR in negative selection of TRA-specific T cells. This might be due to the different models used in the respective studies, such as CD4 versus CD8 T cells (DCs are believed to be the prime antigen-presenting cells (APCs) for CD4 T cells, whereas mTECs are thought to be the prime APCs for CD8 T cells [36]) as well as analysis of different TRAs (with different promoters and mechanisms of regulation) for induction of thymic negative selection. More antigens and more models are needed to resolve this issue.

Can the LTβR pathway control mTEC development and organization?

mTEC development is generally considered to be a stepwise process, where mTEC subsets are defined depending on their maturation status (represented by MHC-II or CD80 expression) and Aire expression ($CD80^{low}Aire^-$, $CD80^{high}Aire^-$, $CD80^{high}Aire^+$) [37]. Different subsets of mTECs are presumed to have different TRA expression patterns and antigen presentation functions as well as different rates of turnover [8,38–40].

The role of the LTβR pathway in the thymus has been largely overlooked compared with its well defined role in peripheral lymphoid organogenesis and development [30,41]. This was partially due to the grossly normal size and architecture of thymi from LT and LTβR deficient mice. However, in a study that examined the thymic medulla from *Ltbr*$^{-/-}$ mice in more detail, reduced numbers of mTEC subsets expressing UEA-1 and the non-polymorphic MHC-II antigen I-O were observed [16]. When defined as $CD45^+$ $G8.8^+$ $CDR1^+$ $B7.1^+$, the total number of mTECs was also dramatically reduced in *Ltbr*$^{-/-}$ thymi. LTβ deficiency was found to have a non-identical phenotype in this regard. In fact, when studying LTβR ligands, it was found that in LTβ and LIGHT double-deficient mice, in which both known ligands of LTβR are ablated, the thymic phenotype found in *Ltbr*$^{-/-}$ mice was only partially reproduced. Thus it was hypothesized that additional unknown ligand(s) of LTβR exist. A role for the LTβR pathway in mTEC development was observed by two other groups [34,42], and the milder effect of LTα deficiency, compared with LTβR, on mTEC development was also noted [34]. Furthermore, the development of the $Aire^+$ MHC-II high mTEC population was also found to be dependent on LTβR [34,42]. It is now generally agreed that LTβR is required for proper mTEC development. It remains unclear, however, exactly how, and at which differentiation stage, LTβR regulates mTEC development (Box 2).

It must be noted that other TNFR superfamily members, CD40 and RANK, are also important for mTEC development and central tolerance [43–45]. This is not surprising, as both CD40 and RANK can deliver signals through the non-canonical NF-κB pathway. However, it is surprising that so many TNFR family members are involved in mTEC development. This coordinated regulation pattern of mTECs by different molecules is probably based on different ligand–receptor spatial and temporal expression patterns [46], which highlights the fact that the finely tuned regulation of mTECs is crucial for establishing central tolerance.

It is important to note the LTβR pathway is also involved in mTEC organization. In immunofluoresence microscopy experiments not all mTEC markers reveal identical defects in thymic medulla organization; obvious disorganization is detected using UEA-1 staining but is less clear with MTS-10 staining [16,32]. Lectin UEA-1-expressing mTECs were found in clumps, and the connective mTEC network was disrupted in mice with LTβR deficiency [16], when compared with the broad and even distribution in wild type (WT) thymi. A similar finding was noted in *Nfkb2*$^{-/-}$, *plt*/*plt* and *Ccr7*$^{-/-}$ mice [47,48]. Thymocyte migration in the thymus is a highly organized process and the developing thymocytes need to patrol the thymic medulla for antigen to undergo negative selection [49,50]. Whether disrupted mTECs/medulla organization itself also influences negative selection of autoreactive thymocytes remains largely unclear and awaits further investigation.

Is expression of Aire and TRA controlled by the LTβR pathway directly or indirectly?

Given the similar autoimmune phenotypes between *Ltbr*$^{-/-}$ and *Aire*$^{-/-}$ mice, it was proposed that LTβR might regulate thymic central tolerance in an Aire-dependent manner or via regulation of TRA gene expression. This led to the initial finding of dramatically reduced *Aire*, *Insulin 1* and *Collagen II* gene expression in total thymi of *Ltbr*$^{-/-}$ or *Lta*$^{-/-}$ mice compared to WT thymi by quantitative real-time (RT)-PCR [32,33]. However, in a separate study,

normal Aire expression was found in mTECs isolated from *Ltbr*$^{-/-}$ thymi by semiquantitative RT-PCR [16]. Supporting the latter, normal Aire and TRA expression in *Lta*$^{-/-}$ thymi was found by semiquantitative PCR and *Lta*$^{-/-}$ thymi showed largely normal Aire^{+} mTEC frequency by tissue immunofluoresence staining [11,51]. Thus, these studies led to the suggestion that the LT-LTβR pathway regulates Aire and TRA expression in thymus through indirect mechanisms.

More recent studies have attempted to clarify this controversial issue by analyzing Aire and TRA gene expression in more detail on a per cell basis [34,52]. In these studies, purified mTECs from *Ltbr*$^{-/-}$ and/or *Lta*$^{-/-}$ mice showed no reduction of Aire or TRA gene expression when compared with WT mTECs using both gene array and quantitative RT-PCR. Thus, these studies concluded that LTβR signaling is not directly required for TRA expression in mTECs. Instead, based on gene profiling, it was proposed that the role of LTβR on Aire expression might not be direct but indirect through the regulation of mTEC development [34,52]. The data suggest that the reduction of Aire or TRA in whole thymic tissues seen in earlier studies is likely to be associated with the reduced number of total or subsets of mTECs rather than reduced Aire expression in individual cells.

To study this further, MHC-II^{hi} (mature) and MHC-II^{lo} (immature) mTECs were isolated, from *Lta*$^{-/-}$ and *Ltb*$^{-/-}$ mice and Aire and TRA expression was measured. While Aire expression was not reduced in either subset of mTECs from *Lta*$^{-/-}$ or *Ltb*$^{-/-}$ mice compared with WT, some Aire-dependent (including *insulin 2*) and Aire-independent TRAs were reduced in both the *Lta*$^{-/-}$ and *Ltb*$^{-/-}$ mTEC subsets [42]. This supports a direct role for LT signaling in regulating expression of some TRAs in mTECs. It is worth noting that this study also found that LT deficiency has a much more pronounced effect on TRA expression in the MHC-II^{lo} mTEC subset than in the MHC-II^{hi} mTEC subset, which raises the possibility that LT signaling might be more important for TRA expression in some mTEC subsets than others [42].

The studies described above focused mostly on the essential role of the LT-LTβR pathway in Aire and TRA expression. Whether the LTβR signaling pathway is sufficient to upregulate Aire and TRA expression is a different question. Efforts to address this issue have been somewhat limited due to the lack of appropriate reagents and the low-level expression of antigens in mTECs. However, an earlier study showed that treatment of mice with the 3C8 clone of agonistic anti-LTβR upregulated in vivo thymic *Aire*, *Insulin 1* and *Collagen II* transcript expression after several hours [32,33]. Furthermore, in vitro experiments showed that 3C8 treatment of the mTEC cell line 427.1 can upregulate the expression of these genes, suggesting a direct impact of LTβR signaling on Aire and TRA expression in mTECs [32]. However, upregulation of Aire by agonisitic LTβR antibody was not found in a recent study using a 2-deoxyglucose (2-DG)-treated fetal thymic organ culture (FTOC) while Crp (an Aire-independent TRA) was significantly upregulated [45]. Thus, different conclusions have been drawn from these two studies. It should be kept in mind that different models, reagents and stimulation time were used in these studies and the role of LT-LTβR might be different in different scenarios.

Box 2. Outstanding questions

- At what stage of differentiation does LTβR regulate mTECs? What are the cellular and molecular mechanisms for regulation of mTEC development by LTβR signaling?
- How does LTβR cooperate with other TNFR superfamily receptors, e.g. CD40 and RANK, to regulate mTEC development and function?
- How does LTβR regulate TRA (both Aire-dependent and -independent) expression?
- Is the LTβR-mediated regulation of mTECs dependent on TCR–pMHC interaction?
- How does LTβR regulate mTEC and thymic medulla organization and is this a factor in the control of central tolerance?

It is worth noting several issues when interpreting the data described above. Firstly, the induction or upregulation of Aire and TRA expression by direct LTβR signaling raises the interesting question of whether TRA-specific TCR–pMHC interactions during thymocyte development feed back on mTECs to upregulate TRA via stabilized LT-LTβR signaling on an individual cell basis. Given the rare interaction events between TRA-specific TCR and TRA presented by mTECs, this crosstalk between individual thymocytes and mTECs, mediated by an LT–LTβR interaction, might help to increase the efficiency of negative selection. Secondly, it is possible that LTβR plays a more essential role in certain subsets of mTECs than in others [42]. This effect could be compromised when the whole mTEC population is analyzed instead of mTEC subsets [34,52]. Thirdly, LTβR appears to be essential for the expression of only a subset of TRAs. Several other TNF family members, similar to LTβR, are essential for mTEC development [43–45]. Do they also control TRA expression directly? If yes, how do they cooperate with LTβR? These are interesting questions that should be answered by future work. Last, but not least, one can argue that agonistic antibodies that regulate TRA expression might provide means for clinical intervention to enhance negative selection thus providing better central tolerance. However, the clinical relevance of 'sufficiency' for TRA expression and the amount of TRA upregulation required for tolerance induction are not known.

As discussed in the previous section, it is clear that LTβR plays an essential role in mTEC development/organization [14–16,34,52] and by doing so the LTβR pathway can control thymic TRA expression indirectly. Thus, although the direct role of LT-LTβR signaling on Aire expression could be limited at steady state, LT-LTβR signaling would indirectly induce Aire to control negative selection by regulating the development and organization of mTECs.

Could the LTβR pathway regulate thymocyte migration?

We have unexpectedly identified another role for LTβR in central tolerance; that is, regulation of mTEC chemokine expression and thymocyte migration [14]. This study originated from an unexpected finding in the OT-I/RIP-mOVA system used to address the role of LTβR in thymic

negative selection. Although thymic mOVA expression remains normal in RIP-mOVAtg/*Ltbr*$^{-/-}$ mice, we still found defective thymic negative selection of OT-I cells when LTβR was deficient. This finding, together with previous data showing that LTβR controls chemokine expression in peripheral tissues, and the important role of chemokines in central tolerance, led us to examine whether LTβR controls chemokine expression in the thymus, thereby altering migration of developing thymocytes. Indeed, we found impaired secondary lymphoid organ chemokine (SLC) and EBI1-ligand chemokine (ELC) expression in mTECs from *Ltbr*$^{-/-}$ mice, which resulted in defective thymocyte migration to the medulla. To further evaluate the role of the SLC and ELC defect itself on thymic negative selection, we used *plt*/*plt* mice, in which SLC and ELC are both deficient, and found that SLC and ELC deficiency alone is sufficient to lead to a thymic negative selection defect and these findings have been confirmed by others [42,48].

Implications of LTβR-regulated negative selection

Although the underlying mechanisms are not fully understood, the role of LTβR on negative selection of TRA-reactive T cells is clear, at least for certain TRA-reactive T cells. Given the significant influence of LTβR on negative selection of TRA-reactive CD8^{+} T cells and the fact that many tumors express organ-restricted self-antigens, a recent study creatively applied this knowledge to the prevention of tumor development [35]. In this study, ablation of LT signaling either by LTα deficiency, or by administering an LTβR-human Ig fusion protein, dramatically rescued most high-affinity tumor/self-specific TCR clones, which was associated with inhibited/reduced spontaneous tumor development in the TRAMP prostate cancer model. This study reveals a significant role for the LTβR pathway in negative selection and, together with other studies as discussed above, suggests that the degree of LTβR involvement in thymic negative selection might depend on the type of TRA and/ or the type of promoter regulating the TRA, as well as the mTEC subsets involved. In fact, LTβR signaling ablation showed a more dramatic rescue in the TAG-I-TRAMP system than that in the OT-I-RIP-mOVA system (20-fold versus 3-fold). There are at least three models that could explain these data: (1) RIP-driven mOVA and probasin promoter-driven SV40-Tag are expressed in different subsets of mTECs; (2) the transcription or translation of the two genes is regulated differentially by LTβR; and (3) the affinity of the antigenic epitopes of the two proteins to TCR is different. Those models remain to be tested in future work.

It is worth noting that blocking the LTβR pathway could have multiple effects in addition to rescue of high-affinity TRA-reactive T cells. Several models have shown blockade of the LTβR pathway reduces inflammation [53–55], and inflammation has been considered a factor in promoting cancer development [56]. Additionally, the LTβR pathway was found to promote tumor growth by inducing angiogenesis [57]. A recent study demonstrated that the LTβR

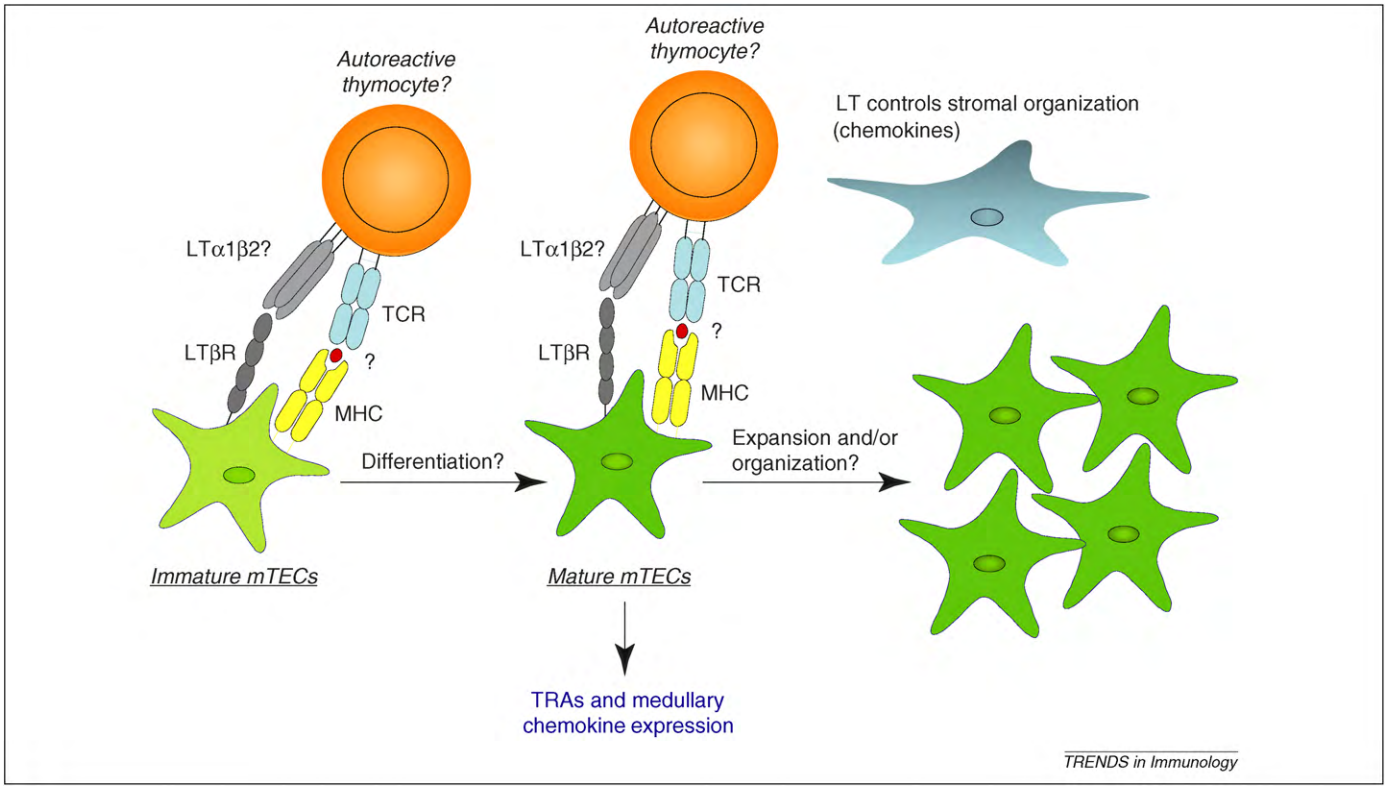

Figure 1. Direct and indirect roles of LTβR in mTEC development and function. LTβR signaling regulates mTEC development and function in various ways. LTβR controls mTEC differentiation; however, it is not known at which stage or how LTβR controls this process. LTβR is not essential for Aire thymic expression but is indeed essential for expression of some Aire-dependent and Aire-independent TRA on a per cell basis. In addition, LTβR might have more impact on TRA expression in some subsets of mTECs than others. The underlying molecular mechanism remains to be determined. LTβR signaling also controls mTEC organization and chemokine production, which might indirectly regulate thymocyte migration or TRA expression and presentation to developing thymocytes. It is unclear which cells deliver which ligand(s) to LTβR for control of mTEC differentiation, TRA and chemokine expression; LT appears to play only a partial role. It is also intriguing to ask whether TCR–pMHC interaction between thymocytes and mTECs is required for LTβR to fulfill its roles.

Table 1. Summary of current data on the role of LTβR in thymic negative selection.

Experimental readout	Data supporting a role for LTβR in thymic negative selection			Data suggesting no role for LTβR in thymic negative selection		
	Experimental model	Observation	Reference	Experimental model	Observation	Reference
mTEC numbers	*Ltbr*$^{-/-}$ thymi tissue staining, mTEC isolation and FACS	Reduced	[14,16,34]	*Lta*$^{-/-}$ mice, tissue staining	No change	[32]
	Ltb$^{-/-}$ mice, UEA1 tissue staining	Reduced	[16]	*LTa*$^{-/-}$ mice, mTEC isolation and FACS	No change	[34]
Aire expression	Whole thymi from *Ltbr*$^{-/-}$, *Lta*$^{-/-}$ mice, RT-PCR	Reduced	[14,32]	Whole thymi from *Lta*$^{-/-}$ mice, semi-quantitative PCR	No change	[11]
	Whole thymi, in vivo agonistic LTβR antibody	Increased	[32,47]	Isolated mTECs from *Lrbr*$^{-/-}$, *Lta*$^{-/-}$ mice, RT-PCR	No change	[34,52]
	2-DG FTOC, in vitro agonistic LTβR antibody	Increased	[32,45]			
Aire-dependent TRAs	Whole thymi from *Ltbr*$^{-/-}$ mice, RT-PCR	Reduced	[14,32]	Isolated mTECs from *Lta*$^{-/-}$ and *Ltbr*$^{-/-}$ mice by CD45^{-} CDR1^{-} G8.8^{+} MHC-II^{hi}, RT-PCR	No change	[34]
	Whole thymi, in vivo agonistic LTβR antibody	Increased	[32,47]	Isolated mTECs from *Ltbr*$^{-/-}$ mice by CD45^{-} CDR1^{-} G8.8^{+}, RT-PCR	No change	[52]
	Isolated mTECs from *Lta*$^{-/-}$, *Ltb*$^{-/-}$ mice by CD45^{-} UEA1^{+} MHC-II^{hi} or CD45^{-} UEA1^{+} MHC-II^{lo}, RT-PCR	Reduced	[42]			
Aire-independent TRAs	Whole thymi from *Ltbr*$^{-/-}$ mice, RT-PCR	Reduced	[33]			
	Isolated mTECs from *Lta*$^{-/-}$, *Ltb*$^{-/-}$ mice by CD45^{-} UEA1^{+} MHC-II^{hi} or CD45^{-} UEA1^{+} MHC-II^{lo}, RT-PCR	Reduced	[42]			
	Whole thymi, in vivo agonistic LTβR antibody	Increased	[33]			
	2-DG FTOC, in vitro agonistic LTβR antibody	Increased	[32,45]			
mTEC chemokines	Isolated mTECs from *Ltbr*$^{-/-}$ mice by CD45^{-} G8.8^{+} CD80^{+}, RT-PCR, SLC and ELC	Reduced	[47]			
	Isolated mTECs from *Lta*$^{-/-}$, *Ltb*$^{-/-}$ mice by CD45^{-} UEA1^{+} MHC-II^{hi} or CD45^{-} UEA1^{+} MHC-II^{lo}, RT-PCR, SLC and ELC	Reduced	[42]			
Negative selection	OT-I/RIP-mOVA, *Ltbr*$^{-/-}$	Reduced	[14]	OT-II/RIP-mOVA, *Ltbr*$^{-/-}$	No change/ reduced	[34]
	TAG-I/TRAMP, *Lta*$^{-/-}$	Reduced	[35]			
	TGB/TRAMP, LTβR-hIg blockade	Reduced	[35]			

signaling pathway is upregulated in chronic HBV or HCV infection-induced hepatitis and hepatocellular carcinoma [58]. Thus, it cannot be excluded that additional mechanisms contributed to tumor prevention.

Concluding remarks

The studies on the role of LTβR in thymic negative selection have raised interesting new questions about how T cells are negatively selected and how LTβR signaling is required for the control of negative selection of some TRA-reactive T cells, but not others. Earlier studies helped clarify the complicated roles of LTβR in various aspects of thymic negative selection (Figure 1). As discussed above, while evaluating the role of LTβR in negative selection, it is worthwhile considering the experimental model and methods used to modulate LTβR signaling (Table 1). Thus, it is not surprising that the role of LTβR in thymic negative selection of TRA-specific T cells is revealed in some studies but not in others. The different results obtained under different scenarios underscore the complicated regulation of thymic negative selection and help to suggest future research directions in which to discover novel factors in this important thymic process. Some key questions that should be addressed in future work are outlined in Box 2. Increased understanding of the mTEC differentiation program, the role of LTβR and, more broadly, all TNFR superfamily receptors, will help us to have a more comprehensive overview of thymic negative selection of various TRA-specific T cells. Furthermore, we can expect to see more preclinical studies using techniques designed to regulate negative selection for the combat of cancer and autoimmune disease.

Acknowledgements
This work was supported by grants from the National Institutes of Health (to Y-X. F.) and the Chinese Academy of Sciences (to M.Z. and Y-X. F).

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