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Lymphotoxin β Receptor Regulates the Development of CCL21-Expressing Subset of Postnatal Medullary Thymic Epithelial Cells

Enkhsaikhan Lkhagvasuren, Mic Sakata, Izumi Ohigashi, and Yousuke Takahama

Medullary thymic epithelial cells (mTECs) play a pivotal role in the establishment of self-tolerance in T cells by ectopically expressing various tissue-restricted self-Ags and by chemoattracting developing thymocytes. The nuclear protein Aire expressed by mTECs contributes to the promiscuous expression of self-Ags, whereas CCR7-ligand (CCR7L) chemokines expressed by mTECs are responsible for the attraction of positively selected thymocytes. It is known that lymphotoxin signals from the positively selected thymocytes preferentially promote the expression of CCR7L rather than Aire in postnatal mTECs. However, it is unknown how lymphotoxin signals differentially regulate the expression of CCR7L and Aire in mTECs and whether CCR7L-expressing mTECs and Aire-expressing mTECs are distinct populations. In this study, we show that the majority of postnatal mTECs that express CCL21, a CCR7L chemokine, represent an mTEC subpopulation distinct from the Aire-expressing mTEC subpopulation. Interestingly, the development of CCL21-expressing mTECs, but not Aire-expressing mTECs, is impaired in mice deficient in the lymphotoxin β receptor. These results indicate that postnatal mTECs consist of heterogeneous subsets that differ in the expression of CCL21 and Aire, and that lymphotoxin β receptor regulates the development of the CCL21-expressing subset rather than the Aire-expressing subset of postnatal mTECs.

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majority of mTECs that express CCL21 represent a late-appearing mTEC subset that is distinct from the Aire-expressing mTEC subset. The number of CCL21-expressing mTECs is reduced in Aire-deficient mice, whereas the number of CCL21-expressing mTECs, but not Aire-expressing mTECs, is reduced in LTβR-deficient mice. These results indicate that postnatal mTECs consist of heterogeneous subsets, including the CCL21-expressing subset and the Aire-expressing subset, and that LTβR regulates the development of the CCL21-expressing subset rather than the Aire-expressing subset of postnatal mTECs.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from SLC (Shizuoka, Japan), B6-plt/plt (14), B6-Aire−/− (6), B6-LTβR−/− (15), and B6-Tcrα−/− (16) mice were described previously. Experiments were performed with the consent of the Animal Experimentation Committee of the University of Tokushima.

Flow cytometric analysis

Minced thymus fragments were digested with 0.125% collagenase D (Roche Applied Science) and 0.01% DNase I (Roche), as previously described (10, 17). Collagenase-digested thymic cells were cell surface stained with PE-conjugated anti-CD205 Ab, PECy5-conjugated anti-CD45 Ab, PECy5-conjugated anti-CD326 (EpCAM), γδ+ conjugated anti-CD326 (EpCAM), and biotinylated Ulex europaeus agglutinin I (UEA1), followed by allophycocyanin-780–conjugated streptavidin. Anti-FcγR mAb (clone 2.4G2) was added before Ab staining to block FcγR-mediated binding of the staining Abs to the cells. After the cell surface staining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin (18). Cells were then stained with rabbit anti-CCL21 Ab (LifeSpan Biosciences), followed by AlexaFluor-488–conjugated goat anti-rabbit IgG Ab (Invitrogen) and AlexaFluor-647–conjugated anti-Aire Ab (eBioscience). Multicolor flow cytometry was performed with a FACS Aria II flow cytometer (BD Biosciences). Forward and side scatter intensities were measured for the identification of the cells.

Immunofluorescence analysis

Frozen thymuses embedded in OCT compound (Sakura Finetek) were sliced into 5-μm–thick sections, fixed with acetone, and stained with AlexaFluor-647–conjugated anti-Aire Ab (eBioscience) and rabbit anti-CCL21 Ab (LifeSpan Biosciences), followed by AlexaFluor-350–conjugated goat anti-rabbit IgG Ab (Invitrogen) and AlexaFluor-647–conjugated anti-Aire Ab (eBioscience). Multicolor flow cytometry was performed with a FACS Aria II flow cytometer (BD Biosciences). Forward and side scatter intensities were measured for the identification of the cells.

Statistical analysis

Statistical analysis was performed with the Student t test using Microsoft Excel software. The p values <0.05 were considered significant.

Results

Flow cytometric detection of CCL21 expression in mTECs

CCL21, a CCR7L chemokine, is strongly expressed by mTECs as compared with other cells in the thymus, including cortical TECs (cTECs) (19, 20). Previous studies examined CCL21 expression profiles in saponin-treated thymic cells specifically detected CCL21 expression in mTECs rather than cTECs only when the cells were permeabilized with saponin (Fig. 1B). The requirement of the saponin treatment for the detection of CCL21 suggested that the detection reflected the intracellular production of CCL21 proteins rather than the possible cell surface association of CCL21 proteins produced by other cells. Thus, flow cytometric measurement of CCL21 expression in saponin-treated thymic cells specifically detected CCL21 expression in mTECs rather than cTECs.

Flow cytometric detection of CCL21-expressing mTEC subset

We then combined the flow cytometric detection of CCL21-expressing mTECs with the detection of Aire-expressing mTECs. It was previously shown using flow cytometry that Aire expression was detected in a fraction of permeabilized mTECs (22). Two-color detection of CCL21 and Aire in mTECs from postnatal B6 mice, along with control staining profiles obtained with normal IgG, identified a distinct population of mTECs that expressed CCL21, but not Aire (Fig. 2A). In contrast, a large fraction of mTECs expressed Aire, but not CCL21, whereas a small fraction of mTECs expressed both CCL21 and Aire (Fig. 2A). The detection of CCL21 in the mTEC fractions was specific, as CCL21 signal caused the lack of CCL21 protein expression (14). We found that CCL21-specific signals in B6 but not B6-plt/plt cells were detected in mTECs rather than cTECs only when the cells were permeabilized with saponin (Fig. 1B). The requirement of the saponin treatment for the detection of CCL21 suggested that the detection reflected the intracellular production of CCL21 proteins rather than the possible cell surface association of CCL21 proteins produced by other cells.
Aire affects the development of CCL21-expressing and Aire-expressing mTEC subsets

It was previously shown that CCL21 mRNA expression was reduced in mTECs from Aire-deficient mice (6, 7). To examine whether Aire affects the cellularity of CCL21-expressing mTECs, we examined mTEC subpopulations in Aire-deficient mice. mTECs from Aire-deficient mice were devoid of the Aire-expressing mTEC subset, including the CCL21<'sub>Aire <sup>a</sup>' and CCL21<'sub>Aire <sup>e</sup>' subpopulations (Fig. 4), reconfirming the specificity of the detection of Aire-expressing mTECs. Interestingly, we found that the cellularity of CCL21-expressing mTECs in Aire-deficient mice was approximately half of that in control mice (Fig. 4). These results indicate that Aire contributes to the postnatal cellularity of CCL21-expressing mTECs.

LTβR affects the development of CCL21-expressing but not Aire-expressing mTEC subset

It was also shown that CCL21 expression, but not Aire expression, was reduced in mTECs of LTβR-deficient mice (8, 9, 12, 13). Using flow cytometric analysis, we found that, among the mTEC subpopulations expressing CCL21 and Aire, the CCL21<'sub>Aire <sup>e</sup>' subpopulation of mTECs showed a significant decrease (<i>p < 0.05</i>; approximately half) in cellularity in LTβR-deficient mice (Fig. 5). In contrast, the deficiency of LTβR did not significantly affect the cellularity of CCL21<'sub>Aire <sup>a</sup>', CCL21<'sub>Aire <sup>e</sup>' and CCL21<'sub>Aire <sup>e</sup>'-subpopulations (Fig. 5). These results indicate that LTβR specifically affects the CCL21-expressing subset, but not the Aire-expressing subset, of postnatal mTECs. Within the CCL21-expressing mTEC subset, LTβR specifically affects the Aire-expressing subset, but not the Aire-expressing subset.

Positive selection affects the development of CCL21-expressing and Aire-expressing mTEC subsets

In the postnatal thymus, lymphotoxin, which regulates the differentiation of mTECs via LTβR signals, is produced by positively selected thymocytes (10, 11, 23). Positively selected thymocytes also produce RANKL, which governs the proliferation of mTECs via RANK signals to form the medullary region in the postnatal thymus (10). Indeed, in the absence of positive selection due to TCRα deficiency, the formation of the thymic medulla was severely impaired and the cellularity of mTECs was greatly reduced (10) (also shown in Fig. 6). Flow cytometric analysis of mTEC subpopulations revealed that the cellularity of the CCL21<'sub>Aire <sup>e</sup>' subpopulation was most severely reduced in 4-wk-old TCRα-deficient mice (Fig. 6), similar to the specific reduction of CCL21<'sub>Aire <sup>e</sup>'-expressing mTECs.
AIRE mTECs in LTβR-deficient mice (Fig. 5). These results suggest that TCR-mediated positive selection regulates postnatal medulla formation not only by promoting the proliferation of mTECs, including Aire-expressing and CCL21-expressing subsets, via RANK signals, but also by promoting the development of the CCL21-expressing subset via LTβR signals.

FIGURE 3. The ontogeny of CCL21-expressing and Aire-expressing mTECs. (A) Averages (symbols) and SEs (bars, n = 3) of the numbers per mouse of indicated mTEC subpopulations (left panel) and the frequencies of indicated mTEC subpopulations in total mTECs (right panel) were determined in B6 mice at indicated ages. (B) Representative flow cytometry profiles of mTEC subpopulations based on the expression of CCL21 and Aire. Numbers indicate the frequencies of cells within the quadrant.

FIGURE 4. Aire affects the cellularity of CCL21-expressing mTEC subset. (A) Representative flow cytometry profiles of mTEC subpopulations based on the expression of CCL21 and Aire. Mice were analyzed at the indicated ages. Numbers indicate the frequencies of cells within the quadrant. (B) Averages and SEs (n = 3) of the numbers per mouse of indicated mTEC subpopulations (top panel) and the frequencies of indicated mTEC subpopulations in total mTECs (bottom panel) were determined in 5- to 8-wk-old mice. NS, p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.
Immunofluorescence detection of CCL21 and Aire in the thymic medulla

To evaluate the results obtained from flow cytometric analysis, we examined the expression of CCL21 and Aire in the thymic sections by immunofluorescence analysis. As shown in Fig. 7, immunofluorescence analysis confirmed the preferential localization of CCL21 and Aire in the medullary region rather than the cortical region of wild-type B6 thymic sections. The signals were specific; CCL21 signals were hardly detected in the thymic sections of B6plt/plt mice, and Aire signals were not detected in the thymic sections of Aire-deficient mice. Both CCL21 and Aire signals were detected in the thymic sections of LTβR-deficient mice and at a low frequency in the thymic sections of TCRα-deficient mice (Fig. 7). However, these immunofluorescence results did not readily reveal whether the CCL21-expressing cells were affected in the thymus of Aire-deficient mice or LTβR-deficient mice. It was even unclear how many CCL21-expressing cells and Aire-expressing cells overlapped on a per cell basis. Nevertheless, the results underscored the advantages of quantitative and single cell-based measurements by flow cytometry, which revealed the presence of the CCL21-expressing subset in addition to the Aire-expressing subset within postnatal mTECs and the preferential regulation of the CCL21-expressing mTEC subset by LTβR.

MHC class II and CD80 expression by CCL21-expressing and Aire-expressing mTEC subsets

It was previously shown that mTECs could be subdivided into two subpopulations according to the cell surface expression of MHC class II and CD80 (17, 24, 25). MHC class IIhigh CD80high mTECs (mTEChigh cells) contain Aire+ cells and efficiently express ectopic self-Ags, thereby representing functionally mature mTECs in the establishment of self-tolerance in T cells (25, 26). Consequently, we finally examined the expression of MHC class II and CD80 in mTEC subpopulations according to CCL21 and Aire. It

FIGURE 5. LTβR affects the cellularity of CCL21-expressing mTEC subset. (A) Representative flow cytometry profiles of mTEC subpopulations based on the expression of CCL21 and Aire. Mice were analyzed at the indicated ages. Numbers indicate the frequencies of cells within the quadrant. (B) Averages and SEs (n = 3) of the numbers per mouse of indicated mTEC subpopulations (top panel) and the frequencies of indicated mTEC subpopulations in total mTECs (bottom panel) were determined in 2- to 3-wk-old mice. NS, $p \geq 0.05$, *$p < 0.05$.

FIGURE 6. Effects of TCR-mediated selection signals on mTEC subsets. (A) Representative flow cytometry profiles of mTEC subpopulations based on the expression of CCL21 and Aire. Mice were analyzed at the indicated ages. Numbers indicate the frequencies of cells within the quadrant. (B) Averages and SEs (n = 3) of the numbers per mouse of indicated mTEC subpopulations (top panel) and the frequencies of indicated mTEC subpopulations in total mTECs (bottom panel) were determined in 4-wk-old mice. NS, $p \geq 0.05$, **$p < 0.01$, ***$p < 0.001$.
was found that most CCL21−Aire− and CCL21−Aire− mTECs were MHC class IIlow and CD80low (mTEClow cells), whereas most CCL21−Aire+ and CCL21−Aire+ mTECs were MHC class IIhigh and CD80high (mTEChigh cells) (Fig. 8). These results indicate that both mTEClow cells and mTEChigh cells show heterogeneity in CCL21 expression. More interestingly, these data indicate that all mTEClow cells are not functionally immature and mTEClow cells contain functionally mature CCL21-expressing mTECs, which contribute to the attraction of developing thymocytes to the thymic medulla and thereby the establishment of self-tolerance in T cells (20, 27).

Discussion
In the current study, we devised a flow cytometric method for the detection of CCL21-expressing mTECs. The results indicate that postnatal mTECs consist of heterogeneous subpopulations based on the expression of CCL21 and Aire. CCL21 and Aire are both functionally important molecules for mTECs in the establishment of self-tolerance in T cells. Thus, our results reveal that postnatal mTECs consist of functionally distinct subsets, as follows: the CCL21-expressing subset and the Aire-expressing subset.

The development of CCL21-expressing mTECs is partially affected by the genetic loss of Aire, whereas the development of Aire-expressing mTECs is not affected by the genetic loss of CCL21. Thus, Aire partially regulates the development of the CCL21-expressing mTEC subset in the postnatal thymus. As CCL21-expressing mTECs show an increase in frequency later in ontogeny than Aire-expressing mTECs, a fraction of the CCL21-expressing mTEC subset may be derived from Aire-expressing mTECs. Alternatively, but not mutually exclusively, Aire-expressing mTECs that are generated earlier in ontogeny may promote the development of CCL21-expressing mTECs that appear...
late in ontogeny. Nonetheless, it should be interesting to note that these late-appearing and functionally mature CCL21-expressing mTECs belong to mTEC\textsuperscript{low} cells.

Aire regulates the promiscuous gene expression of various tissue-restricted self-Ags in mTECs (3). Does the expression of CCL21 in the mTEC subpopulation represent the promiscuous expression of the CCL21 gene? We do not think that is the case. It was reported that the promiscuous expression of tissue-restricted self-Ags exhibited mRNA expression frequencies between 2 and 15% and protein expression frequencies between 1 and 3% (28, 29). On the contrary, our results showed that CCL21 was detectable in >30% of postnatal mTECs. In addition, the majority of CCL21-expressing mTECs did not express Aire. Thus, it is likely that Aire regulates the development of the CCL21-expressing mTEC subset through a mechanism other than the one operative for the promiscuous gene expression of tissue-restricted self-Ags in mTECs. Aire also regulates the mTEC expression of XCL1, another chemokine involved in the attraction of thymic dendritic cells to the medulla (6). Thus, the role of Aire is unlikely limited to the regulation of promiscuous gene expression, but most likely covers various aspects of mTEC functions, including the regulation of the development of the CCL21-expressing mTEC subset, which contributes to the establishment of self-tolerance in T cells.

It was previously shown that LT\beta R signals regulated the expression of CCL21, but not Aire, in postnatal mTECs (8, 9, 12, 13, 30). However, it was not previously known how LT\beta R specifically regulated CCL21 rather than Aire in mTECs. The present results reveal that LT\beta R regulates the development of the CCL21-expressing subset, but not that of the Aire-expressing subset, in postnatal mTECs. Interestingly, our results show that LT\beta R-mediated signals as well as TCR-mediated selection signals preferentially affect the cellularity of the CCL21\textsuperscript{+}Aire\textsuperscript{−} mTECs. In contrast, we detected no compensatory increase in CCL21 expression in CCL21\textsuperscript{−}Aire\textsuperscript{+} mTECs and are functionally mature by attracting positively selected thymocytes (stage 3). Aire partially regulates the development of CCL21-expressing mTECs, so that Aire-expressing mTECs may directly differentiate into (solid line) or indirectly promote the development of (dashed line) CCL21-expressing mTECs. In addition, the development of CCL21-expressing mTECs is partially regulated by LT\beta R, of which the ligand lymphotoxin is produced by positively selected CD4/CD8 single-positive (SP) thymocytes.

White et al. (11) reported that LT\beta R-mediated lymphotoxin signals from positively selected thymocytes regulated the terminal differentiation of mTECs. The terminally differentiated mTECs, the generation of which is promoted by positively selected thymocytes, may contain the CCL21\textsuperscript{+}Aire\textsuperscript{−} subpopulation within the CCL21-expressing mTEC subset. Indeed, our results show that the absence of TCR signals most severely affects the CCL21\textsuperscript{+}Aire\textsuperscript{−} mTEC subpopulation.

CCL21 expression in TECs is detected as early as embryonic day 12.5 (31), prior to the detection of Aire expression in TECs as early as embryonic day 16 (32). CCL21 is expressed even earlier by the parathyroid primordium that neighbors the thymic primordium, under the control of the parathyroid-specific transcription factor Gcm2 (33). The early expression of CCL21 is involved in the seeding of the thymic primordium by T-lymphoid progenitor cells (33). Thus, the development of CCL21-expressing TECs during embryogenesis occurs earlier than that of Aire-expressing TECs and may be regulated independently of Aire. During the embryogenesis, LT\beta R may also assume a different role in TECs by promoting RANK expression (34).

Finally, our results demonstrate the presence of mTECs that express neither CCL21 nor Aire. These CCL21\textsuperscript{−}Aire\textsuperscript{−} cells belong to mTEC\textsuperscript{low} cells. The frequency of these CCL21\textsuperscript{−}Aire\textsuperscript{−} mTECs in total mTECs is >50% during embryogenesis and ∼30% throughout postnatal ontogeny up to 8 wk of age. These cells may contain immature mTECs, possibly including mTEC progenitor cells that are capable of regenerating the thymic medulla after injury of the thymus caused by, for example, chemotherapy.

In conclusion, we have revealed that mTECs consist of subsets with heterogeneous CCL21 and Aire expression and that LT\beta R regulates the development of the CCL21-expressing subset, but not the Aire-expressing subset, in postnatal mTECs. Specifically, we found that LT\beta R specifically affects the cellularity of CCL21\textsuperscript{+}Aire\textsuperscript{−} mTECs, but not CCL21\textsuperscript{+}Aire\textsuperscript{+} mTECs or CCL21\textsuperscript{−}Aire\textsuperscript{+} mTECs. A schema of possible mTEC developmental pathways, pertaining to the CCL21/Aire mTEC subsets, is shown in Fig. 9. In this study, we also devised a flow cytometric method to analyze mTEC subpopulations, in which the expression of individual

**FIGURE 9.** Possible mTEC developmental pathways of CCL21/Aire mTEC subsets. The present results indicate that mTECs consist of heterogeneous subpopulations based on the expression of CCL21 and Aire, which are both functionally important molecules in the establishment of self-tolerance in T cells. The mTEC subpopulation that expresses neither CCL21 nor Aire and belongs to mTEC\textsuperscript{low} cells may represent immature precursor cells (stage 1). The Aire-expressing mTECs, which belong to mTEC\textsuperscript{high} cells, show heterogeneity in CCL21 expression and are functionally mature by ectopically expressing self-Ags (stage 2). The CCL21-expressing mTECs, which belong to mTEC\textsuperscript{low} cells, are accumulated later in ontogeny than the Aire-expressing mTECs and are functionally mature by attracting positively selected thymocytes (stage 3). Aire partially regulates the development of CCL21-expressing mTECs, so that Aire-expressing mTECs may directly differentiate into (solid line) or indirectly promote the development of (dashed line) CCL21-expressing mTECs. In addition, the development of CCL21-expressing mTECs is partially regulated by LT\beta R, of which the ligand lymphotoxin is produced by positively selected CD4/CD8 single-positive (SP) thymocytes.
molecules can be evaluated on a single-cell basis. Aside from revealing the functional heterogeneity of mTEC subsets in the postnatal thymus, flow cytometric analysis of mTEC subpopulations may contribute to improving our understanding of mTEC biology, including development, homeostasis, and regeneration after damages of mTECs and their multiple subsets.

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Disclosures
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