International Immunology, Vol. 34, No. 1, pp. 45–52
https://doi.org/10.1093/intimm/dxab098
Advance Access publication 23 October 2021

The transcription factor Sox4 is required for thymic tuft cell development

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Received 30 June 2021, *editorial decision* 18 October 2021; *accepted* 22 October 2021

Medullary thymic epithelial cells (mTECs) help shape the thymic microenvironment for T-cell development by expressing a variety of peripheral tissue-restricted antigens (TRAs). The self-tolerance of T cells is established by negative selection of autoreactive T cells that bind to TRAs. To increase the diversity of TRAs, a fraction of mTECs terminally differentiates into distinct subsets resembling atypical types of epithelial cells in specific peripheral tissues. As such, thymic tuft cells that express peripheral tuft cell genes have recently emerged. Here, we show that the transcription factor SRY-box transcription factor 4 (Sox4) is highly expressed in mTECs and is essential for the development of thymic tuft cells. Mice lacking Sox4 specifically in TECs had a significantly reduced number of thymic tuft cells with no effect on the differentiation of other mTEC subsets, including autoimmune regulator (Aire)$^+$ and Ccl21a$^+$ mTECs. Furthermore, Sox4 expression was diminished in mice deficient in TEC-specific lymphotoxin β receptor (LTβR), indicating a role for the LTβR-Sox4 axis in the differentiation of thymic tuft cells. Given that Sox4 promotes differentiation of peripheral tuft cells, our findings suggest that mTECs employ the same transcriptional program as peripheral epithelial cells. This mechanism may explain how mTECs diversify peripheral antigen expression to project an immunological self within the thymic medulla.

Keywords: lymphotoxin, mTEC, self-antigen, thymic epithelial cell, thymus

Introduction

In the thymic medulla, virtually all self-antigens are presented so that autoreactive T cells are eliminated, thus establishing central T-cell tolerance (1, 2). Medullary thymic epithelial cells (mTECs) represent specialized machinery for expressing tissue-restricted antigens (TRAs), self-antigens expressed in a limited number of peripheral tissues (3). Among other thymic stromal cells, medullary fibroblasts also contribute to the diversity of self-antigen expression by producing fibroblast-specific antigens in addition to TRAs (4, 5). To diversify gene expression, mTECs employ a variety of transcriptional mechanisms: Fezf2 binds to the proximal promoter of TRAs, and autoimmune regulator (Aire) regulates TRA expression mainly via super enhancers (6), both of which are under the control of Chd4 (7, 8). Therefore, the thymic medulla maximizes self-antigen expression using multiple cell types and transcriptional mechanisms.

TRA diversity is also enhanced by the heterogeneity of mTECs, which are conventionally subdivided into mTEC^{lo} (MHC-II^{lo} CD80^{lo}) cells and mTEC^{hi} (MHC-II^{hi} CD80^{hi}) cells (9). mTEC^{lo} cells include immature mTECs, which differentiate into mTEC^{hi} cells (10, 11). mTEC^{hi} cells express Aire and a large number of TRAs, and so are referred to as mature mTECs. A fraction of mTEC^{hi} cells undergoes further differentiation into atypical types of cells that resemble peripheral epithelial cells (12, 13). These 'terminally differentiated' mTECs down-regulate the expression of MHC-II and CD80 as well as Aire and are classified as mTEC^{lo} cells. In addition, mTEC^{lo} cells include a sub-population expressing CCL21, a chemokine that recruits immature T cells from the cortex to the medulla (14). Therefore, according to the conventional classification of mTECs, mTEC^{hi} cells represent mature mTECs and mTEC^{lo} cells are a heterogeneous population that includes immature mTECs, CCL21-expressing mTECs and terminally differentiated mTECs (15).

Thymic tuft cells, a recently identified subset of terminally differentiated mTECs, exhibit characteristics similar to

the peripheral tuft cells in intestinal or lung epithelium (16, 17). They express several marker proteins (e.g. DCLK1) and chemosensory receptors as well as type 2 cytokines (e.g. IL-25), which are also expressed by peripheral tuft cells. Certain proteins expressed by thymic tuft cells act as self-antigens presented to developing T cells, possibly contributing to the induction of immune tolerance.

SRY-box transcription factor 4 (Sox4) regulates stem cell maintenance and differentiation in various tissues (18) and impacts lymphocyte development (19–21). Although it has been known that Sox4 is highly expressed in mTECs, its functional significance in mTECs has never been shown (9). In this study, we found that Sox4 is highly expressed in thymic tuft cells in addition to Ccl21a^{+} mTECs. Mice lacking Sox4 specifically in TECs exhibited normal development of conventional cortical thymic epithelial cells (cTECs) and mTECs but a reduced number of thymic tuft cells. Furthermore, the expression of Sox4 was under the control of lymphotoxin β receptor (LTβR) signaling in mTECs. These results indicate a role for Sox4 as a transcription factor that links LTβR signaling to the differentiation program of thymic tuft cells.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC. *Foxn1-Cre* mice (22), *Sox4*^{flox} mice (23) and *Ltbr*^{flox} mice (4) were described previously. Mice were bred and maintained under specific pathogen-free conditions in our animal facility and were euthanized by an overdose of inhalational anesthetics. Both male and female mice were used. Animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Tokyo in accordance with institutional procedures.

Quantitative reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from isolated cells using the RNeasy Plus Micro Kit (Qiagen) and reverse transcribed with SuperScript III (Invitrogen, Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) was performed with SYBR Premix ExTaq (TaKaRa) and the StepOne Real-Time PCR System (Life Technologies, Thermo Fisher Scientific). mRNA levels were normalized to that of *Actb* (β-actin) or *Gapdh*.

Flow cytometry

Flow cytometry and cell sorting were performed using FACSCanto II and FACSAria III systems (BD Biosciences). TECs were prepared by digesting thymic fragments with RPMI-1640 (Fujifilm) containing 0.01% Liberase TM (Roche) and 0.01% DNase I (Roche). Before cell staining, Fc blocker (anti-mouse CD16/CD32; clone 2.4G2; TONBO Biosciences) was applied. The monoclonal antibodies against Aire (5H12) and PLZF (Mags.21F7) were purchased from eBioscience. Monoclonal antibodies specific for CD45 (30-F11), EpCAM (G8.8), Ly51 (6C3), MHC class II (M5/114.15.2), CD80 (16-10A1), TCRβ (H57-597) and T-bet (4B10) were purchased from BioLegend. Biotin-labeled ***Ulex europaeus*** agglutinin 1 (UEA1) was purchased from Vector Laboratories. The anti-Dclk1 polyclonal antibody (ab31704) was purchased from Abcam, and Alexa Fluor 488 anti-rabbit IgG was purchased from Thermo Fisher Scientific. The normal rabbit IgG was purchased from Santa Cruz Biotechnology. The anti-RORγt antibody (Q31-378) was purchased from BD Biosciences, and CD1d tetramer (α-GalCer loaded) was purchased from MBL. 7-Aminoactinomycin D (Fujifilm) was used to exclude dead cells. Intracellular staining was performed using the Foxp3 Staining Kit (eBioscience) or IC Fixation Buffer (eBioscience) according to the manufacturers' instructions. Data were analyzed using FlowJo software (Tree Star).

Single-cell RNA sequencing analysis

Single-cell RNA sequencing (RNA-seq) data of thymic stromal cells were extracted from the GSE dataset (accession no. GSE103967), and *k*-means clustering was performed using the Seurat R Package (v. 3.1.2) after normalization and cell cycle correction. The full source code is available in GitHub (https://github.com/nittatakeshi).

Bulk RNA-seq analysis

RNA-seq data of TECs from wild-type (WT) and Aire-knockout (KO) mice were extracted from the GSE dataset (accession no. GSE65617), and data from control (*Ltbr*$^{flox/flox}$) and LTβRcKO (*Foxn1-Cre Ltbr*$^{flox/flox}$) mice were extracted from the GSE dataset (accession no. GSE147357).

Histological analysis

Thymus lobes were embedded in OCT compound (Sakura Finetek), frozen and sectioned at 5–10 μm thickness using a cryostat (Leica). For immunohistochemical analysis of mTECs, sections were air-dried, fixed with acetone and stained with the following antibodies or reagents: anti-keratin 14 (K14) (rabbit polyclonal, BioLegend), anti-rabbit IgG Alexa Fluor 350 (goat polyclonal, Thermo Fisher Scientific), anti-Aire Alexa Fluor 488 (clone 5H12, eBioscience), anti-CD205 APC (clone NLDC-145, BioLegend), UEA1 biotin (Vector Laboratories), Streptavidin Alexa Fluor 350 (Thermo Fisher Scientific), anti-CCL21 (goat polyclonal, R&D Systems) and/or anti-goat IgG Alexa Fluor 488 (donkey polyclonal, Thermo Fisher Scientific). For staining of thymic tuft cells, thymus sections were air-dried, fixed with 4% paraformaldehyde and stained with the following antibodies: anti-CD205 Alexa Fluor 488 (clone 205yekta, Thermo Fisher Scientific), anti-keratin 10 (K10) (clone DE-K10, BioLegend), anti-mouse IgG Alexa Fluor 647 (goat polyclonal, Thermo Fisher Scientific), anti-Dclk1 (rabbit polyclonal, Abcam) and anti-rabbit IgG Alexa Fluor 350 (goat polyclonal, Thermo Fisher Scientific). Images were obtained using a BZ-9000 fluorescence microscope (Keyence) and analyzed using BZ-H2A (Keyence) and ImageJ (National Institutes of Health).

Statistical analysis

Statistical significance was evaluated using unpaired, two-tailed Student's *t*-test using Prism v. 5 (GraphPad Software). For the flow cytometry analysis of invariant natural killer T (iNKT) cells, since the proportion of iNKT cell subsets changes substantially

as mice age (24, 25), we analyzed paired littermates in each experiment and assessed statistical significance using paired, two-tailed Student's *t*-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s., not significant. No exclusion of samples was performed. For animal experiments, sample size was selected based on experience and preliminary experiments, and mice were randomly selected.

Results and discussion

Sox4 is expressed in mTEC subsets including thymic tuft cells

Previous studies have demonstrated that certain transcription factors are highly expressed in mTECs (9). Sox4 is one of such transcription factors although its function in mTECs remains unclear. To gain insight into the expression trait of Sox4, we analyzed the scRNA-seq datasets of thymic stromal cells (16). Sox4 was highly expressed in mTEC subsets, including Ccl21a$^+$ mTECs and thymic tuft cells (Fig. 1A and B).

Sox4 belongs to the SRY-related high-mobility group box (Sox) family, which has 20 members in the mouse. To examine the expression of all Sox family genes in TECs, we analyzed RNA-seq data of isolated cTECs and conventional mTEC subsets (GSE65617) (26). Sox4 was expressed at the highest level in mTEC^{lo} cells (Fig. 1C). Sox9 showed the second highest expression of Sox family members in mTEC^{lo} cells.

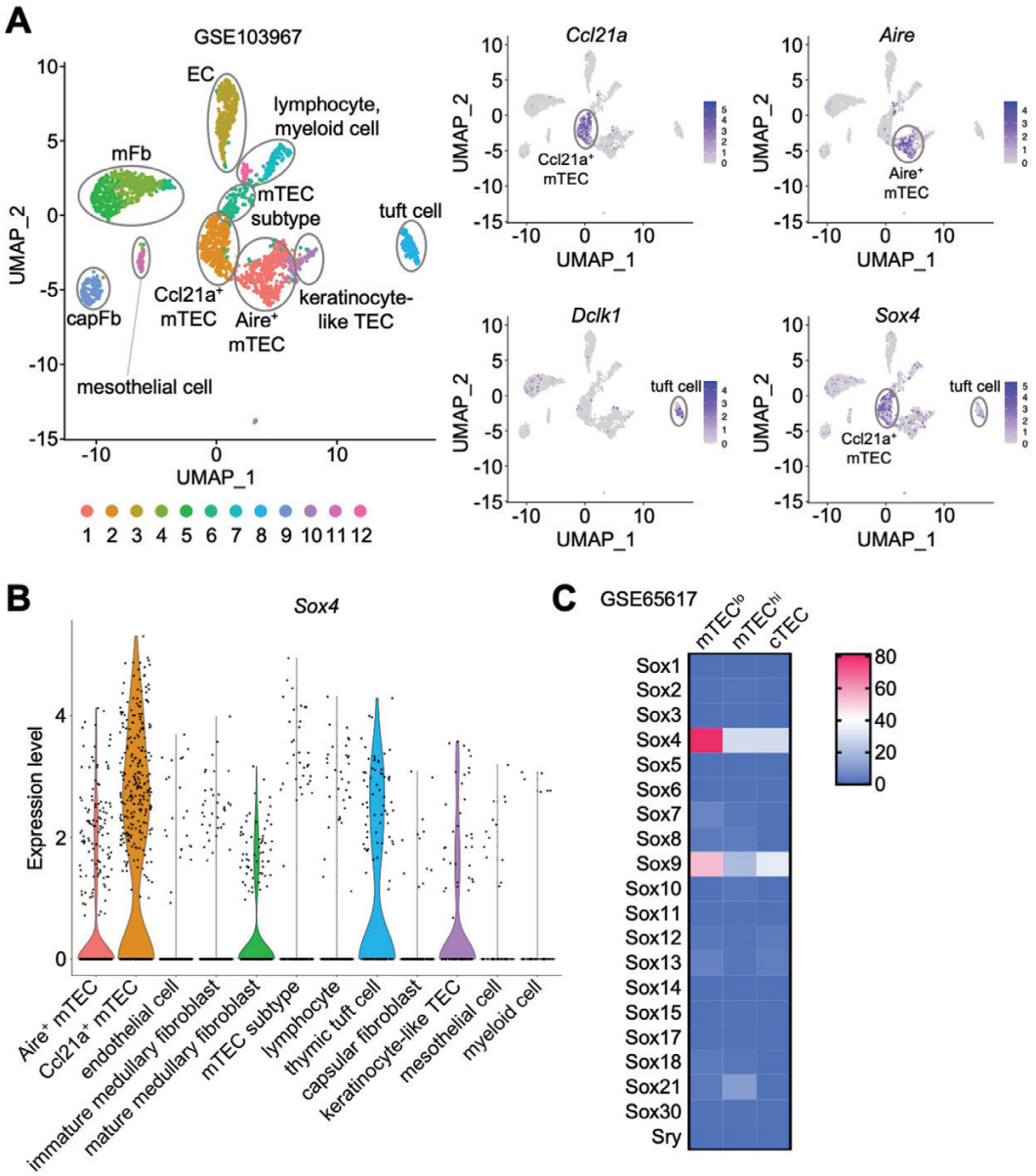

Fig. 1. Expression of Sox4 in subsets of thymic epithelial cells. (A and B) Single-cell RNA-seq data of thymic stromal cells (GSE103967). capFb, capsular fibroblast; EC, endothelial cell; mFb, medullary fibroblast. (A) Two-dimensional representation of cells via UMAP. Each dot represents one cell. (B) Sox4 expression level visualized using a violin plot. (C) Heat map of Sox family genes expressed in mTEC^{lo}, mTEC^{hi} and cTECs (GSE65617).

Sox4 is dispensable for the differentiation of conventional TEC subsets

To investigate the function of Sox4 in TECs, we generated mice lacking Sox4 specifically in TECs (*Foxn1-Cre Sox4*$^{flox/flox}$, 'Sox4cKO'). Whole thymic cells were dissociated by enzymatic digestion and analyzed by flow cytometry (Fig. 2A and B). The frequency and number of cTECs (EpCAM^{+} Ly51^{+} UEA1^{-}) and mTECs (EpCAM^{+} Ly51^{-} UEA1^{+}) were not significantly different between control and Sox4cKO mice. The frequency and number of mTEC$^{lo/hi}$ subsets were not affected in Sox4cKO mice. Furthermore, the number of Aire^{+} mTECs was comparable between control and Sox4cKO mice. Histological analyses detected Aire and Ccl21 expression in the thymic medulla of Sox4cKO mice (Fig. 2C). Therefore, Sox4 deficiency in TECs did not affect the differentiation of conventional TECs.

Next, we examined gene expression in sorted mTEC subsets from control and Sox4cKO mice. qRT-PCR analysis confirmed that Sox4 mRNA expression was completely lost in mTEC^{lo} and mTEC^{hi} cells (Fig. 2D). *Ccl21a* was strongly expressed in mTEC^{lo} cells, and its expression was not affected by Sox4 deficiency. The expression of *Aire* in mTEC^{hi} cells was also not significantly changed in Sox4cKO mice (Fig. 2D). We examined the expression of certain TRA genes. *Csn2* and *Spt2*, representative Aire-dependent TRAs in mTEC^{hi} cells, were expressed at comparable levels in control and Sox4cKO mice. The expression of an Aire-independent TRA, *Crp*, was not decreased by Sox4 deficiency in mTEC^{lo} or mTEC^{hi} cells (Fig. 2D). These results indicate that Sox4 deficiency does not influence the expression of major mTEC-associated genes, including representative TRAs.

Sox4 is required for thymic tuft cell development

In the course of gene expression analysis, we found that mTECs from Sox4cKO mice exhibited reduced expression of tuft cell-associated genes. *Dclk1* and *Pou2f3* are known markers of thymic and peripheral tuft cells, and their mRNA levels were significantly reduced in mTEC^{lo} cells from Sox4cKO mice (Fig. 3A). Other tuft cell-associated genes, *Avil* and *Lrmp* (16), were also significantly underexpressed in Sox4cKO mTEC^{lo} cells compared with control mTEC^{lo} cells. Furthermore, the expression of *Il25*, which is produced by thymic tuft cells and has been shown to be functionally important for the induction of central tolerance (16, 17), was markedly reduced by Sox4 deficiency in mTEC^{lo} cells. These data suggest specific loss of thymic tuft cells in Sox4cKO mice. Indeed, flow cytometry showed that Sox4cKO mice had a significantly reduced frequency and number of Dclk1^{+} mTECs (Fig. 3B and C), indicating that the development of thymic tuft cells is impaired in these mice.

It was previously reported that thymic tuft cells are histologically detectable as DCLK1-expressing cells adjacent to keratin 10-expressing cornified mTECs (17). They sometimes have a bulbous morphology resembling peripheral tuft cells. Our histological analyses showed that the thymus of control mice contained DCLK1-expressing cells with such atypical form in the medullary region (Fig. 3D). The number of DCLK1-expressing cells per medullary area was significantly decreased in Sox4cKO mice compared to controls. These data *in toto* suggest that Sox4 is required for the development of thymic tuft cells.

Previous reports showed that the transcription factor Pou2f3 is a master regulator of thymic tuft cells, as Pou2f3$^{-/-}$ mice exhibited a complete loss of thymic tuft cells (16, 17). Sox4cKO mice showed a significant yet partial impairment of thymic tuft cell development (60% reduction in cell number; Fig. 3C), likely due to the functional redundancy between Sox4 and other Sox family proteins expressed in mTECs, such as Sox9. In addition, Hipk2, a nuclear protein known to interact with Aire and other transcription factors, is also known to promote thymic tuft cell differentiation (17). Molecular interactions and functional coordination between Sox4 and other key regulators including Pou2f3 and Hipk2 remain to be elucidated and require further studies.

Furthermore, it was reported that thymic tuft cells are critical for the development of type 2 iNKT (iNKT2) cells in the thymus (17). Consistent with this, flow cytometry analysis showed that the proportion of iNKT2 cells was significantly reduced in Sox4cKO mice compared with littermate control mice (Fig. 3E).

Sox4 acts downstream of lymphotoxin signaling

To explore the regulatory mechanism of Sox4 expression in mTECs, we investigated the transcriptomes of mTEC subsets. A previous study reported an RNA-seq analysis of mTEC^{lo} and mTEC^{hi} cells isolated from WT and Aire-deficient mice (26). The Sox4 mRNA level was not significantly different between WT and Aire-deficient mTEC^{lo} cells (Fig. 4A), indicating that Aire was dispensable for Sox4 expression in mTEC^{lo} cells. Next, we focused on LTβR, a signaling receptor that controls gene expression in mTEC^{lo} cells (4). In mTEC^{lo} cells lacking LTβR, the Sox4 mRNA level was markedly reduced to the same extent as in mTEC^{hi} cells (Fig. 4A). These results indicate that Sox4 expression is under the control of LTβR signaling in mTEC^{lo} cells.

Furthermore, mice lacking LTβR specifically in TECs (*Foxn1-Cre Ltbr*$^{flox/flox}$, 'LTβRcKO') showed a significant reduction in the frequency and number of Dclk1^{+} thymic tuft cells (Fig. 4B and C). This finding is consistent with a recent report by Lucas *et al.* (27), but the reduction levels vary possibly because of the different origin of *Ltbr*^{flox} mice used in the two studies (4, 28). Our results showed that the decrease in thymic tuft cells in LTβRcKO mice was comparable to that in Sox4cKO mice, strongly suggesting that Sox4 is responsible for the regulation of thymic tuft cell development by LTβR signaling.

Since LTβR signaling in mTECs is activated mainly by T-cell-derived $LT\alpha_1\beta_2$ (29), terminal differentiation of thymic tuft cells might be induced by developing T cells. Formation of the thymic medullary microenvironment is dependent on the 'thymic cross-talk', the interaction between T cells and thymic stromal cells. For example, Aire^{+} mTEC differentiation is induced by T-cell-derived RANKL signaling and T cells in turn migrate to the medullary region in response to mTEC-derived chemokines (30, 31). Our results demonstrate that LTβR signaling induces the expression of Sox4 in mTEC^{lo} cells to drive their differentiation into thymic tuft cells, providing a molecular link between thymic cross-talk signals and mTEC terminal differentiation.

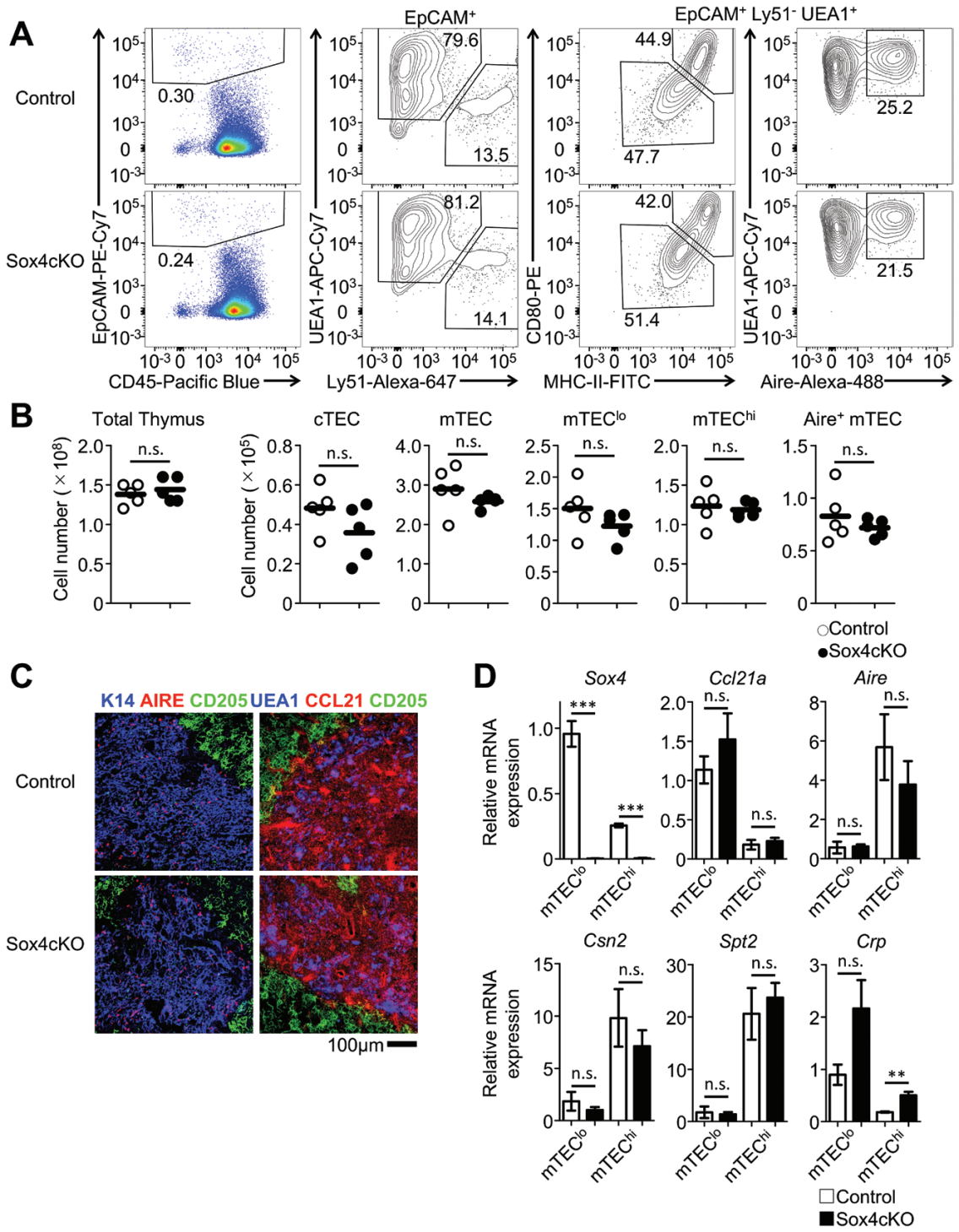

Fig. 2. Sox4 is dispensable for conventional TEC differentiation. (A) The frequency and (B) number of total thymic cells or TECs (EpCAM$^+$), cTECs (EpCAM$^+$Ly51$^+$UEA1$^-$), mTECs (EpCAM$^+$Ly51$^-$UEA1$^+$), mTEC^{lo} cells (EpCAM$^+$Ly51$^-$UEA1$^+$MHC-II^{lo}CD80^{lo}), mTEC^{hi} cells (EpCAM$^+$Ly51$^-$UEA1$^+$MHC-II^{hi}CD80^{hi}) and Aire$^+$ mTECs (EpCAM$^+$Ly51$^-$UEA1$^+$Aire$^+$) as determined by flow cytometry of thymus samples from 4-week-old control (*Foxn1-Cre*, *Sox4*$^{flox/+}$) ($n = 5$) and Sox4cKO (*Foxn1-Cre*, *Sox4*$^{flox/flox}$) ($n = 5$) mice. (C) Thymus sections were stained for keratin 14 (K14, mTEC marker), AIRE, CD205 (cTEC marker), UEA1 (mTEC marker) and CCL21. Data are from three independent experiments with similar results. (D) mTECs sorted from 4-week-old control (*Sox4*$^{flox/flox}$) ($n = 3$) and Sox4cKO (*Foxn1-Cre*, *Sox4*$^{flox/flox}$) ($n = 3$) mice were examined for expression of the indicated genes by qRT-PCR. In (B) and (D), significance was determined using unpaired, two-tailed Student's *t*-tests. $^{**}P < 0.01$; $^{***}P < 0.001$; n.s., not significant.

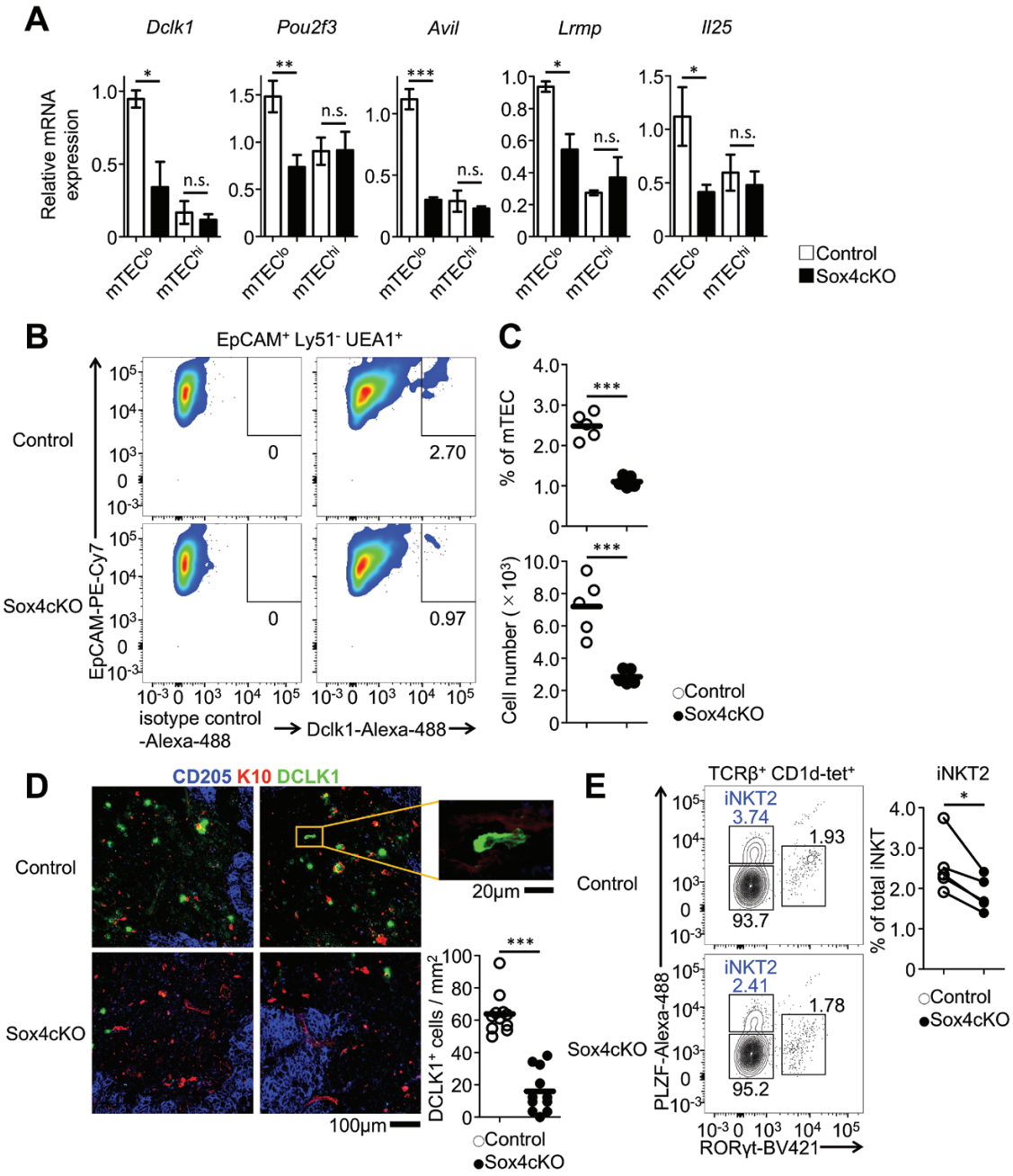

Fig. 3. Sox4 is required for thymic tuft cell development. (A) mTECs sorted from 4- to 16-week-old control (*Sox4*$^{flox/flox}$) (n = 3–8) and Sox4cKO (*Foxn1-Cre*, *Sox4*$^{flox/flox}$) (n = 3–8) mice were examined for expression of the indicated genes by qRT-PCR. (B) Representative plots and (C) the frequency (% of mTEC) and number (cells per thymus lobe) of thymic tuft cells (EpCAM$^+$Ly51$^-$UEA1$^+$Dclk1$^+$) as determined by flow cytometry of gated mTECs (EpCAM$^+$Ly51$^-$UEA1$^+$) from 4-week-old control (*Foxn1-Cre*, *Sox4*$^{flox/+}$) (n = 5) and Sox4cKO (*Foxn1-Cre*, *Sox4*$^{flox/flox}$) (n = 5) mice. (D) Thymus sections from 4-week-old control (*Foxn1-Cre*, *Sox4*$^{flox/+}$) and Sox4cKO (*Foxn1-Cre*, *Sox4*$^{flox/flox}$) mice were stained for the indicated marker proteins. Data are from two independent experiments with similar results. A representative DCLK1$^+$ cell detected in a control thymus is highlighted. The graph shows the number of DCLK1$^+$ cells per unit area of the medullary region. Eleven images from two individual mice per group were examined. (E) Representative plots of thymic iNKT cells (TCRβ$^+$ CD1d-tet$^+$) for RORγt and PLZF from 13- to 16-week-old control (*Sox4*$^{flox/flox}$) (n = 5) and Sox4cKO (*Foxn1-Cre*, *Sox4*$^{flox/flox}$) (n = 5) mice. The graph indicates the frequency of iNKT2 (PLZF^{hi}RORγt^{lo}) in total thymic iNKT cells from littermate control and Sox4cKO mice. In (A), (C) and (D), significance was determined using unpaired, two-tailed Student's *t*-tests. In (E), significance was determined using paired, two-tailed Student's *t*-tests. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant.

Conclusion

The notion that mTECs differentiate into unique epithelial cells resembling skin or intestinal epithelium to produce unique self-antigens was proposed in early studies based on histological and electron microscopic observations (32), prior to the discovery of Aire-dependent TRA expression (33). The recent identification of thymic tuft cells which is based on scRNA-seq and fate-mapping represents rediscovery of

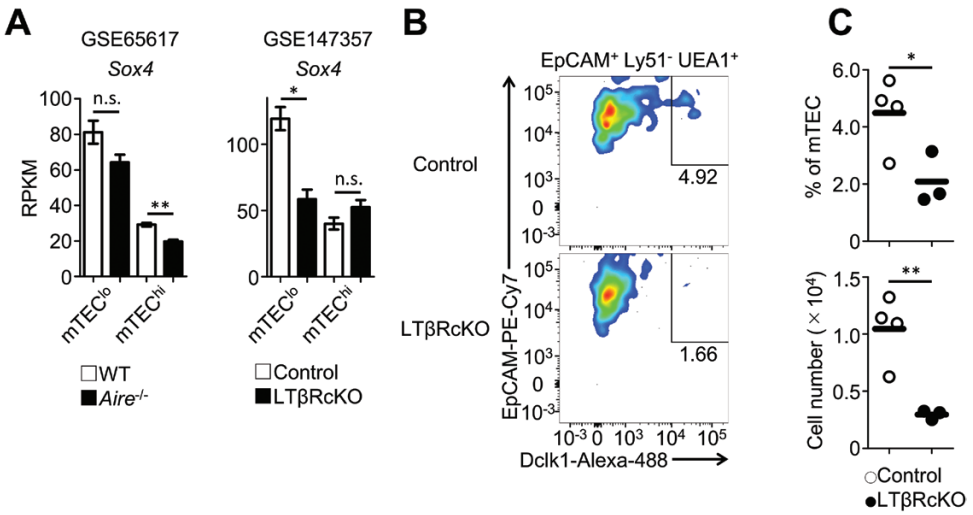

Fig. 4. Sox4 acts downstream of lymphotoxin signaling. (A) The RNA-seq data (GSE65617, GSE147357) were examined for expression of Sox4 by RNA-seq. Control and LTβRcKO represents ***Ltbr***$^{flox/flox}$ mice ($n = 2$) and *Foxn1-Cre*, ***Ltbr***$^{flox/flox}$ mice ($n = 2$), respectively. (B) Representative plot and (C) the frequency (% of mTEC) and number (cells per thymus lobe) of thymic tuft cells (EpCAM$^+$Ly51$^-$UEA1$^+$Dclk1$^+$) as determined by flow cytometry of gated mTECs (EpCAM$^+$Ly51$^-$UEA1$^+$) from 4-week-old control (***Ltbr***$^{flox/flox}$) ($n = 4$) and LTβRcKO (*Foxn1-Cre*, ***Ltbr***$^{flox/flox}$) ($n = 3$) mice. In (A) and (C), significance was determined using unpaired, two-tailed Student's *t*-tests. *$P < 0.05$; **= < 0.01; n.s., not significant.

such cells and the confirmation of this notion (16, 17). In the present study, we found that Sox4, a transcription factor expressed in mTECs, plays a key role in the development of thymic tuft cells. Since Sox4 is also important for the differentiation of intestinal tuft cells (34), our findings suggest that mTECs use specific transcription factors to recapitulate the differentiation of peripheral epithelial cells, in order to expand the variety of self-antigens. Future studies will unveil a whole picture of transcription factors responsible for the differentiation of various mTEC subsets and induction of central immune tolerance.

Funding

This study was supported by the Japan Society for Promotion of Science (JSPS) (KAKENHI 21J11071 to N.M.; 19J00248 and 19K16602 to R.M.; 16H05202, 17H05788, 19H03485 and 19H04802 to T.N.; and 15H05703 to H.T.), the CREST Program of the Japan Agency for Medical Research and Development (AMED) (21gm1210008 to H.T.), the Ichiro Kanehara Foundation for the Promotion of Medical Science and Medical Care (no. 57 to R.M.) and a research grant from the Astellas Foundation for Research on Metabolic Disorders (no. 1102 to R.M.). N.M. and R.M. were supported by a JSPS Research Fellowship for Young Scientists. N.M. was supported by World-leading INnovative Graduate Study Program for Life Science and Technology (WINGS-LST) of the University of Tokyo. A.O. was supported by the MD Scientist Training Program of the University of Tokyo.

Acknowledgements

We thank K. Kubo and T. Tachibana for technical assistance and T. Iguchi for insightful discussions.

Conflicts of interest statement: the authors declared no conflicts of interest.

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