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TGF-β type II receptor expression in thymic epithelial cells inhibits the development of Hassall's corpuscles in mice

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Abstract

Hassall's corpuscles are concentric clusters of keratinized epithelial cells located within the thymic medulla of humans and guinea pigs but are scant in mouse and rat. They are considered to be the terminally differentiated stages of medullary thymic epithelial cells (mTECs) but the mechanisms of their origin are unclear. We have previously deleted the TGF-β type II receptor (TGFβRII) specifically in mouse TECs and reported that these mice have mitigated thymic involution and exhibit earlier reconstitution post-irradiation. In this study, we analyzed the differentiation of mTECs in the TGFβRII-knockout mice. Interestingly, the TGFβRII-knockout mice display enhanced development of Hassall's corpuscles. The expression of Aire, stromal-cell-derived factor 1 and thymic stromal lymphopoietin in the thymi of the TGFβRII-knockout mice was similar to that previously reported for the human thymus. In addition, the putative epithelial progenitor markers MTS20 and MTS24 labeled Hassall's corpuscles in normal mice, but the extent and intensity of this staining were greatly enhanced in Hassall's corpuscles of the TGFβRII-knockout mice. The phosphorylated forms of ERK and JNK were also found in Hassall's corpuscles of the TGFβRII-knockout mice. Taken together, we suggest that TGFβRII-mediated signaling in TECs inhibits their development into Hassall's corpuscles in mice.

Keywords: epithelial cells, Hassall's corpuscle, MTS24, TGF-β type II receptor, thymus

Introduction

The thymus provides a specialized environment uniquely adept in attracting lymphoid precursor cells and inducing their proliferation, differentiation and selection into functionally mature T cells, which are ultimately exported to peripheral lymphoid tissues. Within this microenvironment, thymic epithelial cells (TECs) constitute the most abundant stromal component and are arranged both in the cortex and in the medulla as a three-dimensional scaffold (1–3). Recent reports have indicated that both different types of TECs, cortical TECs and medullary TECs (mTECs), may be derived from common progenitors in ontogeny (4–7). The MTS20 and MTS24 monoclonal antibodies recognize an antigen expressed on a population containing high-efficiency TEC progenitor cells (4, 5). Accumulating evidence indicates that mTECs comprise heterogeneous populations.

Hassall's corpuscles, also known as Hassall's bodies, are found in thymic medulla and form characteristic swirled epithelial structures. In addition to their distinctive histologic appearance, Hassall's corpuscles express the antigens that are detectable in the terminally differentiated upper layers of the epidermis and are therefore thought to be composed of terminally differentiated mTECs (8–10). Hassall's corpuscles are well developed in humans and guinea pigs, but, interestingly, they are not typically seen in mice or rats (2). Hassall's corpuscles have been proposed to act in both the removal of dead thymocytes and the

maturation of medullary thymocytes (11–13). Other studies have provided evidence that Hassall's corpuscles express cytokines, such as IL-7, CD30 ligand, stromal-cell-derived factor 1 (SDF-1), macrophage-derived chemokine and thymic stromal lymphopoietin (TSLP) (14–18). Recently, TSLP produced by Hassall's corpuscles was shown to educate dendritic cells to induce the development of $CD4^+CD25^+$ regulatory T cells (18). These data suggest that Hassall's corpuscles actively communicate with developing T cells within the thymus.

TGF-β superfamily members exert their effects primarily via a receptor complex comprising type I and type II receptors (TGFβRI and TGFβRII) (19, 20). To date, TGFβRII has been identified to be essential for TGF-β binding and for complex assembly with TGFβRI. When TGF-β superfamily members bind to the TGFβRI and TGFβRII complex, activated TGFβRI propagates the signals downstream via phosphorylation of specific receptor-regulated Smad proteins. Phosphorylated Smad2 (p-Smad2) and p-Smad3 form heteromeric complexes with Smad4 and translocate into the nucleus to regulate TGF-β-responsive genes. Several Smad-independent TGF-β signaling pathways have also been identified, including MAPK pathways (19, 20). How they regulate thymopoiesis and the constitution of the TEC compartment is largely unknown. Mice deficient for the expression of TGFβRII die around embryonic day 11.5. Therefore, we have conditionally inactivated TGFβRII using Cre/Lox technology on TECs to identify whether the TGF-β signaling pathway plays a role in TEC development and function (21). We previously demonstrated that the disruption of TGFβRII expression on TECs results in a mitigated thymic involution and an early post-irradiation reconstitution (21).

Here, we performed a detailed analysis of thymic epithelium in the mice deficient for TGFβRII. We found the enhanced development of Hassall's corpuscles in the thymic medulla, suggesting that TGFβRII expression on TECs restricts their cell progression into Hassall's corpuscles in mice. Although previous studies have demonstrated that MTS20 or MTS24 immunostaining is found on a rare subset of mTECs in adult mouse thymus (4, 5), we show that most of the MTS20 or MTS24 staining in the thymus of postnatal mice is found in Hassall's corpuscles. On the basis of our results, we discuss the role of Hassall's corpuscles in thymogenesis.

Methods

Mice

The generation of $TGF\beta RII^{lox/lox}$ and $TGF\beta RII^{lox/lox}$::Foxn1-Cre mice has been previously reported (21). Mice were housed at the center's animal facility in accordance with Institutional and Cantonal review boards and were used at the age of 8 weeks.

Antibodies and reagents

The following antibodies and reagents were used: rabbit anti-keratin 5 (Covance, Berkeley, CA, USA), rabbit anti-involucrin (Covance), rat anti-E-cadherin (clone ECCD2) (a gift from Dr M. Takeichi at the Riken Center for Developmental Biology or a product of Takara Bio Inc., Shiga, Japan), rabbit anti-CXCL12α subunit (SDF-1α) (eBioscience), rat monoclonal anti-Aire (22), rabbit anti-TSLP (Sigma–Aldrich, St Louis, MO, USA), rat monoclonal antibody MTS20 (4, 5), rat monoclonal antibody MTS24 (4, 5), rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology, Inc.), rabbit anti-phospho-p-38 MAPK (Thr180/Tyr182) (Cell Signaling Technology, Inc.) and Alexa Fluor-labeled donkey secondary antibodies (Molecular Probes, Eugene, OR, USA). The binding to biotinylated *Ulex europaeus* agglutinin-1 (UEA-1) (Vector Laboratories, Burlingame, CA, USA) or biotinylated *Tetragonolobus purpureas* agglutinin (TPA) (Sigma–Aldrich) was followed by FITC- or PE-conjugated streptavidin (eBioscience).

Immunofluorescence staining and confocal microscopy

Frozen thymic sections were prepared and immunofluorescence staining was performed on thymic sections as previously described (21, 23, 24). Confocal laser-scanning microscopy analysis was performed on a Zeiss LSM 510 (Carl Zeiss, Oberkochen, Germany). Negative controls were performed by replacement of first-step antibodies with isotype-matched monoclonal antibodies or species-matched antibodies. Representative images were chosen from each experiment (n = 6 for $TGF\beta RII^{lox/lox}$; n = 6 for $TGF\beta RII^{lox/lox}$::Foxn1-Cre mice) for figure preparation.

Results

Hassall's corpuscles are developed in mice deficient for TGFβRII on TECs

To clarify whether the disruption of TGFβRII expression on TECs affects the differentiation of mTECs, we stained thymus tissues from 8-week-old $TGF\beta RII^{lox/lox}$ and $TGF\beta RII^{lox/lox}$::Foxn1-Cre mice with the reagents recognizing mTECs, and we analyzed them by confocal microscopy. The distribution of keratin 5 allows distinction of mTECs, and the mature populations of mTECs bind the lectin UEA-1 (23, 24). It has been demonstrated that the lectin TPA particularly binds to Hassall's corpuscles (25). In $TGF\beta RII^{lox/lox}$ mice, $UEA\text{-}1^+$ epithelial cells formed a network of stellate cells that builds the thymic medulla (Fig. 1A). As expected, the binding of TPA was also restricted to mTECs, and the fractions of TPA-binding TECs were occasionally detected in small globular cell bodies (Fig. 1B). Surprisingly, many TPA-binding spheres were detectable in the thymic medulla of $TGF\beta RII^{lox/lox}$::Foxn1-Cre mice (Fig. 1B). These Hassall's corpuscles in $TGF\beta RII^{lox/lox}$::Foxn1-Cre mice varied in size and expressed the ligands for both UEA-1 and TPA (Fig. 1C). Immunohistochemistry of human thymus using anti-involucrin antibody is known to stain Hassall's corpuscles (26). When thymus sections from $TGF\beta RII^{lox/lox}$::Foxn1-Cre mice were stained with anti-involucrin antibody, we found larger involucrin-expressing structures with a hyalinized degenerated core in the thymic medulla (Fig. 2). Furthermore, immunoglobulins are present in Hassall's corpuscles of the human thymus (27), and we

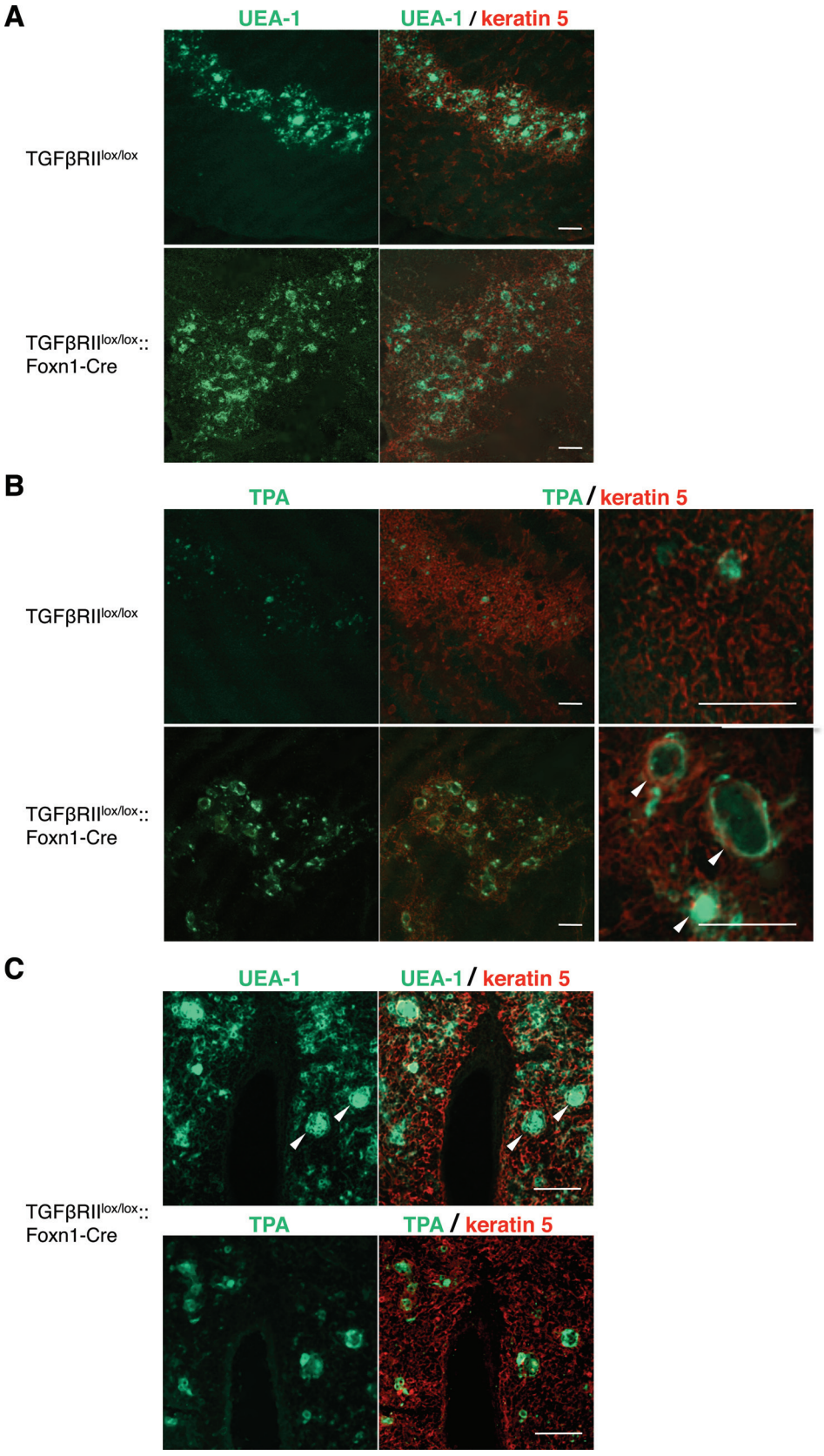

Fig. 1. Hassall's corpuscles are developed in mice deficient for TGFβRII on TECs. Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice at the age of 8 weeks was performed to detect keratin 5 (red) and the binding to (A) UEA-1 or (B) TPA (green).

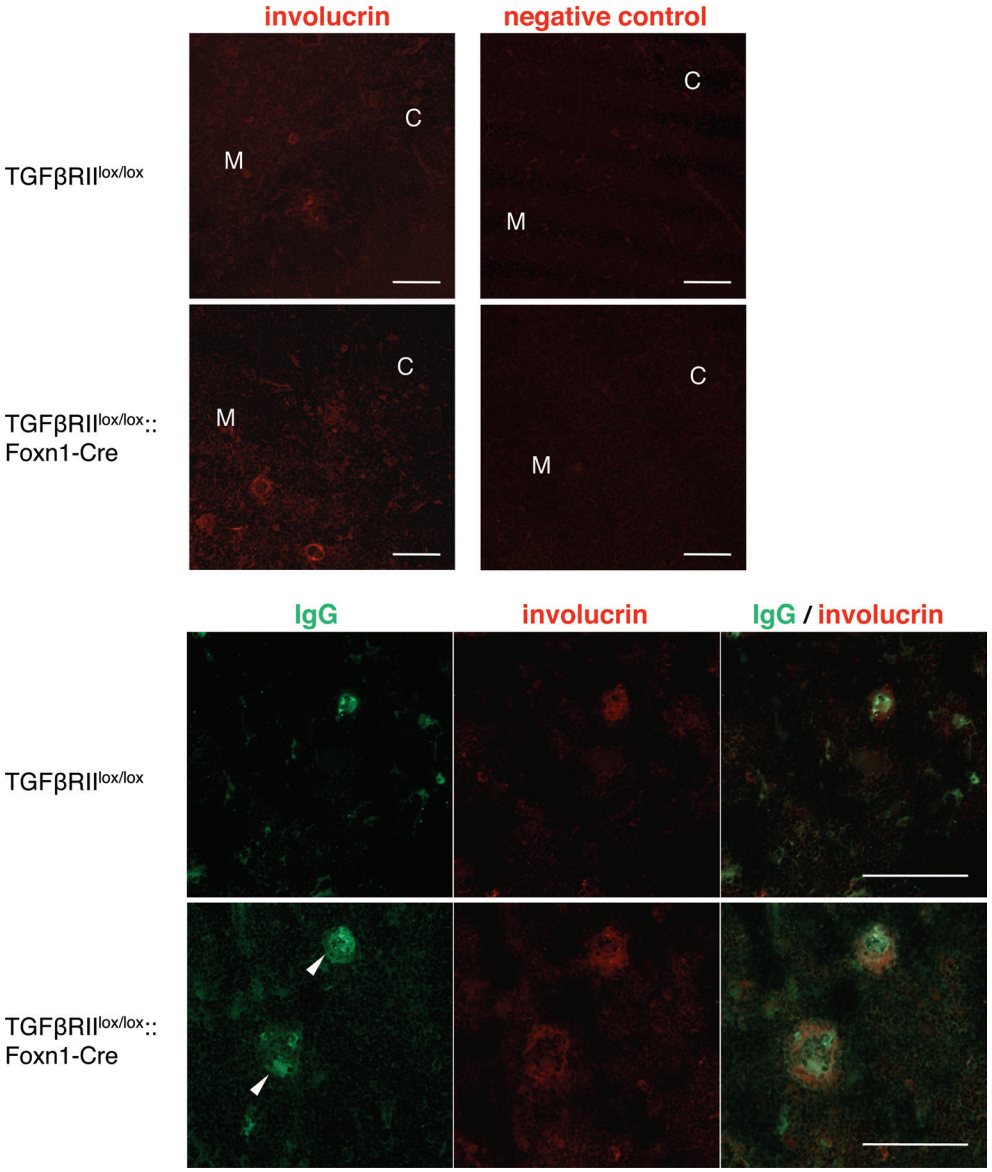

Fig. 2. Hassall's corpuscles in mice deficient for TGFβRII on TECs. Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed to detect involucrin (red) and the binding to IgG (green). Negative controls were performed by replacement of first-step antibodies with isotype-matched antibodies. IgG binds to involucrin-expressing Hassall's corpuscles (arrowheads). Data are representative of three independent experiments with six mice per group. Bars = 100 μm.

also detected IgG in Hassall's corpuscles of TGFβRII$^{lox/lox}$::Foxn1-Cre mice (Fig. 2).

Phenotypic changes of mTEC subsets in mice deficient for TGFβRII on TECs

We further examined the expression of E-cadherin, Aire, SDF-1 and TSLP in the thymi of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice. As reported previously (28), E-cadherin was expressed in all TECs of both cortex and medulla in the thymi of TGFβRII$^{lox/lox}$ mice, and Hassall's corpuscles with globular cell bodies in the medulla were strongly reactive with antibodies against E-cadherin (Fig. 3A). In TGFβRII$^{lox/lox}$::Foxn1-Cre mice, the epithelial cells composing the outer layers of Hassall's corpuscles showed high expression of E-cadherin. A small population of mTECs was shown to express the autoimmune regulator Aire, which is crucial in the induction of T-cell tolerance toward tissue-restricted antigens (29). Previous observations reported that Aire^{+} mTECs

(C) Immunofluorescence staining in serial thymic sections of TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed to detect keratin 5 (red) and the binding to UEA-1 or TPA (green). TPA binds to Hassall's corpuscles (arrowheads) and TPA-binding TECs are concentrically arranged around degenerating cells. Data are representative of three independent experiments with six mice per group. Bars = 100 μm.

are accumulated around Hassall's corpuscles of normal human thymus (30). In wild-type mice, Aire$^+$ cells dispersed within the thymic medulla, whereas a concentration of Aire$^+$ cells was frequently detectable at the margins in the periphery of Hassall's corpuscles in TGFβRII$^{lox/lox}$::Foxn1-Cre mice (Fig. 3B).

SDF-1 is expressed in the thymus and has been reported as a chemoattractant for immature T-cell progenitors in mouse thymus (31). The distribution of SDF-1 was detected in mTEC of TGFβRII$^{lox/lox}$ mice (Fig. 4A). On the other hand, in mutant littermates, high levels of SDF-1 expression were detected in the outer walls of Hassall's corpuscles and the adjacent mTECs as previously reported in human thymus (17). TSLP is a cytokine that was originally identified as a growth factor from mTECs (32). TSLP is produced by stromal cells, epithelial cells and mast cells, but not other hematopoietic cell types or endothelial cells (33). In the mutant mice, TSLP was detectable in the outer layer of Hassall's corpuscles (Fig. 4B).

The putative epithelial progenitor markers MTS20 and MTS24 are expressed in Hassall's corpuscles

Furthermore, the reactivity of MTS20 or MTS24 in the thymi of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was investigated by immunofluorescence staining. As shown in Fig. 5, MTS20 or MTS24 staining was detected in a rare population of

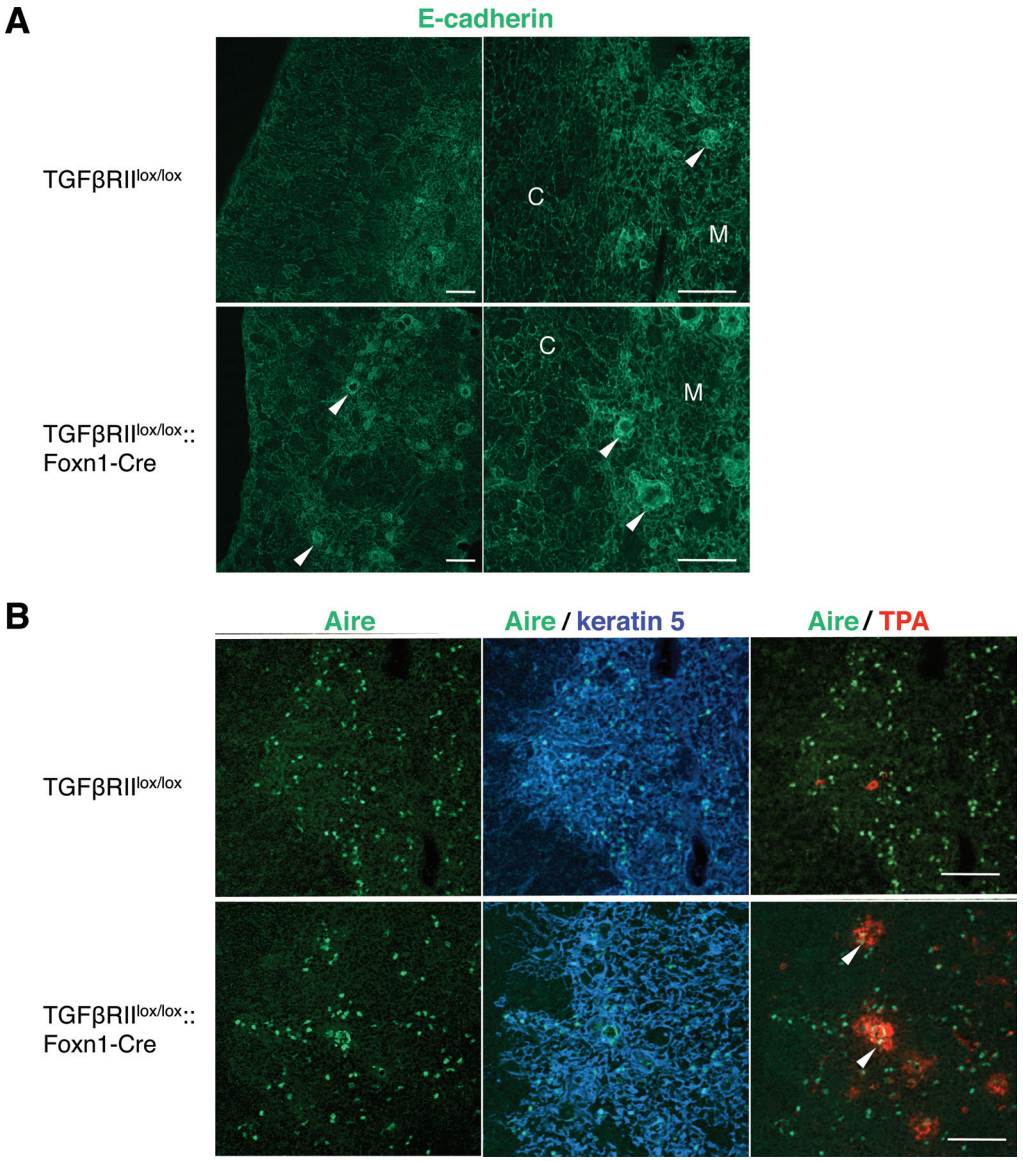

Fig. 3. Localization of E-cadherin and Aire in the thymi of mice deficient for TGFβRII on TECs. (A) Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed to detect E-cadherin (green). E-cadherin is highly expressed in cells forming the outer walls of Hassall's corpuscles (arrowheads) in TGFβRII$^{lox/lox}$::Foxn1-Cre mice. (B) Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed to detect Aire (green), keratin 5 (blue) and the binding to TPA (red). Note the concentration of Aire$^+$ cells in the periphery of Hassall's corpuscles in TGFβRII$^{lox/lox}$::Foxn1-Cre mice (arrowheads). Data are representative of three independent experiments with six mice per group. Bars = 100 μm.

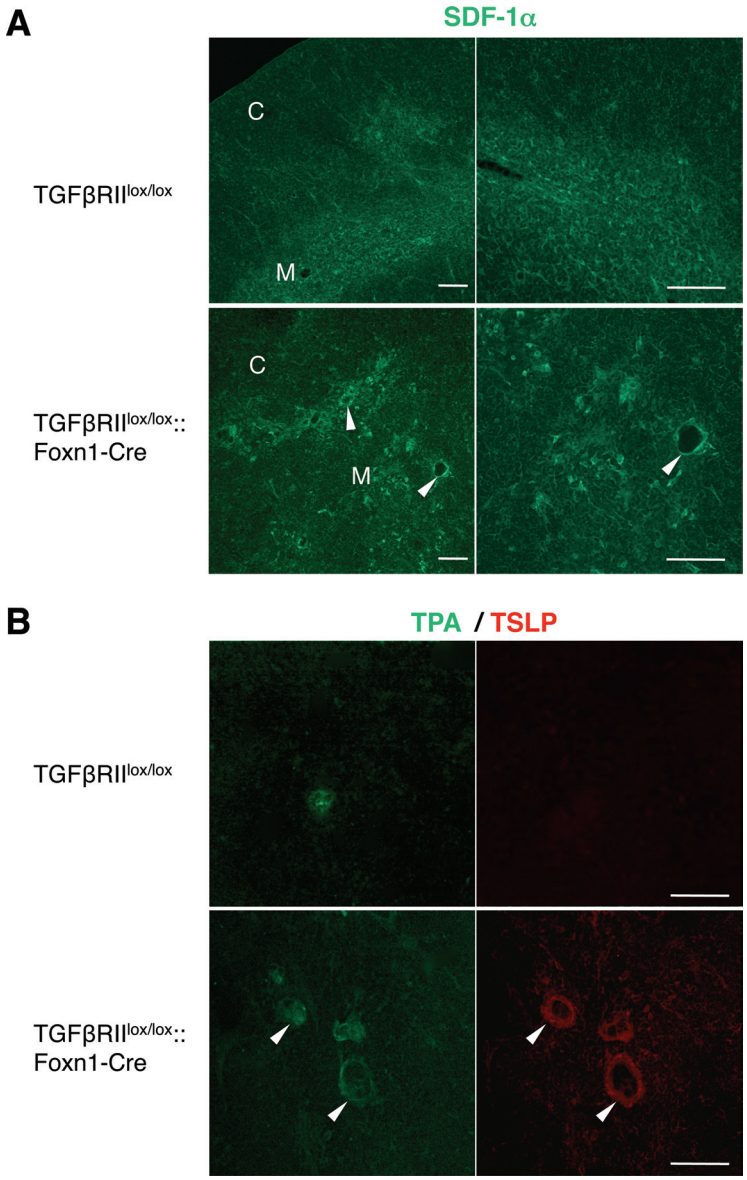

Fig. 4. Localization of SDF-1 and TSLP in the thymi of mice deficient for TGFβRII on TECs. (A) Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed to detect SDF-1 (green). While SDF-1 is expressed in mTECs of TGFβRII$^{lox/lox}$ mice, it is seen in Hassall's corpuscles (arrowheads) and the adjacent mTECs of TGFβRII$^{lox/lox}$::Foxn1-Cre mice. (B) Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed to detect the binding to TPA (green) and TSLP (red). Note the expression of TSLP in the outer layer of Hassall's corpuscles (arrowheads). Data are representative of three independent experiments with six mice per group. Bars = 100 μm.

mTECs and restricted in Hassall's corpuscles of TGFβRII$^{lox/lox}$ mice. Surprisingly, TGFβRII$^{lox/lox}$::Foxn1-Cre mice showed the presence of a high number of MTS20$^+$ or MTS24$^+$ cells in Hassall's corpuscles. The epithelial cells composing the outer layers of involucrin-expressing Hassall's corpuscles were labeled with MTS24 (Fig. 5B).

Activation of MAPK signaling in the thymus deficient for TGFβRII on TECs

Previous studies in human thymus have reported that Hassall's corpuscles are active in ERK and p-38 kinase signaling (34). Activation of MAPK in the thymi of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was investigated by immunofluorescence staining using antibodies that specifically recognize the phosphorylated forms of ERK, JNK or p-38. As shown in Fig. 6, Hassall's corpuscles in the mutant mice frequently displayed positive staining for p-ERK and p-JNK, whereas p-38 kinase was hardly detected in the thymi of TGFβRII$^{lox/lox}$::Foxn1-Cre mice (data not shown). The phosphorylated forms of these three MAPKs were hardly detectable in the thymi of wild-type mice (Fig. 6 and data not shown).

Discussion

In the present study, we found that thymi deficient for the expression of TGFβRII on TECs exhibit markedly enhanced development of Hassall's corpuscles, these structures being

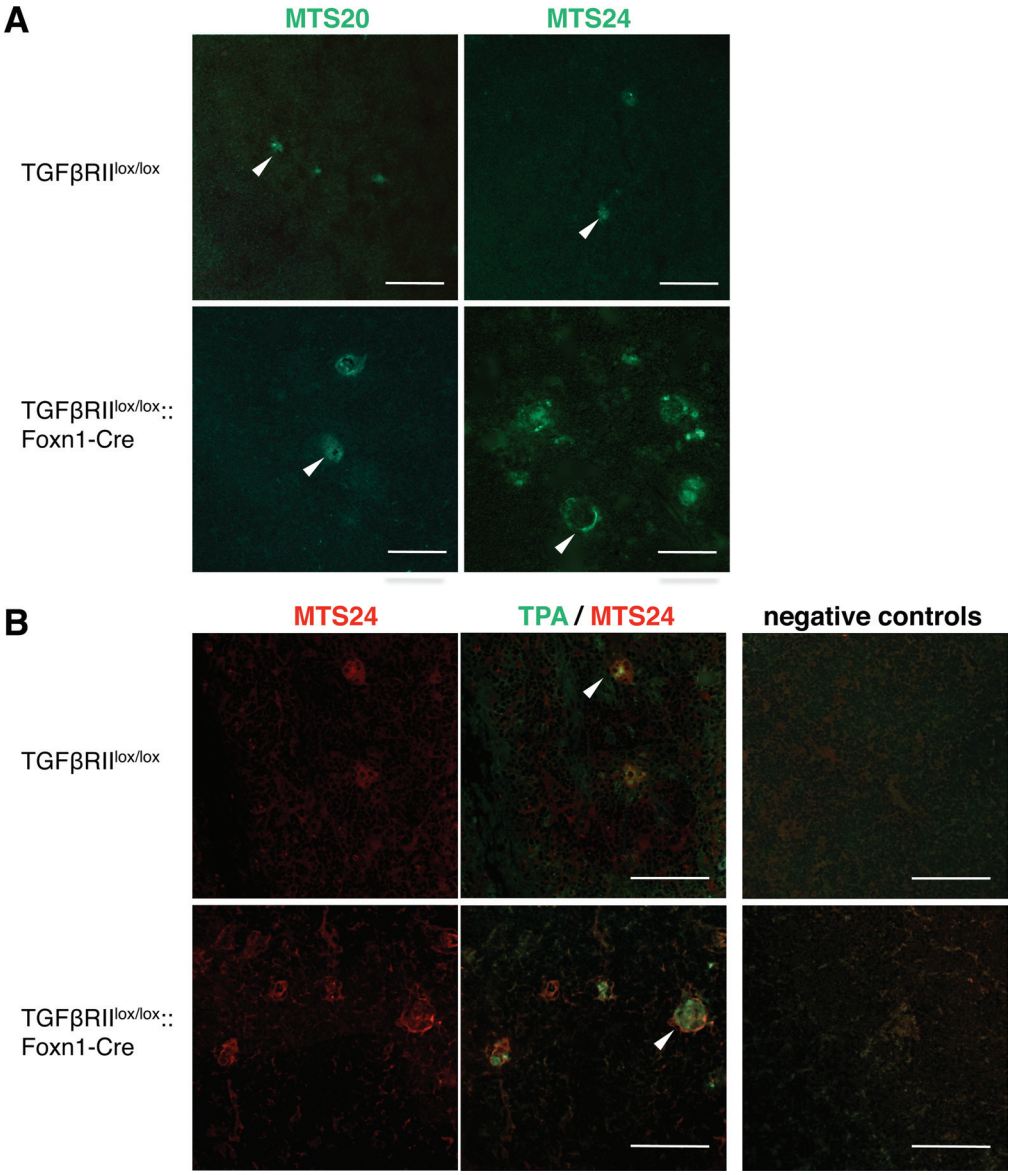

Fig. 5. Immunoreactivity of MTS20 and MTS24 in Hassall's corpuscles. (A) Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed with MTS20 or MTS24 (green). (B) Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed to detect the binding to MTS24 (red) and TPA (green). Negative controls were conducted by replacement of first-step antibodies with isotype-matched antibodies and FITC-conjugated streptavidin alone. Note the positive staining of MTS20 and MTS24 in Hassall's corpuscles (arrowheads). Data are representative of three independent experiments with six mice per group. Bars = 100 μm.

rare or often non-existent in normal mice. The corollary is that TGFβRII-mediated signaling on TECs inhibits the development of Hassall's corpuscles in mice. The distributions of Aire, SDF-1 and TSLP in the thymus of TGFβRII$^{lox/lox}$::Foxn1-Cre mice were similar to those described previously in normal human thymus (17, 18, 30).

The putative epithelial progenitor markers MTS20 and MTS24 were also found to be expressed in Hassall's corpuscles. A high number of MTS20$^+$ or MTS24$^+$ cells was observed in Hassall's corpuscles of TGFβRII$^{lox/lox}$::Foxn1-Cre mice. Indeed, this may be linked to the finding that TGFβRII$^{lox/lox}$::Foxn1-Cre mice display a decelerated age-related thymic involution (21). In addition, the mutant mice exhibit accelerated kinetics in thymopoiesis after irradiation (21). Thymus tissues from immunodeficient patients with a reduced thymopoiesis totally lack Hassall's corpuscles (35–37). Collectively, this implies a role for Hassall's corpuscles in thymic maintenance in the postnatal thymus; these mice will be a vehicle for exploring this possibility. TSLP is also reported to play an important role in expansion of thymocyte progenitors (38, 39), and TSLP was clearly evident in Hassall's corpuscles of TGFβRII$^{lox/lox}$::Foxn1-Cre, potentially contributing to the enhanced thymopoiesis. Nevertheless, we cannot exclude the possibility that Hassall's corpuscles may produce other factors inducing thymopoiesis. In human thymus, mTECs of Hassall's corpuscles produce TSLP, which acts on thymic dendritic cells,

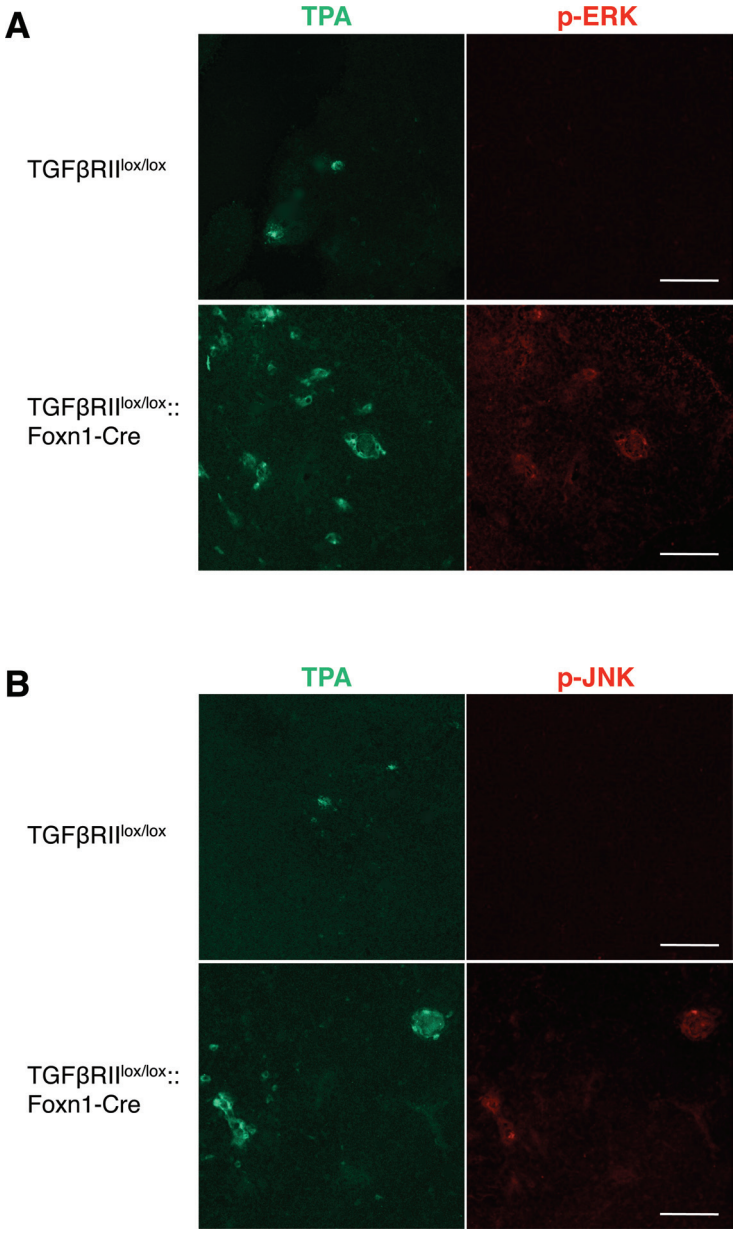

Fig. 6. Phosphorylation of MAPK in Hassall's corpuscles. Immunofluorescence staining in thymic sections of TGFβRII$^{lox/lox}$ and TGFβRII$^{lox/lox}$::Foxn1-Cre mice was performed to detect the binding to TPA (green) and phosphorylated forms of ERK (A, red) and JNK (B, red). TGFβRII$^{lox/lox}$::Foxn1-Cre mice display an increased proportion of cells staining positive for p-ERK and p-JNK in Hassall's corpuscles. Data are representative of three independent experiments with six mice per group. Bars = 100 μm.

and these activated dendritic cells subsequently prime differentiation of CD4$^+$CD8$^-$CD25$^-$ thymocytes into regulatory T cells (18). We examined the localization of regulatory T cells and dendritic cells within the thymi of TGFβRII$^{lox/lox}$::Foxn1-Cre mice, but we could not observe the accumulation of Foxp3$^+$ thymocytes and CD11c$^+$ dendritic cells in Hassall's corpuscles (data not shown).

Interestingly, the phosphorylated forms of ERK and JNK were frequently detectable in Hassall's corpuscles of TGFβRII$^{lox/lox}$::Foxn1-Cre mice. It has been unknown why Hassall's corpuscles are poorly developed in mice and rats. Our findings imply that the absence of the activation of these MAPKs may be contributory. Additional studies will be required to determine whether TGF-β signaling could negatively regulate other signaling-induced MAPK activation in mouse TECs. Since the lack of TGFβRII expression on TECs resulted in the enhanced development of Hassall's corpuscle in mice, it will be interesting to clarify the relationship between TGFβRII expression on TECs and the formation of Hassall's corpuscles in the thymi of patients with primary immunodeficiencies who lack thymopoiesis.

In summary, a striking finding in the loss of TGFβRII expression on TECs was the progressive change in the differentiation

of mTECs leading to the formation of Hassall's corpuscles. To our knowledge, this is the first observation of significant development of Hassall's corpuscles in mice. Further studies will be required to elucidate the mechanism by which TGF-β signaling regulates TEC differentiation, via engagement of other signaling pathways.

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