

Letter to the Editor

ζ Chain-associated protein of 70 kDa (ZAP70) deficiency in human subjects is associated with abnormalities of thymic stromal cells: Implications for T-cell tolerance

To the Editor:

Thymic stromal cells play a critical role in permitting the development, maturation, and selection of T lymphocytes.¹ In particular, cortical thymic epithelial cells (TECs) promote differentiation and positive selection of thymocytes, whereas medullary thymic epithelial cells (mTECs) and dendritic cells (DCs) participate in the negative selection of self-reactive T cells and the generation of regulatory T (Treg) cells (see Fig E1 in this article's Online Repository at www.jacionline.org).^{1,2} Maturation of mTECs is marked by expression of claudin 4 (CLD4), upregulation of HLA-DR, and binding to *Ulex europaeus* agglutinin 1 (UEA-1). A subset of mature mTECs express autoimmune regulator (AIRE), a transcription factor that promotes expression of tissue-restricted antigens, thereby permitting negative selection of T cells recognizing tissue-restricted antigens presented by AIRE⁺ mTECs and thymic DCs.¹ Furthermore, terminally differentiated mTECs in Hassall corpuscles from normal human thymus express involucrin (IVL) and secrete thymic stromal lymphopoietin.^{2,3} This cytokine acts on myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs), which instruct differentiation of natural regulatory T (nTreg) cells expressing the transcription factor forkhead box protein 3 (FOXP3).² Selection of nTreg cells is also contributed by AIRE⁺ mTECs. Therefore cross-talk between thymocytes and thymic stromal cells is essential to establish immune tolerance.

We have previously shown that various forms of severe combined immunodeficiency in human subjects are associated with impaired differentiation of TECs, reduced expression of AIRE, and low numbers of thymic mDCs.⁴ In addition, similar abnormalities are observed in patients with hypomorphic mutations in severe combined immunodeficiency-causing genes and might contribute to the immune dysregulation of Omenn syndrome.⁴ By contrast, little is known on the thymic stromal compartment in patients with late defects in human T-cell development. We have used morphometric analysis and immunohistochemistry to study the thymic microenvironment of 2 infants with ZAP70 deficiency. Both presented with recurrent infections, had palpable lymph nodes, and had a small-sized thymic shadow detectable by imaging. Characteristic of ZAP70 deficiency, both infants had a selective deficiency of CD8⁺ lymphocytes and preserved generation (full Vβ repertoire) but impaired function of CD4⁺ lymphocytes.⁵ Both patients were previously reported and carried a homozygous intronic mutation that resulted in the creation of a cryptic acceptor splice site and a 9-nt insertion in the ZAP70 mRNA. ZAP70 protein was not detected in patients' lymphocytes, indicating that this truly is a null mutation. Thymic biopsy specimens were obtained without complications by using a minimally invasive technique after obtaining informed consent. The study was approved by the Institutional Review Board of the Hospital for Sick Children, Toronto, Ontario, Canada.

Evaluation of thymic architecture in both patients showed preserved corticomedullary demarcation, as well as the presence

of Hassall corpuscles (Fig 1, A), with normal compartmentalization of cytokeratin (CK) 8⁺ CK5[−] cortical TECs and CK8[−] CK5⁺ mTECs (data not shown). However, careful morphometric analysis showed that the thymic medullary area was smaller in ZAP70-deficient (ZAP70^{−/−}) patients than in control subjects (Fig 1, A; ZAP70^{−/−} thymus: 5.52 vs control thymus: 1.05). mTECs from ZAP70^{−/−} patients expressed both CLD4 and the ligand for UEA-1, 2 markers of mature mTECs. Although AIRE⁺ cells were present, their numbers were significantly reduced in ZAP70^{−/−} thymuses (Fig 1, B; ZAP70^{−/−} thymus: 3.3 ± 1.49 vs control thymus: 7.6 ± 1.95; *P* < .001). Hassall corpuscles were present in normal numbers, although some appeared small. Importantly, Hassall corpuscle mTECs from ZAP70^{−/−} thymuses expressed minimal amounts of thymic stromal lymphopoietin and IVL (Fig 1, B), indicating failure to sustain terminal differentiation of mTECs. Of note, staining for AIRE and IVL in control thymuses identified a nonoverlapping set of cells in the thymic medulla, confirming previous observations.³

In the normal thymus FOXP3⁺ thymocytes are abundantly present in the medulla, especially in close proximity to Hassall corpuscles (Fig 1, C, left panels), as previously reported.² A similar pattern was detected also in ZAP70^{−/−} patients, although the number of FOXP3⁺ cells was reduced compared with the number seen in control thymuses (Fig 1, C; ZAP70^{−/−} thymus: 39.3 ± 6.75 vs control thymus: 108.85 ± 10.57; *P* < .001).

Thymic DCs are predominantly located in the medulla and at the corticomedullary junction⁶ and play an important role in negative selection and the generation of nTreg cells.^{1,2} Staining for markers that identify the 3 major thymic DC types (mDCs, pDCs, and Langerhans cells) showed that the number of medullary DCs belonging to each one of these subsets was significantly reduced in ZAP70^{−/−} patients compared with the number seen in healthy control subjects (Fig 2 and see Fig E2 in this article's Online Repository at www.jacionline.org). Depletion of thymic DCs was more prominent in the deep intramedullary area (data not shown) and was especially pronounced for pDCs. Interaction between XCL1 (a chemokine secreted by mature mTECs) and DCs expressing the chemokine receptor XCR1 has been suggested to facilitate trafficking of DCs to the deeper medulla, generation of nTreg cells, and establishment of self-tolerance.⁶ Staining for XCL1 and XCR1 showed no significant differences in the expression of these chemokines in the thymic medullary area of ZAP70^{−/−} patients (Fig 2, C). These data indicate that the reduced numbers of DCs and FOXP3⁺ nTreg cells in the thymic medulla of ZAP70^{−/−} patients is not due to a lack of XCL1-dependent chemotactic signal.

To our knowledge, this is the first detailed analysis of the thymic stromal compartment in human ZAP70 deficiency. Our data demonstrate that human ZAP70 deficiency is permissive for the development of CLD4⁺UEA-1⁺ mature mTECs, which express AIRE. Similar data have been reported also in Zap70^{−/−} mice,³ despite the more severe block in T-cell differentiation. These data are consistent with the notion that signals (RANK ligand and CD40 ligand) delivered by positively selected thymocytes serve primarily to increase the number and maintain the homeostasis of mTECs but are not required to generate AIRE⁺ mTECs.⁷ By contrast, studies in mice have shown that Zap70 is critically required to induce terminal maturation of mTECs, as indicated by lack of IVL

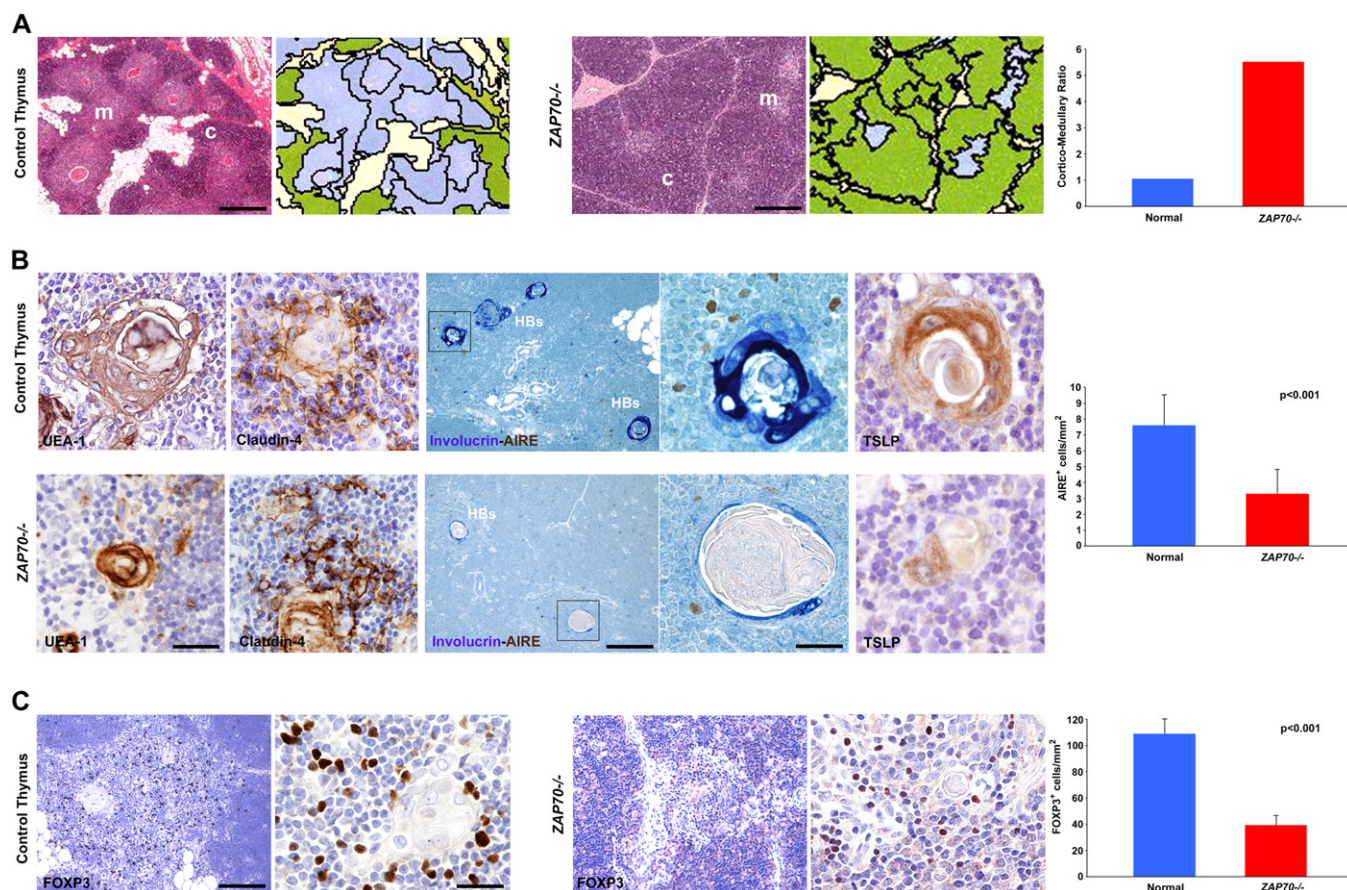


FIG 1. Analysis of the thymic stromal compartment and nTreg cells from biopsy specimens of healthy subjects and ZAP70^{-/-} patients. **A**, Morphometric analysis of normal and ZAP70^{-/-} thymic sections (hematoxylin and eosin staining, $\times 4$ original magnification) by using the Scanscope CS Aperio Scanner (Aperio Technologies, Vista, Calif) and a custom-provided script from Definiens Architect XD 2.0 image analysis software (Definiens, Wilmington, Del) that automatically discriminates cortical (green), medullary (light blue), and connective (yellow) areas. Quantification of corticomedullary ratio is shown in the plot on the right. *c*, Cortex; *m*, medulla. **B**, mTECs expressing UEA-1 and CLD4 are present in both normal and ZAP70^{-/-} thymic biopsy specimens (brown staining, left panels, $\times 40$ original magnification). Double staining for AIRE (nuclear brown staining) and IVL (cytoplasmic blue staining) highlights the presence of AIRE-expressing mTECs around the Hassall corpuscles (central panels, $\times 4$ and $\times 60$ original magnifications), although in ZAP70^{-/-} thymuses AIRE⁺ cell numbers are significantly reduced (graph on the right). Hassall corpuscles in ZAP70^{-/-} thymuses appear smaller and express only minimal amounts of IVL (central panels, blue staining) and thymic stromal lymphopoietin (TSLP; right panel, brown staining, $\times 60$ original magnification). **C**, ZAP70^{-/-} thymuses show a preserved expression of FOXP3⁺ thymocytes within the medullary region (brown nuclear signal, $\times 10$ and $\times 40$ original magnification in the left and right panels, respectively), although in a significantly reduced number compared with control thymus (right graph). Scale bars correspond to 500, 200, 50, and 20 μ m, respectively, for $\times 4$, $\times 10$, $\times 40$, and $\times 60$ original magnification. In the graphs in Fig 1, *B* and *C*, for each of the indicated markers, the mean $\pm$ SD of positive cells per square millimeter measured in 10 different high-power fields for each thymus are shown.

expression in the Hassall-like corpuscles of ZAP70^{-/-} mice.³ This defect might reflect impaired lymphotoxin signals from positively selected thymocytes (and especially CD4⁺ single-positive cells), the generation of which is abrogated in ZAP70^{-/-} mice.³ Although ZAP70 deficiency in human subjects is permissive for CD4⁺ cell development, these cells are hyporesponsive to signaling through the T-cell receptor.⁵ Therefore it is possible that ZAP70^{-/-} CD4⁺ human thymocytes are unable to provide adequate signals to support terminal differentiation of mTECs, thereby explaining phenotypic similarity with ZAP70^{-/-} mice.

Our results also showed that AIRE⁺ mTECs, thymic DCs, and FOXP3⁺ nTreg cells are present in reduced numbers in the thymuses of ZAP70^{-/-} patients. Together with our previous work,

which demonstrated an autoimmune gene expression signature in ZAP70^{-/-} CD4⁺ cells,⁵ these data indicate that ZAP70 deficiency might predispose to autoimmunity. Currently, there is little clinical evidence for this predisposition. This is likely due to the rarity of the disease. Moreover, frank autoimmunity can take several years to develop, whereas most patients receive hematopoietic cell transplantation in infancy. One patient with a hypomorphic ZAP70 mutation appeared to have not developed autoimmunity.⁸ On the other hand, 1 patient with colitis and idiopathic thrombocytopenic purpura was identified at the age of 4 years, and Omenn syndrome, another form of immune dysregulation, has been also described in association with ZAP70 deficiency,⁹ although the frequency of this association appears to be

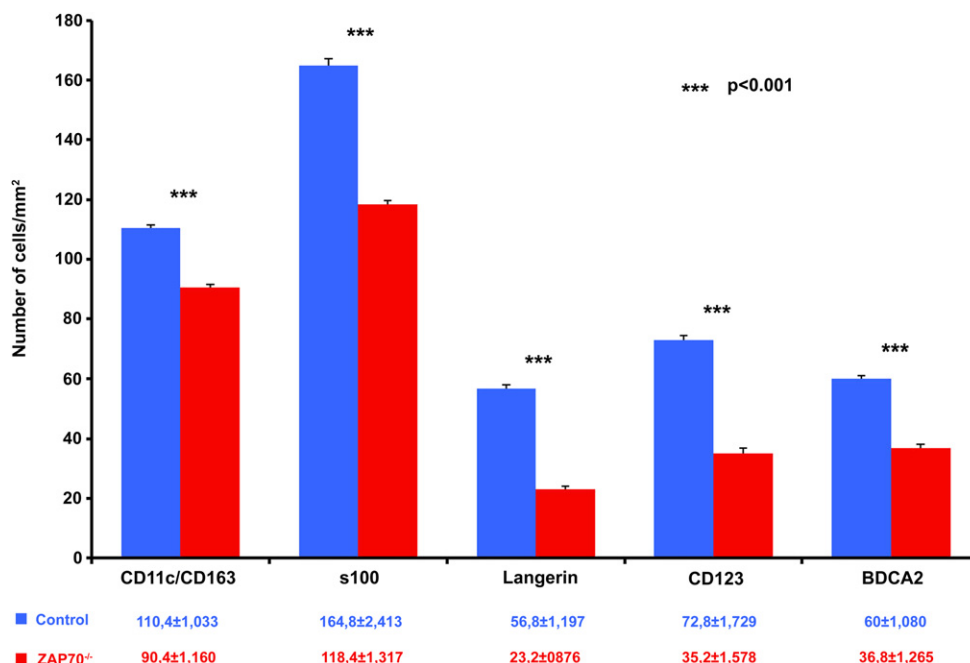


FIG 2. Distribution of DCs in thymic biopsy specimens from healthy subjects and ZAP70^{-/-} patients. All thymic DCs belonging to the 3 major subsets (mDCs, CD11c⁺/CD163⁻; Langerhans cell DCs, S100⁺ and Langerin⁺; and pDCs, CD123⁺/BDCA2⁺) were significantly reduced in ZAP70^{-/-} thymuses compared with those in healthy control subjects. For each marker, the mean ± SD of positive DCs per square millimeter measured in 10 different high-power fields in each thymic biopsy specimen are shown.

less than in other conditions (eg, hypomorphic recombination-activating gene [*RAG*] mutations), which are invariably associated with a severely dysplastic thymus and a very restricted T-cell repertoire.⁴

In summary, ZAP70 deficiency in human subjects is associated with reduced numbers of AIRE⁺ mTECs, impaired terminal differentiation of mTECs, depletion of medullary DCs, and reduced numbers of thymic FOXP3⁺ Treg cells. These abnormalities might contribute to variability of the clinical phenotype in affected patients.

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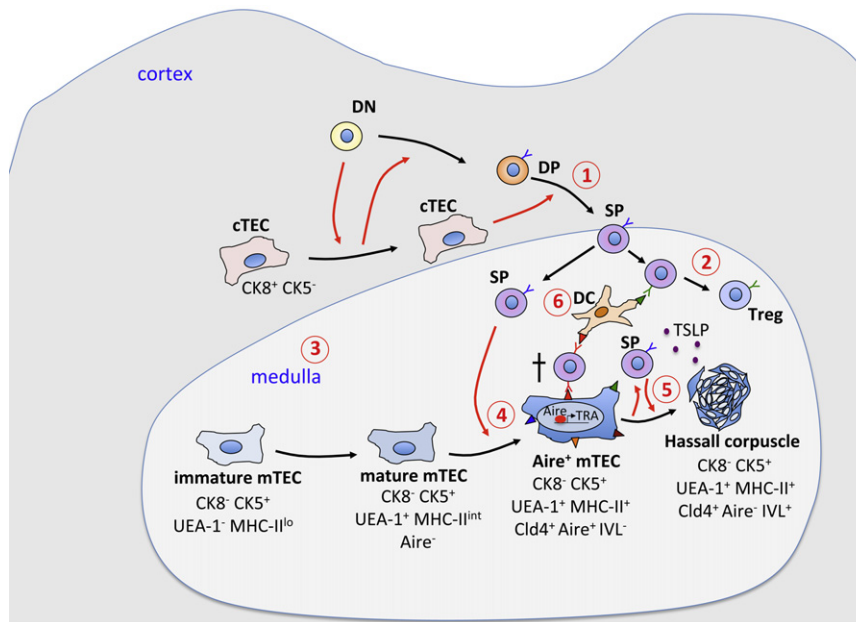


FIG E1. Scheme of thymic lymphostromal cross-talk (indicated by red arrows) and abnormalities observed in the thymuses of *ZAP70*^{-/-} patients. The numbers circled in red identify abnormalities observed in the thymuses of patients with *ZAP70* deficiency, which include (1) defective differentiation of $CD4^+$ single-positive thymocytes and impaired function of $CD4^+$ single-positive cells; (2) reduced number of $FOXP3^+$ Treg cells; (3) reduced size of thymic medulla; (4) reduced number of $Aire^+$ mTECs; (5) lack of terminal maturation of post-AIRE mTECs in the Hassall corpuscles, with lack of IVL expression; and (6) reduced number of medullary DCs. *CK*, Cytokeratin; *cTEC*, cortical thymic epithelial cell; *DN*, double-negative thymocyte; *MHC-II*, MHC class II antigen; *SP*, single-positive thymocytes; *TRA*, tissue-restricted antigen; *TSLP*, thymic stromal lymphopoietin.

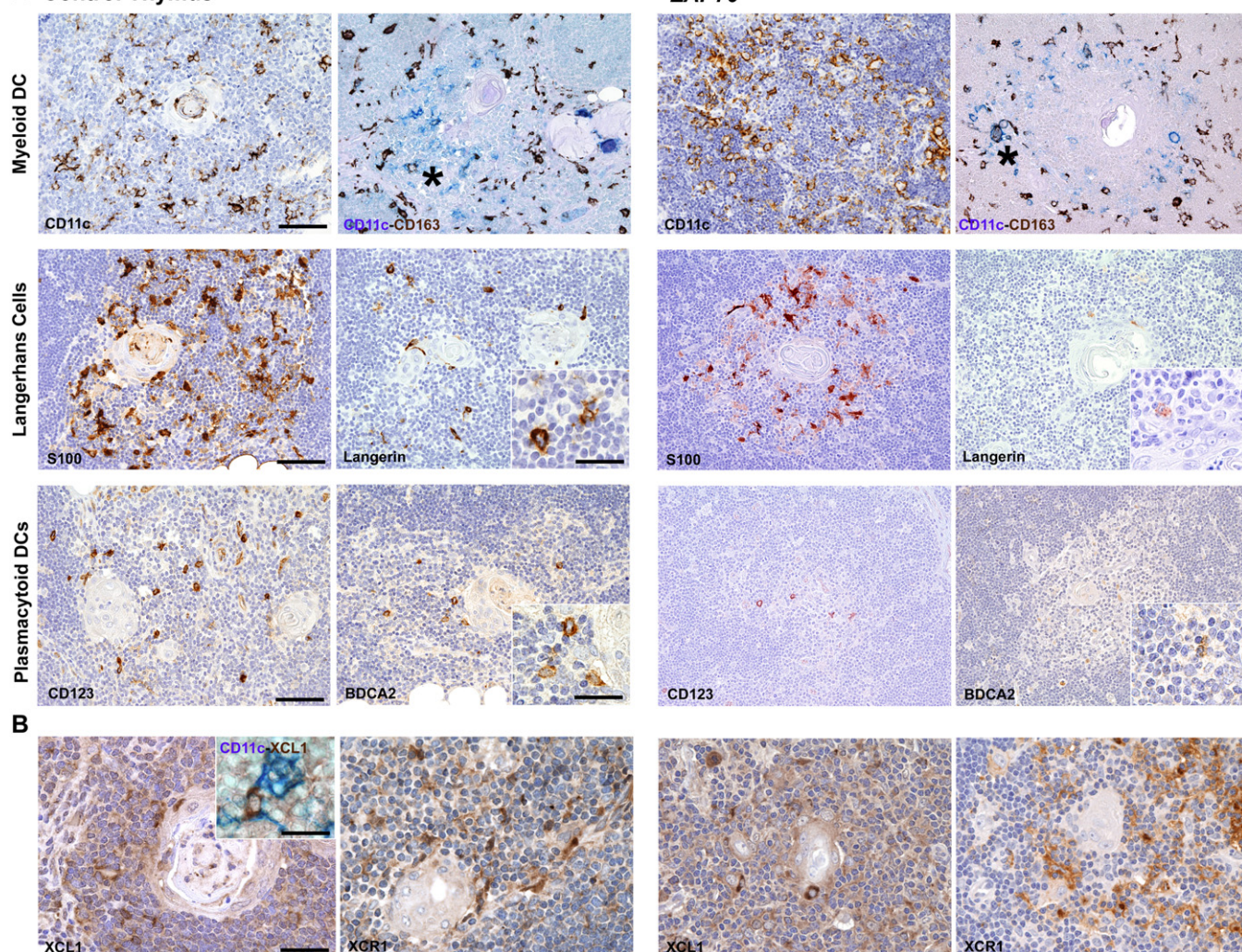
A Control Thymus**ZAP70^{-/-}**

FIG E2. Distribution and relative abundance of DCs in thymic biopsy specimens from healthy subjects and ZAP70^{-/-} patients. **A**, Representative images from both control and ZAP70^{-/-} thymic medullary areas stained for markers of the 3 different major DCs subtypes. Combined CD11c (blue) and CD163 (brown) staining allows one to distinguish CD11c⁺CD163⁻ DCs from CD11c⁺CD163⁺ macrophages, which are primarily distributed in the cortex, with only rare CD11c⁺CD163⁺ macrophages within the medulla (asterisk). **B**, Staining for XCL1 and XCR1 expressed by mature mTECs and DCs, respectively, showed no significant differences between ZAP70^{-/-} and control thymuses. Interaction between XCL1⁺ mTECs and DCs is highlighted by double staining for CD11c (blue) and XCL1 (brown, inset). All staining is from $\times 20$ and $\times 40$ (insets) original magnifications, and the scale bar corresponds to 100 and 50 μm , respectively.