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Targeted deletion of c-Met in thymic epithelial cells leads to an autoimmune phenotype

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Abstract

Hepatocyte growth factor (HGF) and its receptor c-Met signaling have been implicated in regulating various types of cells including epithelial cells. We have previously reported that c-Met is expressed by thymic epithelial cells (TECs), and that *in vivo* administration of hybrid cytokines containing IL-7 and the beta- or alpha-chain of HGF significantly increase the number of TECs. In order to study the role of c-Met signaling in TECs, we generated conditional knockout (cKO) mice in which c-Met was specifically deleted in TECs using a Foxn1-Cre transgene. We show here that c-Met deficiency in TECs results in age-progressive reduction in TEC number and reduced number of regulatory T cells. Consequently, c-Met TEC cKO mice displayed an autoimmune phenotype. Thus, c-Met signaling in TECs is important for the maintenance of TECs and immune self-tolerance.

INTRODUCTION

T cell development in the thymus is dependent on the thymic microenvironment, in which TECs are the major component [1-4]. The importance of TECs in T cell development has been underscored by the fact that defects in TECs result in immunodeficiency and autoimmunity [1, 2]. However, the molecular mechanisms of TEC development and maintenance remain largely unknown.

HGF (also called scatter factor) is a pleiotropic factor that regulates cell growth, motility, morphogenesis and regeneration in various types of cells [5]. The receptor for HGF is the tyrosine kinase encoded by the c-Met. HGF consists of a 60 KD alpha and 30 KD beta chain. We have reported that *in vivo* administration of hybrid cytokines containing IL-7 and

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the beta- or alpha-chain of HGF significantly increases the number of TECs [6-8], suggesting that c-Met signaling may play a role in the development and maintenance of TECs.

Because homozygous c-Met null mice are embryonic lethal [9], there are technical difficulties in analyzing the role of c-Met in TECs. Thus, we generated cKO mice in which c-Met was specifically deleted in TECs using the Cre-loxP system. We report here that TEC-specific c-Met cKO mice had a severe infiltration of lymphocytes into multiple organs and increased activity of autoantibodies to peripheral tissue antigens. This was associated with age-progressive reduction in TEC cellularity and a decreased number of regulatory T cells (Tregs).

RESULTS

c-Met deletion in TECs results in age-progressive reduction in TECs

It has been shown that *Foxn1-Cre* knock-in mice express Cre recombinase in all TECs without disrupting Foxn1 function [10]. To determine whether c-Met is required for TEC hemostasis, we deleted c-Met in TECs using the *Foxn1-cre* transgene. We crossed *c-Met*$^{ft/ft}$ mice with *Foxn1-Cre* mice to generate c-Met cKO mice (*c-Met*$^{ft/ft}$; Foxn1-Cre^{+}) and Ctrl mice (*c-Met*$^{ft/ft}$; *Foxn1-Cre*$^{-}$ or *c-Met*$^{ft/+}$; *Foxn1-Cre*$^{-}$ or *c-Met*$^{ft/+}$; *Foxn1-Cre*$^{+}$). qRT-PCR and Western blot confirmed that almost all floxed alleles were inactivated by Cre recombinase in TECs (Supplementary figure 1A, B).

We then analyzed the number of TECs. At 1 month of age, cKO mice had a slight reduction in the number of total TECs (CD45^{-}EpCAM1^{+}MHC II^{+}), and their subsets cortical TECs (cTECs) (CD45^{-}EpCAM1^{+} MHC II^{+}Ly51^{+}) and medullary TECs (mTECs) (CD45^{-} EpCAM1^{+} MHC II^{+}Ly51^{-}), as compared with Ctrl mice, but the differences did not reach statistical significance (Figure 1A). At 4 months of age, total TEC number in cKO mice was reduced by nearly 43%. Among them, cTEC number had a 31% reduction although differences also did not reach statistical significance, whereas mTEC number had a significant 49% reduction (Figure 1B). The reduction was further increased at 8 months of age with a significant 44% and 57% reduction in cTECs and mTECs, respectively (Figure 1C).

mTECs can be further divided into mTECs^{lo} and mTECs^{hi} subsets based on the expression level of MHC II [4, 11]. It has been suggested that the mTECs^{lo} subset consists of immature precursors of a mature mTECs^{hi} subpopulation although studies also showed that both mTECs^{lo} and mTECs^{hi} subsets contain mature mTECs [4]. As shown in Figure 1C, the number of both mTECs^{lo} and mTECs^{hi} was decreased in cKO mice.

To determine the cellular mechanisms by which c-Met TEC cKO mice had a reduced number of TECs, we examined the survival and proliferation of TECs. We first analyzed cell apoptosis in TECs using a TUNEL apoptosis detection assay. As shown in Figure 1D, the percentages of TUNEL^{+} cells in cTECs and mTECs were increased in cKO mice as compared to Ctrl mice. We then investigated TEC proliferation and found that the percentage of Ki67^{+} cells in mTECs, but not in cTECs, was decreased in cKO mice, as

compared to Ctrl (Figure 1E). The results suggest that cKO mice have a decreased survival of both cTECs and mTECs, as well as a decreased proliferation of mTECs.

We also examined the thymic architecture. Hematoxylin and eosin (H&E) staining revealed that c-Met cKO mice displayed a medullar reduction at 4 months of age (Figure 1F). It is well known that cTECs and mTECs are characterized by differential expression of cytokeratins ($K5^{-}K8^{+}$ for cTECs and $K5^{+}K8^{-}$ for mTECs). Corresponding to the H&E data, immunofluorescent staining showed that the areas for $K5^{+}K8^{-}$ mTECs in the cKO mice were greatly reduced compared to Ctrl mice (Figure 1G). Taken together, the data suggest that c-Met signaling plays an important role in maintaining TEC number.

It is well known that Aire plays a key role in central tolerance by regulating the expression of tissue-specific antigens (TSAs) [12, 13]. We questioned whether c-Met deletion also affected the number of $Aire^{+}$ mTECs. Immunofluorescent staining displayed that the percentage of $Aire^{+}$ cells in medullary area was decreased in cKO mice (Figure 1H and 1I).

Because Foxn1-Cre can delete c-Met in the epidermis, to rule out an indirect effect on the thymus caused by loss of epithelial integrity in the skin, we performed histological analysis for the skin. We found that the deletion of c-Met in Foxn1 expressing cells did not result in the loss of epithelial integrity in the skin (Supplementary figure 2). Therefore, the possible indirect effect on thymus by the effect in the skin can be ruled out.

c-Met deficiency in TECs leads to an autoimmune phenotype

Since TECs play a critical role in establishing self-tolerance, we then examined whether the reduced TEC cellularity in c-Met cKO mice would have an autoimmune phenotype. As shown in Figure 2A, cKO mice aged 4-6 months revealed a severe lymphocytic infiltration in the pancreas, lung, stomach and liver, while such infiltrates were absent in Ctrl mice of similar age.

Development of autoimmunity in the cKO mice was further demonstrated by the production of autoantibodies against various organs. Immunostaining of $RAG1^{-/-}$ tissue section with sera from the cKO mice revealed autoantibodies against the pancreas, lung, stomach, and salivary gland (Figure 2B), whereas sera from Ctrl mice had no autoantibodies against these organs. These results suggest c-Met deficiency in TECs leads to an autoimmune phenotype.

c-Met deletion in TECs results in reduced number of thymocytes including Tregs

The reduced TEC cellularity suggested that thymocyte development could be affected by TEC-specific c-Met-deficiency. As shown in Figure 3, a slight reduction of the thymocyte number was observed in 1-month-old cKO mice, and significant ~43% and ~54% reduction in thymic cellularity was seen in 4-month- and 8-month-old cKO mice, respectively. The results are consistent with reduced TEC cellularity in cKO mice. However, the relative frequencies of CD4 and CD8 double negative (DN), double positive (DP), and single positive (SP) were not affected in cKO mice. Consequently, the numbers of all thymocyte subsets were proportionally reduced. The results suggest that, despite a reduction in TECs, the remaining TECs are sufficient to support thymocyte development.

It is well known that Tregs can suppress autoimmune responses. The development of natural Tregs in the thymus is also supported by TECs [14, 15]. As shown in Figure 3D, the number of thymic Tregs in cKO mice was also significantly decreased, beginning from 1 month of age, earlier than the decrease in number of thymocytes (Figure 3A).

Reduced expression of NF-κB2 in mTECs of c-Met cKO mice

It has been reported that NF-κB2 inducing kinase (NIK), IκB kinase α (IKKα), NF-κB2, tumor necrosis factor receptor-associated factor 6 (TRAF-6) and smad4 are involved in the development and/or maintenance of mTECs [16-21]. To determine whether these molecules are the downstream targets of c-Met, we analyzed their expression in mTECs from c-Met cKO and Ctrl mice by qRT-PCR. As shown in Figure 4, the expression of NF-κB2 in was reduced in cKO mTECs, while the expression of NIK, IKKα, TRAF-6 and smad4 were comparable between cKO and Ctrl mTECs.

DISCUSSION

Thymocyte development undergoes positive and negative selection, generating T cells that are tolerant to self-antigens, but able to react with alloantigens. cTECs support the positive selection of thymocytes that have undergone TCR rearrangements capable of recognizing self-MHC. mTECs mediate the negative selection, leading to clonal deletion of self-reactive T cells. In this report, we show that ablation of c-Met in TECs results in reduced number of cTECs and mTECs and disturbed thymic architecture, which are associated with a reduced number of thymocytes including Tregs. Although the remaining TECs still support the development of thymocytes, c-Met cKO mice displayed autoimmune phenotypes, suggesting a breakdown in self-tolerance. It is likely that the autoimmune phenotype in c-Met cKO mice is due to impaired negative selection and reduced number of Tregs. Future studies using TCR transgenic mice should further confirm this conclusion.

It has been reported that turnover time for TECs is about 10-14 days [11]. The reduced number of TECs, especially mTECs, in cKO mice, could be caused by a change in TEC turnover. Indeed, we have shown that c-Met deletion in TECs results in increased apoptosis of both cTECs and mTECs, as well as decreased proliferation of mTECs. It remains to be determined whether c-Met deletion affects TEC progenitor populations [22, 23].

We have also shown that c-Met deletion in TECs results in significant reduction in the number of $Aire^+$ mTECs. It is well known the Aire plays a critical role in mediating negative selection by regulating TSA expression [12, 13]. It is possible that the reduced number of $Aire^+$ mTECs is due to decreased number of mTECs. However, we cannot exclude the possibility that the decreased number of $Aire^+$ mTECs in c-Met cKO mice is due to reduced transcriptional regulation of Aire expression.

Several signal molecules, such as NF-κB2, NIK, IKKα, TRAF-6 and smad4, have been shown to be involved in the development and/or maintenance of mTECs [16-21]. However, the ligands and receptors that trigger these molecules are not well understood. We have shown that the expression of NF-κB2 in mTECs was reduced in c-Met cKO mice, indicating that NF-κB2 is a downstream target of c-Met signaling in mTECs. Our results are consistent

with previous reports that NF-κB2-deficient mice had a reduced number of mTECs and autoimmune phenotypes [20, 21].

Studies have shown that mTECs produce multiple chemokines, such as CCL21 and XCL1, which attract positively selected thymocytes, dendritic cells and Tregs to the medullary region so that T cells undergo medullary selection and establish self-tolerance [4, 24, 25]. It has been reported that the expression of CCL21 and XCL1 in mTECs of Aire-deficient mice was reduced [4, 24, 25]. It is possible that the reduced number of Aire$^+$ mTECs in c-Met cKO mice results in reduced expression of the chemokines in the medullary region, which also contributes to the breakdown of self-tolerance in the mice.

Many studies have demonstrated that during aging, TECs undergo both a qualitative and quantitative loss that is believed to be the major factor responsible for age-dependent thymic involution [1, 2, 26, 27]. There is increased incidence of autoimmune diseases in the elderly, which is likely due to their degenerated TECs [26]. We have shown that ablation of c-Met in TECs results in age-progressive decrease in the number of TECs, suggesting that a decrease in c-Met signaling in TECs may also contribute to age-dependent thymic involution.

In summary, we have demonstrated that TEC-specific c-Met cKO mice have an age-progressive reduction in TEC number, and reduced number of regulatory T cells. Furthermore, these mice have a severe infiltration of lymphocytes into multiple organs and increased activity of autoantibodies to peripheral tissue antigens. Thus, c-Met signaling in TECs is important for maintaining these cells and supporting immune self-tolerance.

METHODS

Mice

c-Met$^{ft/ft}$ mice were kindly provided from Dr. S. Thorgeirsson [9]. We backcrossed the *c-Met$^{ft/ft}$* mice to a C57BL/6 background for over 10 generations. The *c-Met$^{ft/ft}$* mice were then backcrossed with *Foxn1-Cre* mice (in a C57BL/6 background) [10] to generate *c-Met* cKO and control (Ctrl) mice. Mice were used according to protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut. For all experiments mice of both sexes were used in each experimental group and matched for numbers of males and females in cKO and Ctrl groups.

Genotyping

Genotyping was performed on tail biopsies using a PCR–based method developed by Transnetyx (Cordova, TN). Primers specific for the genotyping were used as described [9].

Real-time quantitative RT-PCR (qRT-PCR)

CD45$^-$EpCAM1$^+$ TECs or CD45$^-$EpCAM1$^+$Ly51$^-$ mTECs were isolated from mice by immunomagnetic cell separation (Miltenyi Biotec., Auburn, CA). Total RNA was isolated from the cells using the Nucleo Spin RNA II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. cDNA was synthesized using total RNA with the High Capacity cDNA Reverse Transcription Kit (Invitrogen). qRT-PCR was performed by the

7500 real-time PCR system (Applied Biosystems, UK) using the Power SYBR green mastermix (Applied Biosystems, UK). Primers are summarized in Supplementary table 1.

Flow Cytometry analysis

Single cell suspensions of thymocytes and splenic cells were stained with fluorochrome-conjugated antibodies as described [6, 8, 28, 29]. For intracellular staining, the cells were first permeabilized with a BD Cytofix/Cytoperm solution for 20 minutes at 4°C. Direct or indirect staining of fluorochrome-conjugated antibodies included: CD4 (BioLegend, San Diego, CA, USA; cat. no. 100412), CD8 (BioLegend; cat. no. 100705), CD45 (BioLegend; cat. no. 103111), CD25 (BioLegend; cat. no. 102029), Foxp3 (BioLegend; cat. no. 320011), EpCAM1 (BioLegend; cat. no. 118219), Ly51 (BioLegend; cat. no. 108305), I-A^b (BioLegend; cat. no. 116407), and Ki67 (Dako, Carpinteria, CA; cat. no. M7249). The samples were analyzed on a FACSCalibur or LSRFortessa X-20 Cell Analyzer (BD Biosciences). Data analysis was done using FlowJo software (Ashland, OR).

TUNEL Assay

Cells were labeled with TEC cell surface antibodies, and then stained with an *in situ* cell death detection kit (Roche Applied Science). A negative control without the terminal deoxynucleotidyl transferase was included with each sample. The cells were then analyzed by flow cytometry.

Immunohistology

Immunohistological analysis of thymus was performed as described previously[29, 30]. Briefly, tissues were incubated in 4% paraformaldeyde for 4 hours followed by incubation in 30% sucrose solution overnight. The tissues were embedded, and subsequently cut into 5 micrometer sections. The sections were stained with rabbit anti-mouse K5 polyclonal antibody (Covance Research Products, Denver, PA), and rat anti-mouse K8 monoclonal antibody (mAb) (Throma I mAb, raised by P. Brulet and R. Kemler and obtained from the Developmental Studies Hybridoma Bank, University of Iowa, IA), or antibody against Aire, followed by AlexaFluor-488-, and 546-conjugated goat anti-rat IgG, and goat anti- rabbit IgG (Invitrogen). To detect autoantibodies, frozen cryosections of various organs from *RAG1*-/- mice were fixed with ice-cold acetone and incubated with sera (1:150) obtained from cKO and Ctrl mice. The sections were then incubated with Alexa 488-conjugated anti-mouse Ig (Molecular Probes), and analyzed under a Nikon A1R confocal microscope (Nikon, Kanagawa, Japan). Histological analysis was performed blind. The percentage of the total area of $Aire^+$ cells was automatically calculated using Adobe Photoshop 7.0 software (Adobe Systems Inc, San Jose, CA, USA).

Western Blot

Cells were washed with PBS, resuspended in a sample buffer, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were incubated with antibody against mouse c-Met (Santa Cruz Biotechnology, Inc., Dallas, TX), washed, incubated with HRP-linked

secondary antibody, and then developed with enhanced chemiluminescence (GE Healthcare Biosciences, Pittsburgh, PA).

Statistical analysis

P-values were based on the two-sided Student's t test. A confidence level above 95% ($p<0.05$) was determined to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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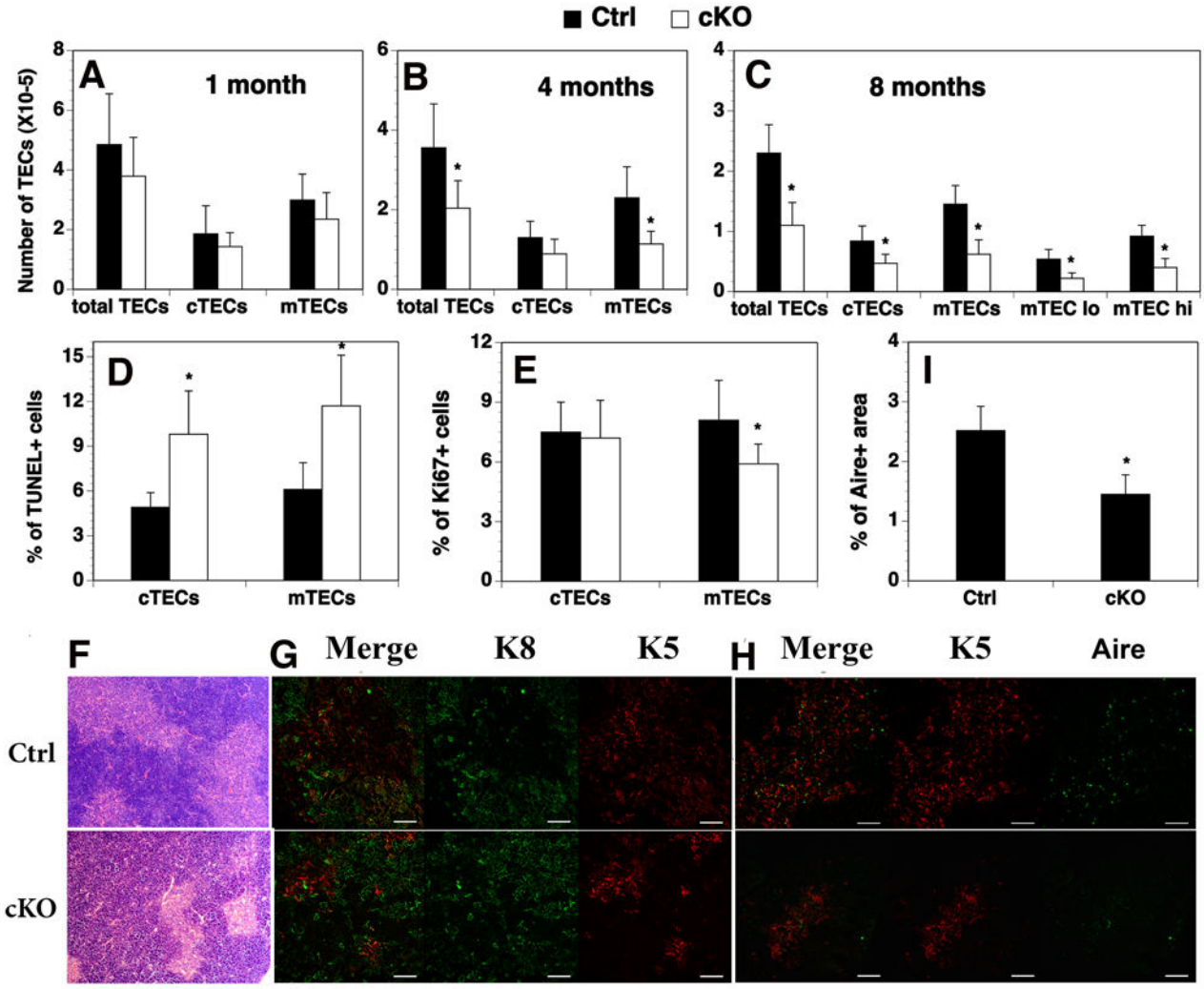

Figure 1.

c-Met cKO mice have a reduced number of TECs. The number of total TECs (CD45$^-$ EpCAM1$^+$ MHC II$^+$), and their subsets cTECs (CD45$^-$EpCAM1$^+$ MHC II$^+$Ly51$^+$) and mTECs (CD45$^-$EpCAM1$^+$ MHC II$^+$Ly51$^-$) from c-Met cKO and Ctrl mice at the age of (A) 1-month, (B) 4-months, and (C) 8-months was analyzed by flow cytometry. (C) The number of mTEC^{lo} (CD45$^-$EpCAM$^+$Ly51$^-$MHC II^{lo}) and mTEC^{hi} (CD45$^-$EpCAM$^+$Ly51$^-$MHC II^{hi}) in 8-month-old mice was also analyzed. (D, E) The percentages of (D) TUNEL$^+$ and (E) Ki67$^+$ cells in cTECs and mTECs of 8-month-old mice as analyzed by flow cytometry. (F-H) The thymi were subjected to (F) H&E, and (G, H) immunofluorescent staining with (G) anti-K5 and K8 antibodies, and (H) anti-K5 and Aire antibodies. Shown are representative thymi from 4-month old of cKO and Ctrl mice. (F) Magnification, X100. (G, H) Scale bar = 100 μm. (I) Quantitative evaluation of Aire-positive areas. Means ± S.D. are presented. The data are representative of 3 independent experiments with similar results (6 mice per group). * P<0.05 compared with Ctrl mice.

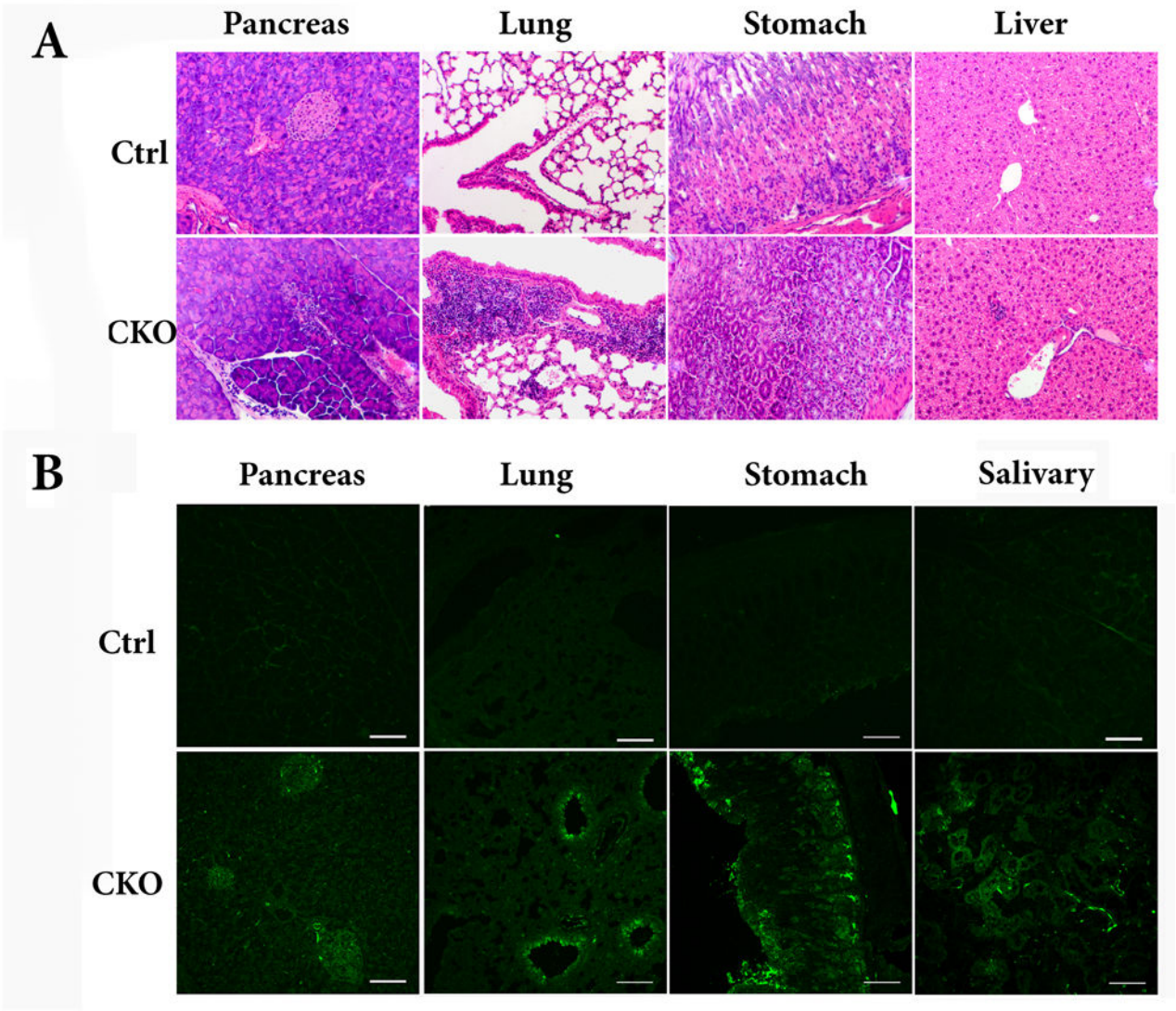

Figure 2.

c-Met cKO mice have an autoimmune phenotype. (A) H&E staining of the pancreas, lung, stomach, and liver from 4-6 month-old cKO and Ctrl mice. Magnification, X200. (B) Autoantibodies were determined by immunofluorescent staining the indicated organs from $Rag1^{-/-}$ mice with sera from 4-6 month-old cKO and Ctrl mice. Scale bar = 100 μm. Data are representative of 3 experiments.

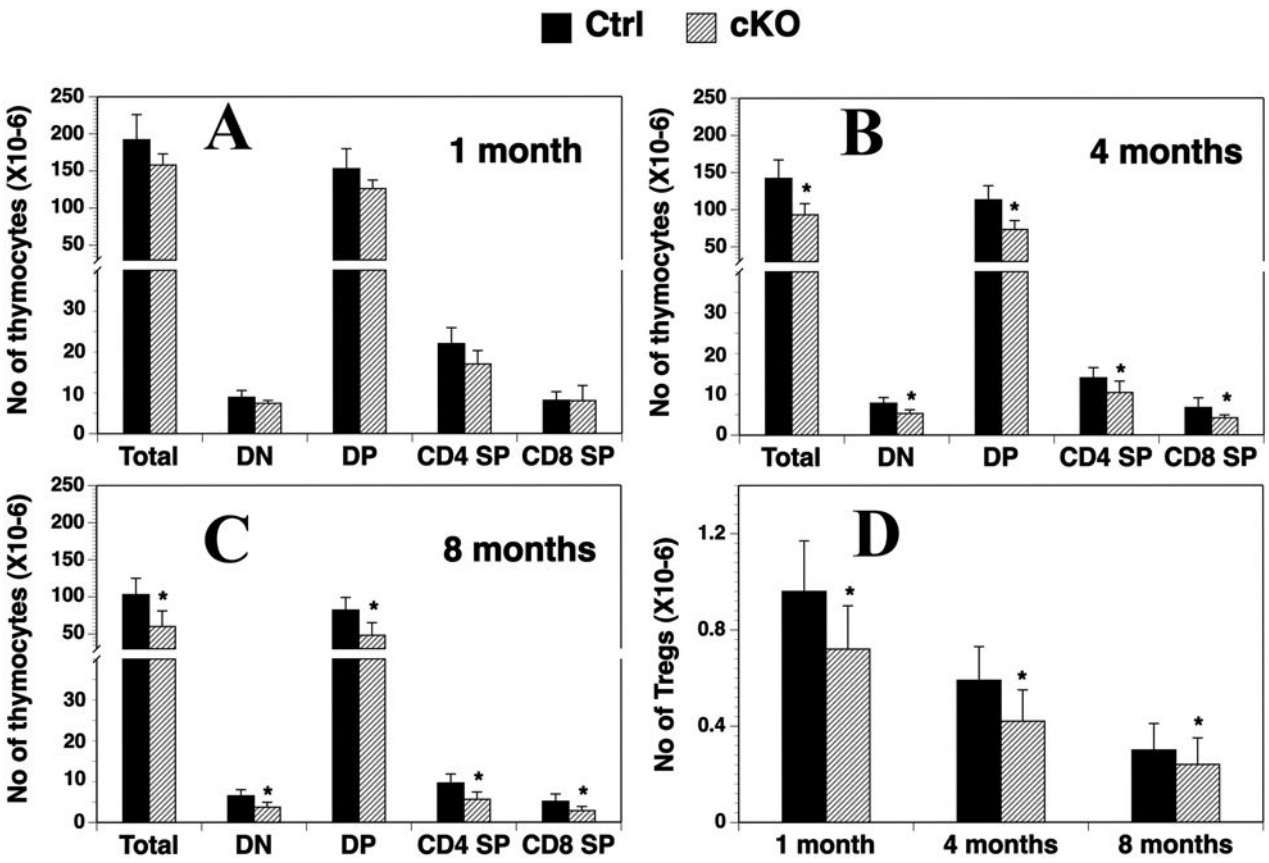

Figure 3.

c-Met cKO mice have a reduced number of thymocytes including Tregs. The thymi were harvested from cKO and Ctrl mice. The number of total thymocytes, CD4 and CD8 DN, DP, CD4 SP, and CD8 SP thymocytes in c-Met cKO and Ctrl mice at the age of (A) 1-month, (B) 4-months, and (C) 8-months, as well as (D) $CD4^{+}FoxP3^{+}$ Tregs was analyzed by flow cytometry. Means ± S.D. are presented. The data representative are of 3 independent experiments with 6 mice per group. * $P<0.05$ compared with Ctrl mice.

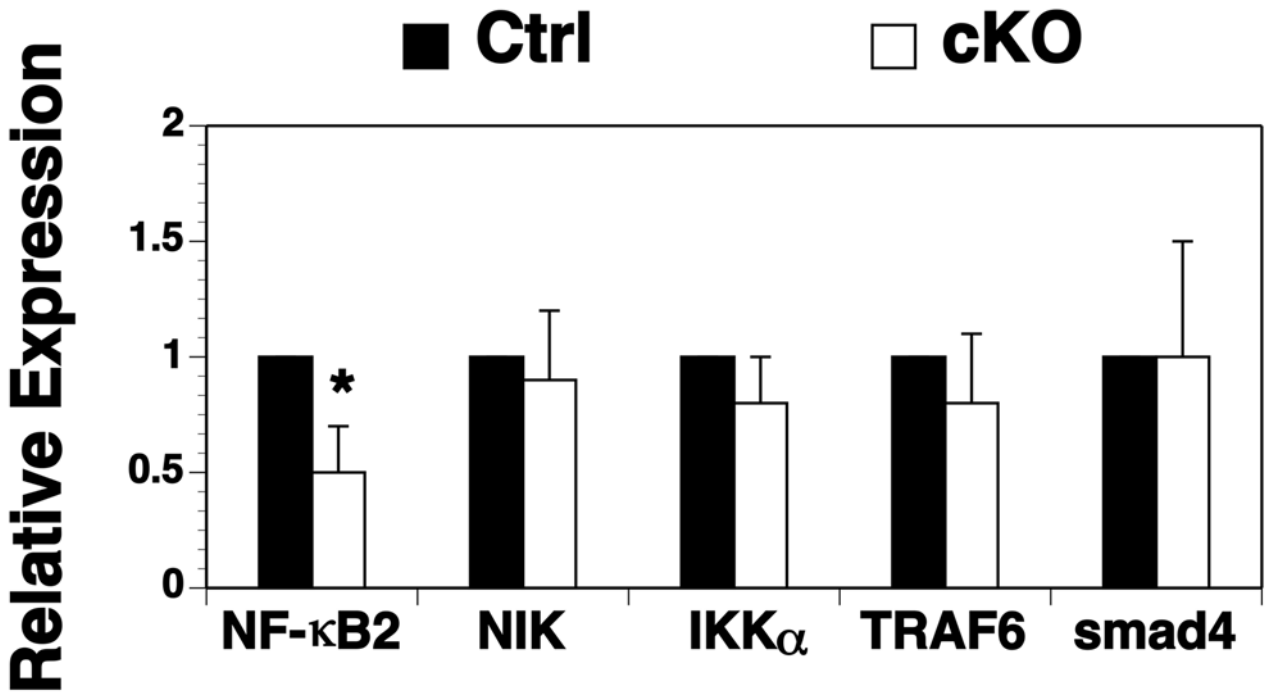

Figure 4.

c-Met cKO mice have a reduced expression of NF-κB2 in mTECs. $CD45^{-}EpCAM1^{+}$ $Ly51^{-}$ mTECs were isolated from 4-month old c-Met cKO and Ctrl mice. The expression of NF-κB2, NIK, IKKα, TRAF-6 and smad4 in the mTECs was analyzed by qRT-PCR. Expression levels for each gene were normalized to the housekeeping gene GAPDH and are presented as relative expression compared with Ctrl mice. The data are representative of 2 independent experiments with 5 mice per group. * $P<0.05$ compared with Ctrl mice.