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IRF7-Dependent IFN-β Production in Response to RANKL Promotes Medullary Thymic Epithelial Cell Development

Dennis C. Otero,*† Darren P. Baker,‡ and Michael David*†

The contributions of IFN regulatory factor (IRF) 3/7 and the type I IFNs IFN-α/β to the innate host defense have been extensively investigated; however, their role in thymic development is less clear. In this study, we show that mice lacking the type I IFN receptor IFN-α/β receptor (IFNAR) or the downstream transcription factor STAT1 harbor a significant reduction in self-Ag-presenting, autoimmune regulator (AIRE)+ medullary thymic epithelial cells (mTECs). Constitutive IFNAR signaling occurs in a receptor IFN-α; however, their role in thymic development is less clear. In this study, we show that mice lacking the type I IFN ligand; RFP, red fluorescence protein; TCR MBP, TCR specific for myelin basic protein; RANK, receptor activator for NF-κB; MHC II, MHC class II; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; EpCAM, epithelial cell adhesion molecule; IFNAR, IFN-α/β receptor; IFNGR, IFN-γ receptor; IRF, IFN regulatory factor; K5, keratin 5; K8, keratin 8; LTβ, lymphotxin β; MHC II, MHC class II; MS, multiple sclerosis; mTEC, medullary thymic epithelial cell; RANKL, receptor activator for NF-κB ligand; RFP, red fluorescence protein; TCRMBP, TCR specific for myelin basic protein; Treg, regulatory T cell; UEA-1, Ulex europaeus agglutinin-1; WT, wild-type; YFP, yellow fluorescent protein.

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thymic medulla and established a cross talk between the RANK and IFN signaling cascades crucial for the development of AIRE-expressing mTECs.

In summary, our findings endorse the notion that the type I IFN system influences thymic structure and function and consequently affects T cell selection by promoting the development of mTECs, a process that involves the temporally and spatially coordinated interplay between the RANK and IFN signaling pathways.

Materials and Methods

Animals and cell culture

STAT1−/− (17), IFNAR1−/− (23), IRF3−/− (24), and IRF7−/− (25) mice have been described previously. Transgenic Mx1-Cre mice and mTmG mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were between 6 and 10 wk of age at the time of the experiments. All mice used in these experiments were housed in a pathogen-free environment and bred and cared for in accordance with University of California, San Diego Animal Care Facility regulations. All studies involving animal environment and bred and cared in accordance with University of California,

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demonstrate a vital contribution of the IFN-α/β–STAT1 axis in the functional development of self-Ag–expressing mTECs.

IFN-β control over RANK signaling is required to maintain AIRE levels in thymic stromal cells

The TNF family members LTβ, CD40, and RANK all contribute the development of mTECs, and deletion of any of the genes encoding these cell-surface molecules or their downstream effectors results in defects of medullary development. As a cross talk between the RANK ligand (RANKL) and IFN signaling pathways had been demonstrated previously in the maturation process of osteoclasts (29), we elected to investigate RANK signaling in thymic stromal cells. Similar to osteoclasts, treatment of freshly isolated thymic stromal cells or the mTEC cell line TE-71 with RANKL resulted in the induction of IFN-β mRNA expression (Fig. 2B). In accordance with previous reports, mTECs rapidly
lose AIRE expression in culture (Fig. 2C, left panel) as determined by intracellular staining for AIRE and flow cytometric analysis. Although RANKL treatment alone of thymic stromal cells slightly increased the number of UEA-1hi cells (Fig. 2C, middle panel), costimulation with both IFN-β and RANKL resulted in a pronounced increase in AIRE+ cells (Fig. 2C, right panel). IFN-β by itself was capable of inducing AIRE expression in both thymic stromal cells (Fig. 2D) as well as in the TE-71 cells (Fig. 2E). In addition, costimulation with IFN-β and RANKL did not result in a reduction of c-fos, p65, or Traf6 (Fig. 2D), indicating that IFN is not causing the degradation of components of the RANK signal transduction pathway in mTECs. This presents a contrast to the mechanism of RANKL and IFN-β opposition during osteoclast differentiation, in which IFN-β treatment causes the loss of c-fos induction by RANKL (29). Finally, analysis of self-Ag expression following stromal cell stimulation indicates that IFN alone can induce expression of the AIRE-dependent self-Ag INS2 (Fig. 2F, top panel), but had little effect on the expression of the AIRE-independent self-Ag CRP (Fig. 2F, bottom panel). These findings not only demonstrate that IFN-β is induced by RANK signaling on thymic stromal cells, but also that a cross talk exists between the RANK- and

FIGURE 2. IFN-β promotes AIRE expression following RANKL stimulation. (A) AIRE, INS2, and CRP mRNA levels in thymic stromal cells from WT and STAT1−/− mice (average ± SD; n = 4). (B) Thymic epithelial cells from WT mice (left panel) or TE-71 cells (right panel) were stimulated with 500 ng/ml RANKL for the indicated times and IFN-β mRNA levels were determined by quantitative PCR (qPCR) (n = 4). (C) Thymic epithelial cells were left untreated or pretreated with 1000 U/ml IFN-β prior to stimulation with 500 ng/ml RANKL for 24 h. UEA-1+ cells were analyzed for intracellular AIRE expression by flow cytometry. (D) Thymic epithelial cells were depleted of CD80+ cells and then stimulated with 500 ng/ml RANKL, 1000 U/ml IFN-β, or both for 24 h. Lysates were probed for AIRE, ISG15, IRF7, cFOS, p65, and Traf6 by Western blotting (representative of three experiments). (E) TE-71 cells were treated as indicated, and mRNA levels for AIRE and ISG15 were determined by qPCR (average ± SD; n = 3). (F) Thymic epithelial cells were treated as indicated and mRNA levels for CRP and INS2 were determined by qPCR (average ± SD; n = 4).
IFNAR-initiated signaling pathways that governs AIRE expression in the thymus.

Previously selected CD4+ T cells are required to promote mTEC development through RANKL expression (6). As STAT1−/− T cells were unable to restore impaired mTEC development in RAG−/− mice in complementation chimera assays (data not shown), we reasoned that RANKL expression on activated thymocytes might be influenced by STAT1 and that consequently a T cell–intrinsic defect might contribute to the impaired development of the medullary compartment in the thymi of IFNAR1−/− and STAT1−/− mice. Using OTII-transgenic mice with Ag specificity toward OVA-derived peptides, we exposed WT thymic stromal cells to OVA peptide and cocultured them with either WT or STAT1−/− OTII+ thymocytes. Twenty-four hours following culture initiation, RANKL expression on thymocytes was analyzed by flow cytometry. As illustrated in Supplemental Fig. 1, activation of both WT and STAT1-deficient thymocytes resulted in increased RANKL expression on their cell surface; however, high levels of RANKL were only ob-

**FIGURE 3.** Constitutive IFN signaling in the thymus is restricted to the medullary region. (A) Schematic diagram of the IFN response reporter mice. IFN-dependent Cre expression results in the excision of the mT sequence coding RFP and expression of mG encoding GFP [Muzumdar et al. (30)]. (B) RFP and GFP expression in sections of thymi from WT and IFNAR1−/− mice carrying mT/mG and Mx1-Cre transgenes (top panel; scale bars, 500 μm). Sections were also stained for UEA-1 (bottom panel, pseudocolored purple; scale bars, 100 μm). (C) Flow cytometric analysis of CD45°UEA-1+ mTEC cells (top panel) and CD45°UEA-1+ hematopoietic cells (bottom panel) from thymi of either Mx1-Cre− or Mx1-Cre+ mT/mG mice. (D) Histogram comparing GFP expression levels in CD45°UEA-1+ mTECs of Mx1-Cre− or Mx1-Cre+ mT/mG mice. Arrow indicates GFP levels in CD45° hematopoietic cells.
served in WT thymocytes (Supplemental Fig. 1, bottom right panel). Consequently, the failure of mature STAT1$^{-/}$ T cells to support the development of mTECs in RAG$^{-/-}$ chimeras is likely due to their inability to express sufficient levels of RANKL upon activation. In summary, these observations imply both a T cell–intrinsic and –extrinsic role for STAT1 in mTEC development and function.

Constitutive IFN signaling in the thymic medulla

The phenotype observed in STAT1$^{-/-}$ and IFNAR$^{-/-}$ animals is highly indicative of tonic IFN-α/β signaling in the thymus even in the absence of infectious events. Indeed, IFN-β expression in the thymus had been reported and suggested that type I IFN signaling might be taking place in the thymus. To determine the exact compartment in which IFN signaling occurs in the thymus of uninfected, nonimmunized animals, we devised a transgenic reporter mouse strain. There, a tandem red fluorescent protein (RFP) called tdTomato is followed by a stop codon and a subsequent EGFP-coding sequence (30). LoxP sites flank the tdTomato gene including the stop codon such that expression of Cre recombinase results in the excision of the tdTomato-coding sequence and the stop codon, releasing expression of the downstream GFP. We crossed these animals to mice harboring the Cre transgene under the control of the type I IFN-inducible Mx-1 promoter, which has been widely used to inducibly delete LoxP-flanked alleles by treating mice with either IFN-β or the IFN-inducing TLR3 ligand polyinosinic-polycytidylic acid (31). Conceptually, all cells in these animals display red fluorescence until exposure to type I IFN leads to their transition into green fluorescence (Fig. 3A). Analysis of these double-transgenic mice (mT/mG; Mx1-Cre) revealed the abundant, Mx1-Cre transgene-dependent emergence of GFP-positive cells in the thymic medulla, but not in the cortical region (Fig. 3B, top middle panel), indicating that constitutive IFN signaling in the thymus is highly restricted to the thymic medulla. As anticipated, further immunohistochemical analysis detected UEA-1–positive mTECs only among the GFP-positive cell population (Fig. 3B, bottom panel, and Fig. 3C). Fig. 3C illustrates that CD45$^b$UEA-1$^b$ cells express a wider range of GFP as compared with CD45$^b$ cells that display one uniform peak (indicated by arrow) on histograms for GFP expression (Fig. 3D). Unexpectedly, only a very small fraction of thymic T cells expressed GFP in double-transgenic mice (Supplemental Fig. 2, middle panel), prompting us to examine the expression levels of the IFN receptor during thymocyte development. Intriguingly, we observed that both double- and single-positive thymocytes expressed reduced levels of IFNAR1 on their surface (Supplemental Fig. 3A) as compared with CD4 or CD8 single-positive splenocytes. This finding offers an explanation for the previously reported reduced ability of thymocytes to respond to type I IFNs (32). Accordingly, thymocytes do not exhibit the same responsiveness to IFN-β stimulation as splenic T cells, as revealed by reduced phosphorylation of STAT1 (Supplemental Fig. 3B). Thus, although apparently significant amounts of IFN are being produced in the thymic medulla, only the thymic stromal cells, but not thymocytes, are capable of responding to it. mTECs exhibit promiscuous gene expression through AIRE, which could be an alternative explanation to IFN signaling for the expression of the Mx1-Cre transgene in the medulla. Indeed, Mx-1 Cre was suggested to exhibit “leaky” expression (31, 33). To verify that tdTomato excision and subsequent GFP expression in double-transgenic mice was dependent on IFN signaling rather than promiscuous, AIRE-mediated Mx1-Cre expression, we bred the double-transgenic mice onto an IFNAR1$^{-/-}$ background. Elimination of IFNAR1 in mT/mG; Mx1-Cre mice resulted in a dramatic reduction in the number of GFP-positive mTECs (Fig. 3B, right panels). In accordance, fewer UEA-1–positive cells were detected in the absence of IFNAR1 (compare Fig. 3B, bottom middle and bottom right panels). The fact that a few residual GFP-expressing cells can be identified in IFNAR1$^{-/-}$ thymi could possibly be attributed to signaling via the IFN-α receptor, which is expressed in cells of the epithelial lineage and shares signaling mediators such as STAT1 and -2 with IFNAR1. Thus, even though type I IFNs are predominately responsible for thymic Mx1-Cre expression, the possibility remains that mTEC-specific Mx1-Cre expression might also occur as a consequence of minute IFN-α– or AIRE-initiated events, albeit in a significantly less competent manner. Importantly, no GFP$^+$ cells were detected among the thymocyte population in the periphery of mT/mG; Mx1-Cre; IFNAR1$^{-/-}$ mice (Supplemental Fig. 2, bottom panel, and data not shown), demonstrating that Mx1-Cre expression in lymphocytes was dependent on type I IFN signaling.

RANKL induces IFN-β expression in UEA-1$^+$ mTECs

To identify the cellular sources of IFN-β, the type I IFN commonly produced first, we used knockin mice that express yellow fluorescent protein (YFP) from a bicistronic IFN-β:YFP mRNA (34). Flow cytometric analysis of YFP expression in total cells from thymus of naive IFN-β$^{MOB/MOB}$ mice revealed that ~6% of thymocytes constitutively express IFN-β (Fig. 4, top panel). The vast majority of the YFP$^+$ cells were characterized as UEA-1$^+$ and
FIGURE 5. IRF7 is necessary for thymic IFN-β expression and mTEC development. (A) Thymic stromal cells from WT or IRF7<sup>−/−</sup> mice were purified and stimulated with 500 ng/ml RANKL for the indicated time points. Expression of IFN-β and IκBα mRNAs was determined by quantitative PCR (qPCR). (B) Thymic architecture in WT and IRF7<sup>−/−</sup> mice revealed by H&E staining of thymic sections (scale bar, 500 μm). (C) Ratio of medullary to cortical cellularity in thymic sections from WT and IRF7<sup>−/−</sup> mice (data collected using ImageJ [National Institutes of Health] and (Figure legend continues)
MHC II\textsuperscript{high} cells (Fig. 4A, bottom panel), consistent with our hypothesis that AIRE\textsuperscript{−} immature mTECs are the major source of type I IFN in the thymus. As previously indicated, IFN-β is typically produced by plasmacytoid dendritic cells and other cell types after recognition of pathogen-associated molecular patterns via pattern recognition receptors. However, IFN-β production by UEA-1\textsuperscript{+} mTECs occurs independent of such stimulation, thus raising the question as to the identity of the factor(s) that control type I IFN release in the thymus. To this end, thymic stromal cells were purified from thymus of IFN-β\textsuperscript{−/−}MOs mice and treated with RANKL for 24 h. As shown in Fig. 4B, a clear upregulation of YFP expression in response to RANKL could be observed in UEA-1\textsuperscript{+} mTECs, consistent with our earlier findings illustrated in Fig. 2B. In contrast, no induction of YFP:IFN-β was noticeable when the cells were stimulated through CD40 or the LTβ receptor (Fig. 4B), indicating that RANKL is indeed responsible for the presence of IFN-β in the thymic medulla.

**IRF7-deficient mice display defects in mTEC development and IFN-β expression after RANK stimulation**

Our results demonstrated a need for tonic IFN-α/β signaling in promoting the development and maturation of AIRE\textsuperscript{+} mTECs, and revealed that UEA-1\textsuperscript{−}AIRE\textsuperscript{−} immature mTECs are the primary source of IFN-β in the thymus. Previous work with osteoclasts demonstrated that IFN-β production following RANK signaling was dependent on c-Fos and independent of the expression of IRFs (29). However, no IRF7\textsuperscript{−/−} cells were employed in that study, and in light of the fact that IRF3 and -7 are required for induction of IFN-β in response to pathogens, we decided to investigate IFN production following RANKL stimulation in mTECs derived from IRF3- and IRF7-deficient mice. As shown in Fig. 5A, IRF7-deficient thymic stromal cells were incapable of inducing IFN-β messages following RANKL stimulation, whereas significant IFN-β messages could be induced in WT cells (Fig. 5A, left panel; p < 0.05) or IRF3-deficient stroma (not shown). Furthermore, no difference in the RANK signaling pathway leading up to induction of IkBα was observed (Fig. 5A, right panel; p > 0.05). Thus, IRF7 appears necessary for IFN production in response to RANKL stimulation in the thymus.

Based on the above findings, it seemed reasonable to expect that the loss of IRF7 would have a detrimental impact on the mTEC population. Indeed, H&E staining revealed a clear reduction in the thymic medullary region in IRF7-deficient mice as compared with WT animals (Fig. 5B), which is highlighted by a significant alteration of the mTEC/cortical TEC (cTEC) ratio (Fig. 5C). Accordingly, we noted a reduction in the IFN signature in freshly isolated thymic stroma (Fig. 5D). Immunostaining for K5 indicated the presence of immature mTECs in the IRF7-deficient thymus; however, there was a drastic reduction in the number of UEA-1\textsuperscript{+} cells (Fig. 5E, middle panels), with a concomitant diminution of mature AIRE\textsuperscript{+} cells (Fig. 5E, right panel). Accordingly, total stromal cells isolated from IRF7\textsuperscript{−/−} thymi contained lower levels of AIRE mRNA, accompanied by a severe reduction in the expression of the AIRE-dependent self-Ag INS2 (Fig. 5F). Flow cytometric analysis of the few remaining mature mTECs revealed that the amount of AIRE protein in the IRF7\textsuperscript{−/−} cells is identical to that found in their WT counterparts (Fig. 5G). However, the total numbers of UEA-1\textsuperscript{+} and AIRE\textsuperscript{+} cells were dramatically reduced in the thymi of IRF7\textsuperscript{−/−} mice compared with those of WT animals (Fig. 5H, 5I). Thus, we conclude that the lack of IFN-β production by IRF7\textsuperscript{−/−} stromal cells illustrated in Fig. 5A is the result of severely diminished numbers of the IFN-β–producing cells.

In summary, our findings indicate that mTECs experience a hindrance in the progression from the immature K14\textsuperscript{+}K5\textsuperscript{hi}UEA-1\textsuperscript{−} population to mature UEA-1\textsuperscript{+} and subsequently AIRE expressing cells in the absence of IRF7. We find that the presence of IRF7 is required in order for RANKL to facilitate the production of IFN-β, which in turn is crucial for the differentiation of immature mTECs into mature AIRE\textsuperscript{+} cells capable of promiscuous self-Ag expression.

**Discussion**

Our previous work demonstrated that STAT1 deficiency dramatically increases the incidence of autoimmune disease (18). In addition to decreased CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} function, it seemed conceivable that the absence of STAT1 also leads to impaired deletion of autoreactive T cells in the thymus. Indeed, we found that both STAT1 and the type I IFN receptor chain IFNAR, but not the IFN-γR, are crucial for the processes governing the deletion of autoreactive CD8\textsuperscript{+} cells in the TCR\textsuperscript{HY} model system (22). Importantly, we discovered that the coexistence of WT thymocytes restored the impaired elimination of TCR\textsuperscript{HY}STAT1\textsuperscript{−/−} thymocytes in male bone marrow chimeric mice, whereas bone marrow of TCR\textsuperscript{−/−} mice that lack mature T cells failed to support the purging of the autoreactive TCR\textsuperscript{HY}STAT1\textsuperscript{−/−} T cells, thus strongly supporting the notion that mature T cells contribute to the efficiency of the selection process in a STAT1-dependent manner. It is of interest to note that a similar effect was described in mice lacking CD40 (35). Specifically, it was shown that CD40L plays a non–cell-intrinsic role in deletion of self-reactive thymocytes. Although the molecular mechanism behind this effect was not known at the time, we now know that CD40 plays a role in mTEC development (26). As development of mTECs, which exhibit a short t\textsubscript{1/2} (36) and are indispensable for negative selection of self-reactive T cells, is dependent on their T cell interactions, we decided to analyze the architecture of STAT1\textsuperscript{−/−} thymi. Indeed, STAT1\textsuperscript{−/−} mice display a reduced medullary compartment as well as a reduction in the number of mature mTECs expressing CD80, UEA-1, and AIRE (Fig. 1). The defects in mTEC development in STAT1\textsuperscript{−/−} mice were strikingly similar to defects found in the absence of the TNF family members RANK, CD40, and LTβ (7, 8, 26). The importance of cross-talk between the RANK and IFN signaling cascades had previously been described in osteoclast differentiation and maturation (29). There, RANKL presented by mature T cells stimulates RANK on osteoclast precursors, resulting in their proliferation and simultaneous production of IFN-β. Autocrine stimulation via IFNAR subsequently leads to the

represent average ± SD of three sections each from three mice (p < 0.05). (D) mRNA levels of IFN-β, IkBα, and ISG54 in thymic stromal cells from WT or IRF7\textsuperscript{−/−} mice as determined by qPCR. (E) Thymic architecture in WT and IRF7\textsuperscript{−/−} mice as revealed by H&E staining of thymic sections (left panel), K5 and UEA-1 expression (second panel from left; scale bars, 100 μm), UEA-1 only (second panel from right), as well as EpCAM and AIRE expression (right panel; scale bars, 50 μm). (F) Relative mRNA levels of AIRE and INS2 in purified stromal cells from WT and IRF7\textsuperscript{−/−} thymi measured by qPCR (n = 3). (G) Histogram shows percentage of AIRE\textsuperscript{+} cells in the CD45\textsuperscript{hi}EpCAM\textsuperscript{−}UAE-1\textsuperscript{−} gate of thymic stromal cells from WT and IRF7\textsuperscript{−/−} mice. (H) Total number of UEA-1\textsuperscript{hi} cells as determined by flow cytometric analysis of CD45\textsuperscript{hi}EpCAM\textsuperscript{−}UAE-1\textsuperscript{−} mTEC populations from WT and IRF7\textsuperscript{−/−} thymi. (I) Total number of AIRE\textsuperscript{+} cells based on flow cytometric analysis of CD45\textsuperscript{hi}EpCAM\textsuperscript{−}UAE-1\textsuperscript{−}AIRE\textsuperscript{+} mTEC populations in WT and IRF7\textsuperscript{−/−} thymi (n = 4). (J) Hypothetical model of IFN-β production and function in the thymus.
termination of RANK signaling, causing the terminal differentiation of the precursors into mature, nonproliferating osteoclasts. We reasoned that a similar interplay might govern the development of thymic epithelial cells. Indeed, we noted that IFN-β is induced by treatment of thymic stromal cells with soluble RANKL. However, addition of exogenous IFN-β was able to promote the maintenance of AIRE expression during RANKL stimulation, consistent with the notion that RANKL promotes expansion of the mTEC population, whereas IFN-β mediates their differentiation and/or survival. In accordance with this concept, we noticed elevated K5 expression (characteristic of UEA-1-negative, immature mTECs) in STAT1-deficient thymi with a parallel reduction in the expression of cell-surface markers associated with mature mTECs (Fig. 1E). We therefore conclude that IFN production following RANK signaling provides a feedback loop that facilitates the terminal differentiation of mTECs, which is impaired in the absence of IFNAR or STAT1. These striking observations offer a new model for the development of medullary thymic epithelia that involves cross talk between the RANK and type I IFN signaling pathways.

Under the above-outlined hypothesis, one has to assume the continuous production of type I IFNs in the thymic medulla independent of infectious processes or other immune-response provoking events. In contrast to IFN-γ, of which the role in T cell development has been studied extensively, comparatively little is known about the contributions of IFN-α/β to this process. We therefore designed an animal model that allows for the identification of cells that have been exposed to type I IFN. By means of this mT/mG; Mx1-Cre system, we observe spatially tightly restricted IFN responses in the thymus that are almost exclusively limited to the medulla (Fig. 3). Our studies using mT/mG; Mx1-Cre mice lacking either IFNAR or STAT1 revealed a dramatic decline of GFP-positive mTECs compared with thymi from mice with an intact IFN-α/β signaling cascade, corroborating the vital contributions of this cytokine to the development of the thymic medullary compartment. Intriguingly, IFNAR<sup>-/-</sup> reporter mice still exhibited some GFP expression in the remaining UEA-1<sup>+</sup> cells. One possible explanation might be redundancy to type I IFN signaling in mTECs, as epithelial cells in general are responsive to IL-29 (IFN-λ). Although IFN-λ signals through a receptor distinct from IFNAR1/2, it also employs STAT1/2 heterodimers and stimulates an overlapping gene profile (37). Nevertheless, such IFN-λ expression in the thymus is apparently insufficient to promote mTEC maturation in IFNAR<sup>-/-</sup> mice (Figs. 1, 3). Alternatively, an IRF family member could be mediating residual IFN-λ expression downstream of RANK, but also that IRF7-deficient mice were found to have normal thymic compartments and mTECs that involves RANK and type I IFN signaling. It is possible that viruses that infect thymic cell populations might evoke the innate immune response. Lastly, our findings might have additional implications due to the fact that many viruses are capable of inhibiting IFN signaling and thus evading the immune response. Therefore, viruses that infect thymic cell populations might evoke immune disorders by repressing IFN signaling in medullary thymic stromal cells, consequently breaking tolerance of self-reactive thymocytes or upsetting T<sub>reg</sub> development.

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Disclosures
The authors have no financial conflicts of interest.

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