

Regulation of medullary thymic epithelial cell differentiation and function by the signaling protein Sin

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Medullary thymic epithelial cells (mTECs) play an important role in T cell tolerance and prevention of autoimmunity. Mice deficient in expression of the signaling protein Sin exhibit exaggerated immune responses and multitissue inflammation. Here, we show that Sin is expressed in the thymic stroma, specifically in mTECs. Sin deficiency led to thymic stroma-dependent autoimmune manifestations shown by radiation chimeras and thymic transplants in nude mice, and associated with defective mTEC-mediated elimination of thymocytes in a T cell receptor transgenic model of negative selection. Lack of Sin expression correlated with a disorganized medullary architecture and fewer functionally mature mTECs under steady-state conditions. Additionally, Sin deficiency inhibited the expansion of mTECs in response to *in vivo* administration of keratinocyte growth factor (KGF). These results identify Sin as a novel regulator of mTEC development and T cell tolerance, and suggest that Sin is important for homeostatic maintenance of the medullary epithelium in the adult thymus.

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Abbreviations used: cTEC, cortical thymic epithelial cell; DSS, dextran sodium sulfate; ERK, extracellular signal-regulated kinase; KGF, keratinocyte growth factor; LT β R, lymphotoxin β receptor; mTEC, medullary thymic epithelial cell; qPCR, quantitative PCR; RANKL, receptor activator of NF- κ B ligand; RIP, rat insulin promoter; TRA, tissue-restricted antigen; UEA-1, *ulex europaeus* agglutinin-1.

Immunological self-tolerance is defined as a state in which the immune system does not react destructively against self-molecules, cells, or tissues. T cell tolerance is established in the thymus (central tolerance), as well as in peripheral lymphoid and nonlymphoid organs (peripheral tolerance), and involves mechanisms such as physical elimination of autoreactive T cell clones (clonal deletion) or their functional diversion into regulatory T (T reg) cells (Sprent and Webb, 1995; Kyewski et al., 2002; Sprent and Kishimoto, 2002; Anderson et al., 2007; Boehm, 2008). Within the thymic cortex, successful interactions between the TCRs of developing lymphocytes and antigens presented by MHC molecules on epithelial cells lead to positive selection of thymocytes. Surviving lymphocytes that express TCRs with inappropriate reactivity to self-peptides are subsequently eliminated via induction of apoptosis (negative selection) in the thymic medulla (Lo et al., 1997). Recent evidence demonstrates that a large number of proteins with tissue-restricted expression are ectopically expressed

in rare cells in the thymic medulla, which are of epithelial rather than hematopoietic origin (Kyewski et al., 2002; Kyewski and Derbinski, 2004; Kyewski and Klein, 2006). These cells are tolerogenic medullary thymic epithelial cell (mTECs) resembling BM-derived cells in their ability to present self-peptide and eliminate autoreactive thymocytes. mTECs also contribute to the intrathymic development of CD4⁺CD25⁺ T reg cells (Jordan et al., 2001; Apostolou et al., 2002; Aschenbrenner et al., 2007), which control autoimmunity by dampening the immune responses of self-reactive T cells in peripheral tissues.

Genetic mutations that affect the development and function of mTECs can compromise T cell tolerance. For example, deletion or mutation of RelB (Burkly et al., 1995), the TRAF6, the NIK (Kajiura et al., 2004), the lymphotoxin β receptor (LT β R; Boehm et al., 2003), the receptor activator of NF- κ B ligand (RANKL), and its receptor RANK (Akiyama

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et al., 2008; Hikosaka et al., 2008) leads to defective differentiation of mTECs phenotypically, resulting in a reduced or absent medulla in mice (Derbinski and Kyewski, 2005). The absence or disruption of the three-dimensional medullary architecture in these mice correlates with development of autoimmunity, manifested as lymphocytic infiltration in multiple tissues and autoantibody production. In addition, the absence of tissue-restricted antigen (TRA) expression and presentation by mTECs in mice lacking the transcriptional regulator Aire results in similar autoimmune manifestations (Anderson et al., 2002; DeVoss et al., 2006). In humans, genetic lesions within the *aire* gene result in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. This syndrome is characterized by chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency (Mathis and Benoist, 2004; Mathis and Benoist, 2007). Thus, proper development of and antigen presentation by mTECs plays a key role in the establishment of central tolerance and prevention of autoimmunity.

Our studies have concentrated on elucidating the role of the adapter molecule Sin in T lymphocyte function. As a member of the Cas family of adapter proteins (Alexandropoulos et al., 2003), Sin (Src interacting protein), also known as Efs (embryonal fyn substrate; Alexandropoulos and Baltimore, 1996; Ishino et al., 1997), promotes protein-protein interactions via conserved modular domains. All Cas proteins are substrates for Src kinases, and Src kinase-mediated phosphorylation of these proteins is important for their adapter function (Alexandropoulos et al., 2003; Alexandropoulos and Regelman, 2009). Sin is highly expressed in the thymus, and we previously generated knockout mice to study the role of Sin in thymic and immune system function. Although thymocyte development in Sin^{-/-} mice is normal, young Sin knockout mice exhibit exaggerated T cell-mediated immune responses. More importantly, aged knockout mice spontaneously develop T cell-containing inflammatory lesions and tissue damage (Donlin et al., 2005). These data suggest that Sin normally acts to suppress T lymphocyte activation, and that Sin deficiency leads to aberrant lymphocyte responses and inflammation.

In this study, we examined the mechanism through which Sin deficiency causes inflammation and autoimmunity. Sin expression was confined to the thymic stroma and was particularly enriched in mTECs. Sin deficiency in this compartment associated with increased presence of T cell-containing inflammatory infiltrates in radiation chimeras and thymus-transplanted nude mice. Absence of Sin expression associated with a disorganized thymic medulla reduced total numbers of functionally mature Aire⁺ mTECs and TRA expression, and defective clonal deletion of antigen-specific thymocytes. The reduction of mature mTECs in knockout animals associated with abnormal steady-state mTEC proliferation. In addition, lack of Sin expression led to impaired expansion of mTECs induced by in vivo administration of keratinocyte growth factor (KGF). These experiments identify Sin as a signaling regulator of mTEC

differentiation and provide novel insight into the molecular mechanisms that regulate mTEC differentiation and establishment of self-tolerance.

RESULTS

Sin deficiency results in autoantibody production in aged animals

We previously showed that genetic ablation of Sin expression led to inflammatory, T cell-containing infiltrates in several tissues, including the small intestine, liver, kidney, and lung in older animals (Donlin et al., 2005). Because tissue inflammation in many mouse models also correlates with autoantibody production, we tested for the presence of autoantibodies in the serum of young and aged animals (4 and 7–12 mo old, respectively). Autoantibodies directed against the small intestine, kidney, ovary, and adrenal gland were present in the sera of the majority (~80%) of aged knockout mice, as compared with ~30% of wild-type controls (Fig. S1 A and Table S2). Autoantibody production against the liver and thyroid gland was less prevalent and was found in ~60% of the knockout mice versus 30% of controls, whereas no difference in autoantibody production against the pancreas and stomach was observed in the two experimental groups (unpublished data). Autoantibodies against the same tissues were absent in the sera of young (4-mo-old) knockout animals (Fig. S1 B and unpublished data), suggesting that symptoms of autoimmunity in Sin knockout mice develop as the animals age.

Sin expression is enriched in mTECs

Endogenous Sin expression was readily detectable in the whole thymus (Fig. 1 A, top). Fractionation of the thymus into thymocyte and stromal cell suspensions revealed that Sin expression was enriched in the thymic stroma as compared with thymocytes (Fig. 1 A, middle and bottom). Sin-specific antibody staining of thymic sections localized Sin expression to the thymic medulla (Fig. 1 B, top). The punctate staining pattern suggested that Sin is expressed in a sub-population of cells residing in the medulla. Sin expression was not evident in the cortex and no staining was observed in thymic sections prepared from Sin^{-/-} mice (Fig. 1 B).

Given the high expression of Sin in the thymic stroma we next examined whether Sin was expressed in thymic epithelial cells. Thymic stroma cell suspensions were stained with different antibodies, and medullary and cortical TECs (cTECs) were sorted by gating on the nonhematopoietic (CD45⁻) cell population as G8.8⁺LY51^{lo} and G8.8⁺LY51^{hi} cells, respectively (Fig. 1 C, dot plots). The expression of Sin was analyzed by semiquantitative RT-PCR using RNA isolated from sorted mTECs and cTECs. Consistent with the staining pattern of Sin in thymic sections, Sin expression was readily detectable in mTECs, whereas nearly undetectable levels of Sin were present in cTECs (Fig. 1 C, bottom). Our results are consistent with previously published gene array data showing that *sin* is one of ~450 genes encoding self-antigens

and differentiation genes expressed in mouse and human mTECs that were not detectable in cTECs, DCs, or thymocytes (Gotter et al., 2004).

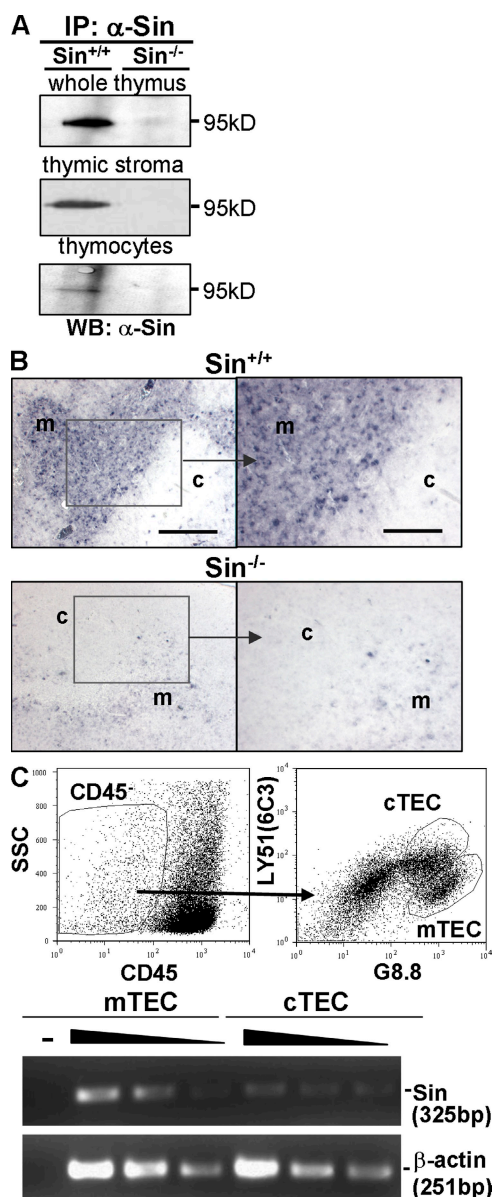


Figure 1. Sin is expressed in mTECs within the thymic medulla. (A) A whole thymus (top) or thymic stroma and thymocytes (from the same thymus; middle and bottom, respectively), were solubilized in lysis buffer and immunoprecipitations were performed using a Sin-specific antibody. One of two representative experiments is shown. (B) Thymic sections isolated from adult wild-type and Sin^{-/-} animals were stained with Sin-specific rabbit polyclonal antibody and analyzed by light microscopy. c, cortex; m, medulla. Bars: (left) 200 μ m; (right) 100 μ m. One of two representative experiments is shown. (C) mTECs and cTECs were sort purified from wild-type mice by gating on CD45⁺ cells as G8.8⁺LY51^{int} and CD45⁺G8.8⁺LY51^{hi}, respectively, as shown. RNA purified from these cells was used in RT-PCR reactions to assess expression of Sin, using Sin- and β -actin-specific primers. One of three independent purifications is shown. — indicates no enzyme control.

Sin plays a functional role in the thymic stroma

The enriched expression of Sin in the thymic stroma as compared with thymocytes prompted us to examine whether Sin played an intrinsic role in stromal function. Hematopoietic stem cell-enriched BM harvested from wild-type or Sin^{-/-} animals was injected into the tail vein of irradiated congenic recipients. Chimeras were analyzed \sim 4 mo after reconstitution. Staining of splenocytes with CD45.1 and CD45.2-specific antibodies revealed that >90% of the cells in recipient mice were donor-derived (unpublished data). We previously showed that Sin^{-/-} animals develop higher percentages of activated and effector/memory T cells correlating to the presence of inflammatory infiltrates (Donlin et al., 2005), and therefore chose these aberrant manifestations as functional readouts of the Sin^{-/-} phenotype. Sin^{-/-} animals that received wild-type BM exhibited an accumulation of infiltrates in the liver (Fig. 2 A, left), one of the tissues most commonly affected in aged Sin^{-/-} mice, and CD69⁺ activated T cells in the spleen (Fig. 2 A, histogram and bar graph). These manifestations were accompanied by a significant increase in the percentages and total cell numbers of CD44^{hi}CD62^{lo} effector/memory T cells (Fig. 2 B, contour plots and bar graphs). In contrast, no infiltrates and normal levels of CD69 and CD44^{hi}CD62^{lo} cells were present in wild-type mice reconstituted with wild-type or Sin^{-/-} BM (Fig. 2, A and B). Analysis of chimeras at 3 mo after reconstitution revealed an intermediate effect on CD69 marker expression in Sin^{-/-} recipients of wild-type BM, but not the wild-type controls (unpublished data), suggesting that these symptoms increase in severity as the BM graft ages. Additionally, no autoantibodies were detected at \sim 4 mo after reconstitution (unpublished data), consistent with the late onset (>7 mo) of autoimmunity in aged knockout mice.

Similar results were obtained when 2-deoxyguanosine-treated E15 Sin^{-/-} or wild-type thymic lobes were transplanted under the kidney capsule of nude mice. Specifically, transplantation of Sin^{-/-} thymi resulted in increased numbers of total CD3⁺ T cells in the spleen and lymph nodes (Fig. S2 A) and increased inflammatory infiltrates in the liver and kidney of recipient nude mice as compared with controls (Fig. 2 C and Fig. S2 B). These results, together with the expression pattern of Sin and data showing no discernible differences in the intrinsic signaling properties of Sin^{-/-} CD3⁺ T cells as compared with controls (assayed by proliferation in response to TCR activation [Donlin et al., 2005], total phosphotyrosine levels, PLC- γ phosphorylation, MAP-kinase activation, and intracellular calcium release [unpublished data]), strongly suggest that the absence of Sin from the thymic stroma and not the hematopoietic compartment contributes to autoimmunity in Sin^{-/-} mice.

Sin deficiency leads to defective clonal deletion of antigen-specific T cells

The results from BM and thymic transfers suggested that dysfunction of Sin^{-/-} thymic stroma is causal to autoimmune symptoms. Therefore, we hypothesized that the production of inflammatory infiltrates and autoantibodies in aged Sin^{-/-}

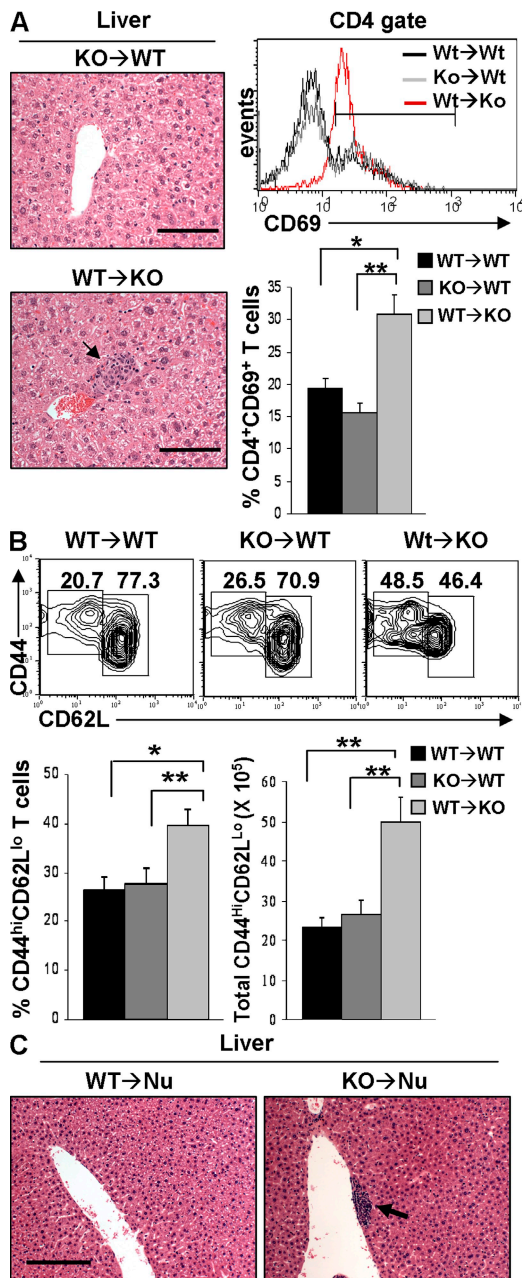


Figure 2. Sin deficiency in the thymic stroma mediates production of activated T cells and inflammatory infiltrates. (A) H&E-stained sections of the liver from BM recipients ~4 mo after reconstitution. Images are representative of at least three animals per group. Bar, 100 μ m. Expression of CD69 (histogram and bar graph) and the percentages and total numbers of effector memory donor T cells (B) in the different chimeras were determined from the CD4⁺ splenocyte gate using CD45.1- or CD45.2-antibodies and flow cytometry. Bar graphs in A and B depict mean \pm SE of at least five animals per group from at least two independent injection/irradiation experiments. *, $P < 0.05$; **, $P < 0.005$. (C) Thymic lobes from Sin^{+/+} ($n = 5$) and Sin^{-/-} E15 embryos ($n = 6$) were transplanted 1:1 into nude (Nu) recipients in three independent surgical groups. ~4 mo after transplant liver sections were analyzed for inflammatory infiltrates (arrow) with H&E. 5/6 and 0/5 nude recipients of knockout or wild-type thymic lobes, respectively, scored positive for infiltrates in the liver. Bar, 200 μ m.

mice could be caused by abnormal mTEC-mediated negative selection of autoreactive T cells. To examine this hypothesis, we crossed Sin^{-/-} animals to OTII mice that express a transgenic V α 2V β 5 TCR specific for an epitope in the ovalbumin protein (OVA₃₂₃₋₃₂₉) in the context of I-A^b (Barnden et al., 1994). A second transgene encoding a membrane-bound form of OVA (mOVA) under the control of the rat insulin promoter (RIP), which is active in mTECs, was crossed into Sin^{-/-}/OTII background (Kurts et al., 1996). These transgenic lines were previously used to show that clonotypic cell deletion was impaired in the absence of the transcriptional regulator Aire (Anderson et al., 2005). Wild-type and Sin knockout animals generated similar percentages of OTII clonotypic CD4⁺ T cells in the absence of cognate OVA antigen (Fig. 3, A and C). Dramatic deletion of clonotypic T cells in the thymus was observed in wild-type RIP-mOVA/OTII animals (Fig. 3, B, left, and C). In contrast, double transgenic mice in the Sin^{-/-} background had significantly more clonotypic cells (Fig. 3, B, right, and C), suggesting defective clonal deletion of antigen-specific T cells in the absence of Sin. A significant rescue of clonotypic cells was also evident in the spleen of RIP-mOVA/OTII knockout animals as compared with controls (Fig. 3 D). Similarly, Sin^{-/-} RIP-mOVA/OTI mice displayed increased numbers of clonotypic CD4⁺CD8⁺ cells in the thymus (unpublished data), suggesting that Sin controls the negative selection of both the CD4⁺ and CD8⁺ cell lineages by mTECs.

In addition to the OTII animal model, and because we detected low levels of Sin expression in cTECs (Fig. 1 C), we used the H-Y TCR transgenic system to determine if the effect of Sin deletion on negative selection of thymocytes was strictly confined to the medullary TECs. H-Y mice express class I MHC-restricted TCR α and β chains that recognize the male H-Y antigen presented by H-2D^b MHC class I molecules on cortical epithelial cells. In female H-Y animals, positive selection of CD4⁺CD8⁺ clonotypic cells proceeds unimpeded. In male mice, expression of the H-Y peptide in the cTEC population leads to high-affinity interaction and elimination of the majority of clonotypic thymocytes at the double-negative (CD4⁻CD8⁻) to double-positive (CD4⁺CD8⁺) transition (Kisielow et al., 1988). Both wild-type and Sin^{-/-} female H-Y transgenic mice displayed normal production of clonotypic T cells, suggesting normal positive selection in these animals (Fig. S3, A and B). Elimination of H-Y transgenic cells in male Sin^{-/-} animals was also normal compared with male wild-type H-Y controls (Fig. S3, A and B), suggesting that Sin does not affect early deletion of thymocytes in this transgenic model, and that the role of Sin is restricted to later stages in thymocyte negative selection that occur in the medulla.

Absence of Sin expression in mTECs has no effect on T reg cell development and function

In addition to clonal elimination of autoreactive T cells, self-antigen-expressing mTECs also support the development of CD4⁺CD25⁺ T reg cells (Jordan et al., 2001; Apostolou

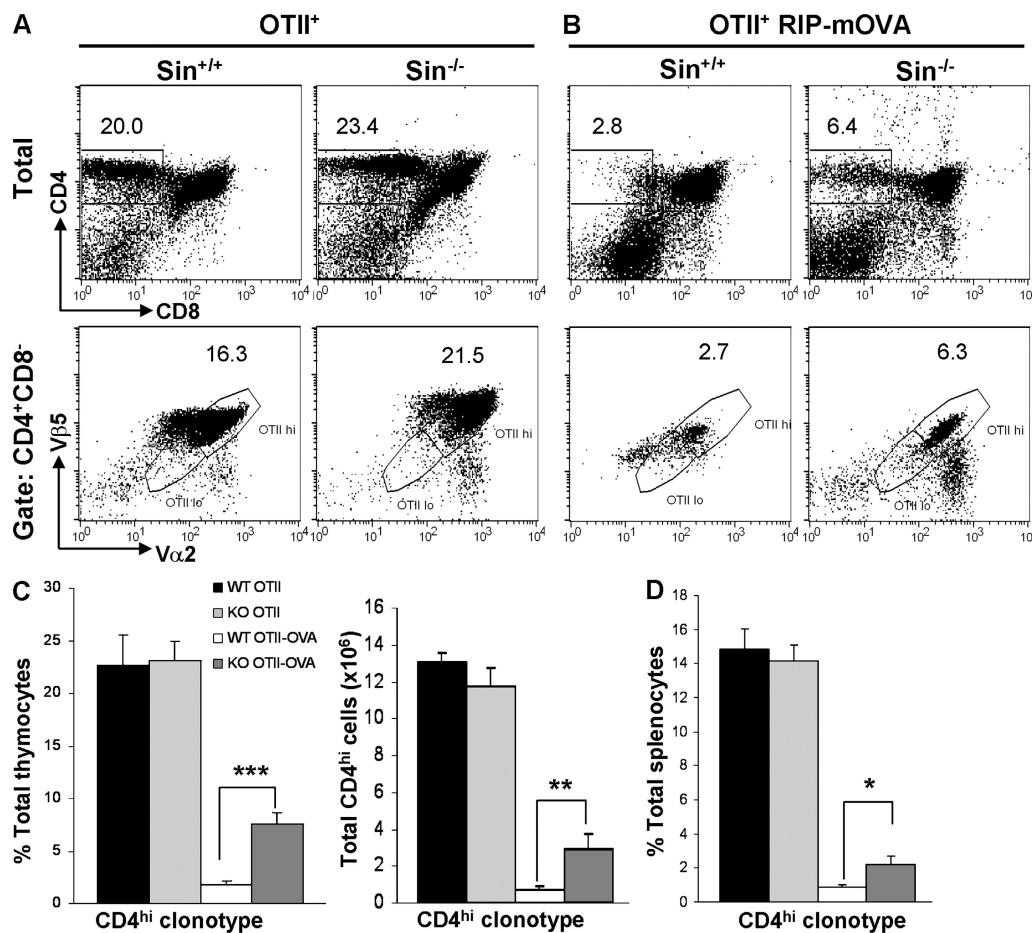


Figure 3. Defective negative selection of antigen-specific T cells in the absence of Sin expression. (A–C) OTII transgenic mice were crossed into the Sin^{-/-} background in the absence (A) or presence (B) of the cognate antigen in the form of transgenic mice expressing mOVA from the rat insulin promoter. Cell suspensions from the thymus and spleen were stained with antibodies against CD4, CD8, and the transgenic Vβ5, Vα2 TCR chains. The percentages and total numbers of clonotypic cells were determined by flow cytometry (C). Student's *t* test was used to compare wild-type and Sin knock-out OTII/RIP-mOVA mice. ***, *P* < 0.00005; **, *P* < 0.01; *, *P* < 0.05. Bar graphs represent mean + SE. *n* ≥ 5 animals per group from at least three independent experiments.

et al., 2002; Aschenbrenner et al., 2007). We thus investigated whether, in addition to defective negative selection of antigen-specific T cells, Sin ablation also interfered with T reg cell development and/or function. To this end, lymph node T cells were stained with antibodies against CD25 and Foxp3, a transcription factor specifically expressed in T reg cells, and percentages of CD4⁺CD25⁺Foxp3⁺ T cells were determined by flow cytometry (Brunkow et al., 2001). Wild-type and Sin^{-/-} mice exhibited similar percentages and total numbers of CD4⁺CD25⁺Foxp3⁺ T cells in the lymph node, suggesting Sin expression is not required for in T reg cell production (Fig. 4 A).

The suppressor function of Sin^{-/-} T reg cells was tested in two different in vivo colitis models. In the first approach, colitis was chemically induced in wild-type mice through administration of dextran sodium sulfate (DSS) in the drinking water. DSS administration disrupts the barrier of mucosal epithelial cells in the colon, leading to bacterial invasion and activation of innate and adaptive immune responses

(Heller et al., 2002). Injection of CD4⁺CD25⁺ T reg cells into animals before DSS treatment suppresses symptoms of colitis (Huber et al., 2004). CD4⁺CD25⁺ T reg cells isolated from wild-type or Sin^{-/-} animals were injected into the peritoneal cavity of wild-type mice, which were subsequently fed DSS for 9 d. Animals injected with PBS lost >20% of their body weight, a characteristic of severe intestinal inflammation, and exhibited visible signs of disease (Fig. 4 B). In contrast, mice injected with wild-type or Sin^{-/-} CD4⁺CD25⁺ T cells maintained their weight and displayed no signs of disease (Fig. 4 B). Thus, wild-type and Sin-knockout T reg cells were equally efficient in suppressing DSS-induced colitis, suggesting that T reg cell function is normal in the absence of Sin.

In the second approach, we used the murine model of inflammatory bowel disease (IBD) in immunodeficient Rag1^{-/-} mice to test the suppressor function of Sin^{-/-} T reg cells. Injection of effector CD4⁺CD25⁻ cells into Rag1^{-/-} animals causes a wasting disease associated with

progressive weight loss and colonic inflammation, whereas coinjection of suppressor $CD4^+CD25^+$ T reg cells prevents this disease (Powrie et al., 1996). $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations from wild-type and $Sin^{-/-}$ animals were sorted and transferred intravenously into $Rag1^{-/-}$ recipients in the indicated combinations (Fig. 4 C). $Rag1^{-/-}$ mice reconstituted with wild-type or $Sin^{-/-}$ $CD4^+CD25^-$ T cells alone, exhibited wasting disease as expected, whereas cotransfer of wild-type or knockout $CD4^+CD25^+$ T cells prevented weight loss (Fig. 4 C). Although in the experiment shown (Fig. 4 C) transfer of $Sin^{-/-}$ $CD4^+CD25^-$ effector T cells into $Rag1^{-/-}$ recipients led to more severe weight loss than the wild-type effector cells, this difference was not consistent over the eight experiments that were performed. In summary, results from both colitis induction experiments suggest that the *in vivo* suppressor function of $Sin^{-/-}$ T reg cells is intact and that Sin plays no apparent role in the development or function of these cells.

Sin deficiency in the thymic stroma correlates with altered medullary architecture and reduced numbers of UEA⁺MHCII^{hi}Aire⁺ mTECs

Given the expression of Sin in mTECs and the defective mTEC-mediated negative selection in the absence of Sin, we examined whether ablation of Sin expression affected development of the thymic medulla. Previous experiments have shown that small medullary islets in newborn mice expand and fuse to form a cohesive, centrally located medullary region within the first weeks of age (Rodewald et al., 2001; Yang et al., 2006). Hematoxylin and eosin (H&E) staining of paraffin-embedded thymic sections revealed small medullary islets in newborn wild-type and $Sin^{-/-}$ animals (Fig. 5 A, top), which expanded and coalesced in centrally located structures a week after birth in wild-type mice (Fig. 5 A, left middle). In contrast, Sin deficiency correlated with dispersed/disorganized medullary regions, which persisted in adult knockout mice (Fig. 5 A, right middle and bottom). A histological survey sampling five sections of the thymus over a

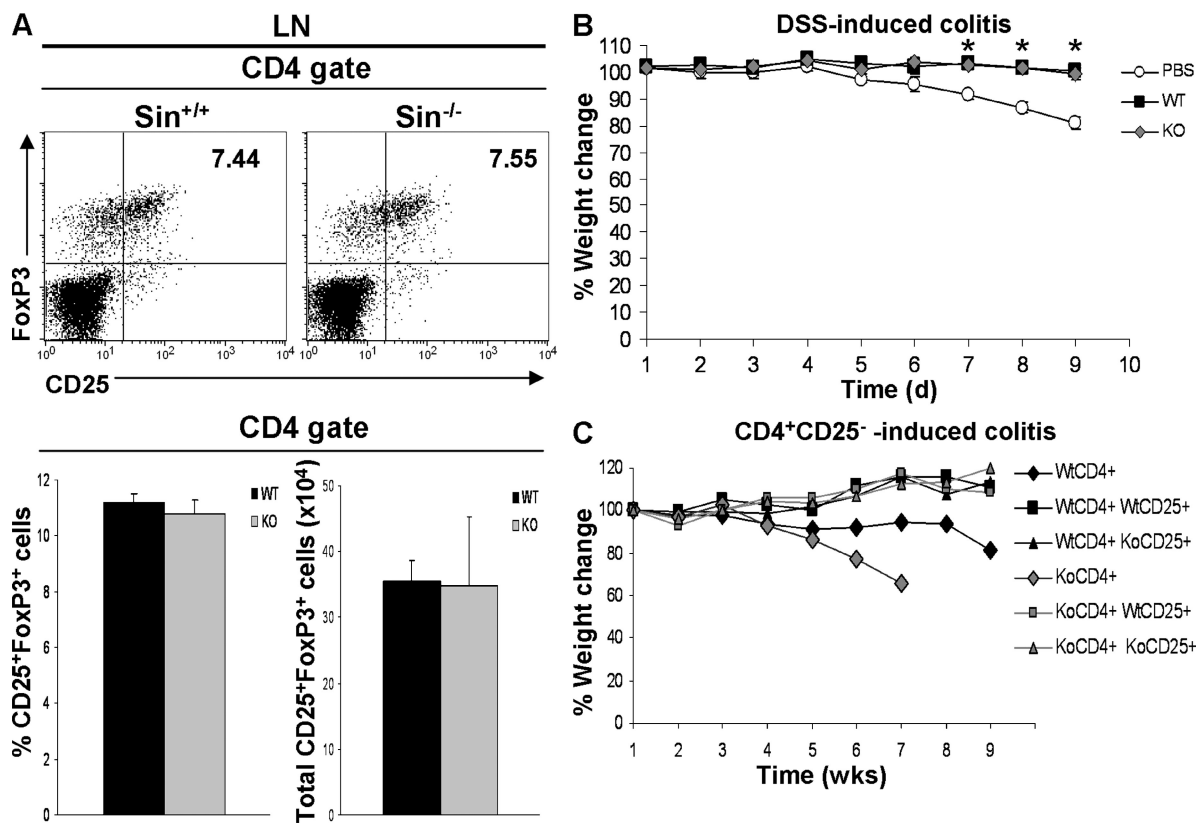


Figure 4. Normal development and function of T reg cells isolated from $Sin^{-/-}$ animals. (A) T reg cell percentages and total numbers in lymph nodes (LN) of wild-type and $Sin^{-/-}$ mice were analyzed by gating on $CD4^+CD25^+FoxP3^+$ cells. Bar graphs represent mean \pm SE. $n = 5$ in two independent experiments. (B) 7.5×10^5 $CD4^+CD25^+$ T cells from wild-type or $Sin^{-/-}$ donors were injected into wild-type mice, and colitis was induced with 3% DSS. Graph shows mean \pm SE. PBS ($n = 9$), wild-type ($n = 6$), or $Sin^{-/-}$ $CD4^+CD25^+$ T cells ($n = 11$) from at least three independent experiments. Repeated measures analysis of variance was used to determine main effects ($P < 0.05$) between injection groups, and post hoc Fisher PLSD was used to compare injection groups by day. Stars indicate significant differences ($P < 0.001$) from day 7–9 between each injection group and PBS control. (C) 4×10^5 $CD4^+CD25^-$ effector T cells with or without 10^5 wild-type or $Sin^{-/-}$ $CD4^+CD25^+$ T reg cells were injected into $Rag1^{-/-}$ recipients. Percentage weight loss or gain in B and C was determined by assigning the weight at day 1 as 100% and calculating percent total body weight measured by daily (B) or weekly (C) intervals. One of eight representative experiments is shown in C.

200- μ m distance, revealed a reduction in total medullary area and a higher number of medullary islets in the thymi of adult $\text{Sin}^{-/-}$ animals as compared with controls (Fig. 5 B).

Medullary TECs display terminal L-fucosyl residues that bind to plant lectin ulex europaeus agglutinin-1 (UEA-1; Farr and Anderson, 1985). Functional maturation of UEA^+ mTECs is marked by up-regulation of MHCII molecules and the transcriptional regulator Aire (Tykocinski et al., 2008). Thymic sections from the same time points depicted in Fig. 5 A stained with UEA-1, or adult thymi stained with the medullary and cortical markers Keratin 5 and Keratin 8, respectively, revealed similar medullary structures as noted with H&E (Fig. S4, A and B). Thymic sections from adult animals stained with UEA-1 or antibodies against MHCII and Aire revealed reduced staining for $\text{UEA}^+\text{MHCII}^+\text{Aire}^+$ mTECs in $\text{Sin}^{-/-}$ thymi as compared with controls (Fig. 5, C and D). Collectively, these results suggest that Sin deficiency leads to a disorganized medullary architecture and possibly reduced numbers of functionally mature UEA^+ mTEC subpopulations.

To determine if the apparent medullary reduction in $\text{Sin}^{-/-}$ thymi correlated with a quantitative reduction in UEA^+ mTECs, single thymi were dispersed and the total numbers of UEA^+ TECs were determined by flow cytometry. Indeed, $\text{Sin}^{-/-}$ mice exhibited a significant decrease in the total numbers of $\text{UEA}^+\text{MHCII}^+$ mTECs, whereas no differences were observed in the $\text{UEA}^-\text{MHCII}^+$ cTEC population as compared with wild-type animals (Fig. 6 A). Within the UEA^+ mTEC compartment MHCII, CD80/86 and Aire are used to distinguish a functionally distinct population. For example, recent studies suggest that $\text{CD80}^+\text{MHCII}^+\text{Aire}^-$ mTECs give rise to $\text{CD80}^+\text{MHCII}^+\text{Aire}^+$ cells which are regarded as functionally mature mTECs (Gäbler et al., 2007; Gray et al., 2007; Rossi et al., 2007b). Sin deficiency led to a significant reduction in the numbers of $\text{UEA}^+\text{MHCII}^+$ mTECs, whereas a modest reduction in the numbers of $\text{UEA}^+\text{MHCII}^+$ cells was not statistically significant (Fig. 6 B). Similar results were observed when stromal cell suspensions were stained and mTECs were

identified as $\text{LY51}^{\text{lo}}\text{G8.8}^+\text{MHCII}^+$ cells (Fig. S5, A and B). Although the $\text{LY51}^{\text{lo}}\text{G8.8}^+\text{MHCII}^{\text{hi}}$ mTEC population was significantly reduced in the absence of Sin expression (Fig. S5 C, left graph), the $\text{LY51}^{\text{lo}}\text{G8.8}^+\text{MHCII}^{\text{lo}}$ mTEC (Fig. S5 C, right graph) and $\text{LY51}^{\text{hi}}\text{G8.8}^+\text{MHCII}^+$ cTEC populations (Fig. S5 D) were unaffected.

In separate experiments, and given the reduced Aire staining in thymic sections observed in Fig. 5 (C and D), we examined the effect of Sin deficiency on the numbers of functionally mature Aire-expressing mTECs. Lack of Sin expression led

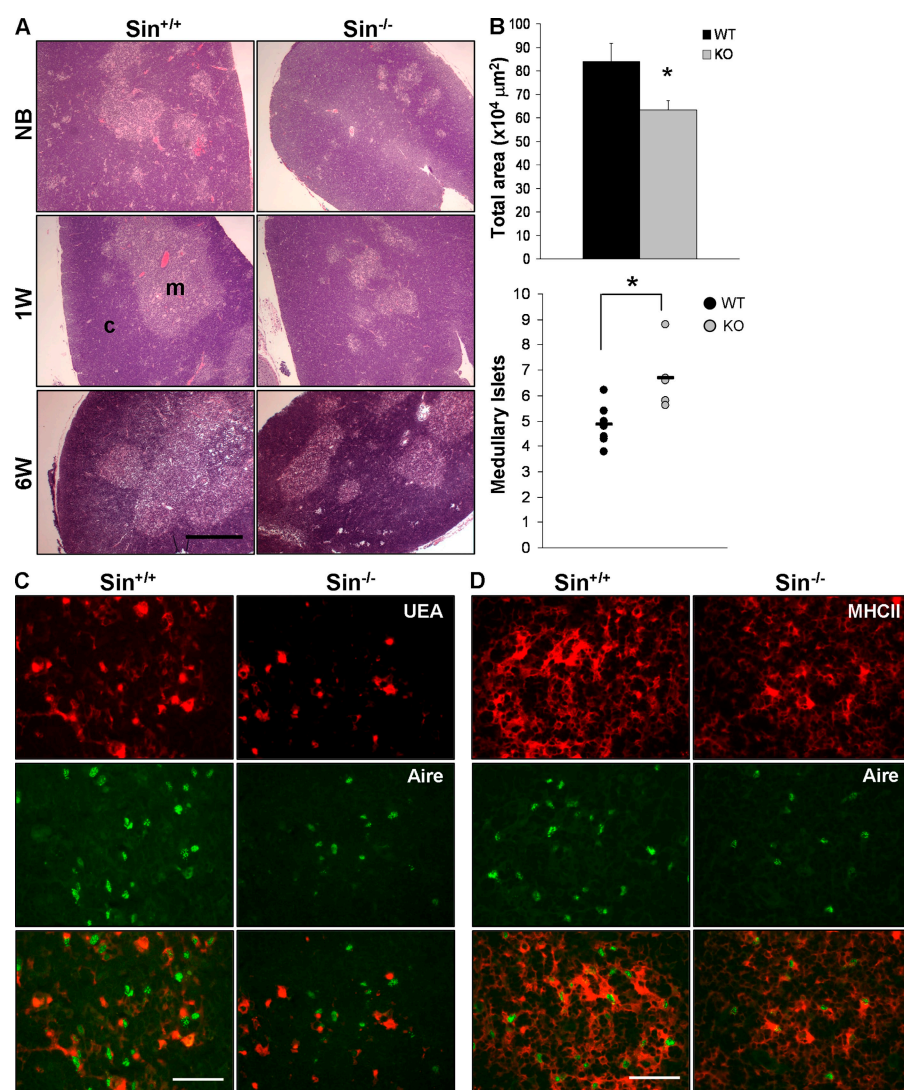


Figure 5. Sin deficiency results in a disorganized medullary architecture. (A) Thymi

were isolated from wild-type and $\text{Sin}^{-/-}$ animals at different ages (NB, new born; W, week), and sections of thymic lobes stained with H&E were analyzed by light microscopy. Bar, 200 μ m. (B) Total medullary area and number of islets were calculated over 5 slices spanning a 200- μ m thymic section from 5–6-wk-old wild-type and $\text{Sin}^{-/-}$ animals. For A and B, at least five mice per group were analyzed in three separate experimental groups. Mean \pm SE is depicted on graphs. *, $P < 0.05$. (C and D) Frozen thymic sections from wild-type and $\text{Sin}^{-/-}$ mice were stained with UEA (red, left) or MHCII (red, right) to reveal medullary area, and co-stained with anti-Aire (green). Images are representative of four independent experiments. Bar, 30 μ m.

to reduced numbers of UEA⁺MHCII⁺Aire⁺ cells (Fig. 7 A), suggesting that Sin is required for normal maturation of mTECs. Consistent with the reduction in mature mTECs, quantitative PCR (qPCR) analysis using mRNA obtained from total TEC preparations revealed reduced levels of MHCII, CD80 and Aire in Sin^{-/-} TECs as compared with wild-type controls (Fig. 7 B and C, left graph). These analyses also revealed reduced expression of most but not all Aire-dependent and

Aire-independent antigens tested (Fig. 7 C). In contrast, Sin deficiency had no effect on expression of the chemokine CCL21 or the cTEC Cathepsin-L- and mTEC cathepsin-S-specific proteases (Fig. 7 B, right graph). Further qPCR analyses using mRNA from sorted mature UEA⁺MHCII^{hi} mTEC populations derived from age-matched groups of either wild-type or Sin knockout animals revealed no significant effect of Sin deficiency on MHCII, CD80, Aire, and representative TRA expression (Fig. 7 D). These data suggest that reduced surface marker and TRA expression observed in the whole TEC fraction was caused by reduced numbers of mature mTECs, rather than a direct effect of Sin deficiency on TRA expression. Collectively, the experiments presented above show that Sin deficiency results in loss of cohesive medullary regions, defective maturation of mTECs and as a consequence reduced TRA expression. These effects are perhaps causal to the defective negative selection of antigen-specific T cells and development of autoimmunity observed in Sin knockout mice.

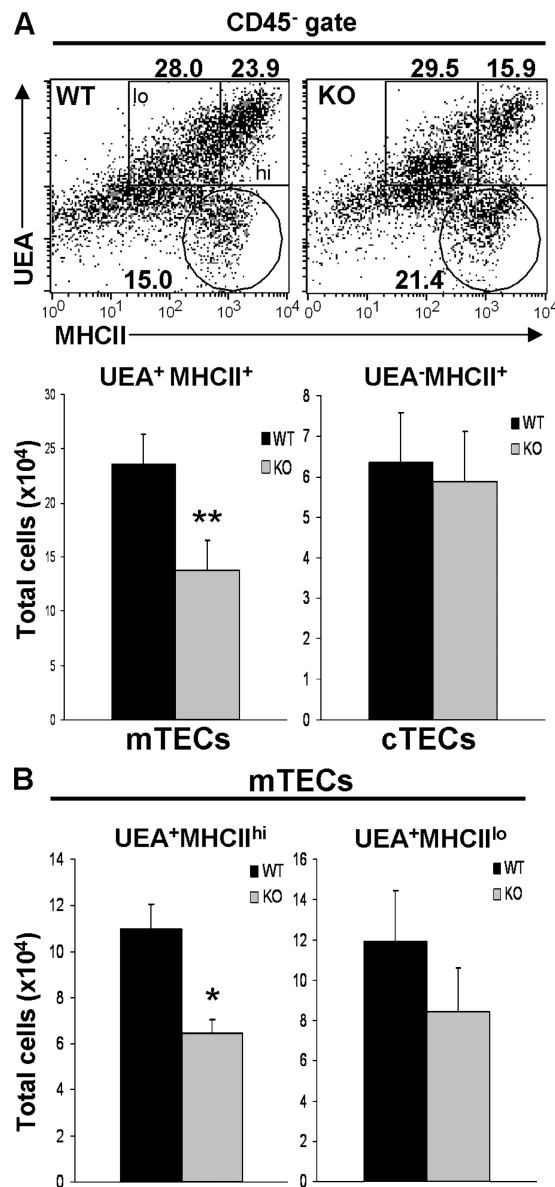


Figure 6. Sin^{-/-} animals have reduced total numbers of UEA⁺MHCII^{hi} mTECs. (A) Individual thymi from wild-type and knockout mice were analyzed for total CD45⁻UEA⁺MHCII⁺ mTECs (rectangle gate) and CD45⁻UEA⁻MHCII⁺ cTECs (circular gate) by flow cytometry. (B) Total numbers of CD45⁻UEA⁺MHCII^{hi} and CD45⁻UEA⁺MHCII^{lo} mTECs were determined from the same cell preparations as in A. Graphs show the mean + SE. *, P < 0.01; **, P < 0.005. At least six animals were analyzed per genotype, from two independent experiments.

Sin regulates growth factor-mediated mTEC expansion

Given the reduced numbers of mature mTECs in Sin knockout mice, we hypothesized that Sin regulated steady state and/or growth factor-induced proliferation of mTECs in the adult thymus. To examine this hypothesis, TECs purified from wild-type and Sin^{-/-} mice were stained with anti-CD45 and MHCII antibodies, UEA-1, and antibodies against the proliferation marker Ki67. These experiments revealed a significant reduction in the percentages and total numbers of proliferating UEA⁺MHCII⁺Ki67⁺ mTECs in knockout animals compared with controls (Fig. 8 A). The previously described rapid turnover of mTEC populations in the adult thymus (Gray et al., 2007) prompted investigation into the cumulative steady-state expansion of the mTEC populations in the absence of Sin. As with the Ki67 labeling experiments, mice injected with the proliferation marker BrdU with continuous exposure to BrdU for 3 d, exhibited significantly reduced percentages and total numbers of UEA⁺MHCII⁺BrdU⁺ mTECs (Fig. S6). In contrast, there was no significant difference in UEA⁻MHCII⁺Ki67⁺ or UEA⁻MHCII⁺BrdU⁺ cTECs in Sin knockout mice as compared with controls (Fig. 8 A and Fig. S6). Together, these results suggest that Sin is required to maintain the normal rate of mTEC proliferation and mTEC homeostasis in the adult thymus.

KGF (also known as fibroblast growth factor-7) is a soluble factor that regulates mitogenesis and differentiation of epithelial cells (Finch and Rubin, 2004). KGF exerts its effects by binding to a splice variant of the fibroblast growth factor receptor family, the FGFR2IIIb receptor, expressed in keratinocytes as well as in TECs (auf dem Keller et al., 2004; Rossi et al., 2007a). In adult thymi, KGF is expressed by mesenchymal cells and thymocytes and controls postnatal thymic development (Alpdogan et al., 2006; Rossi et al., 2007a). In vivo KGF administration leads to expansion of the hypoplastic medullary compartment in the thymic rudiment of Rag^{-/-} mice (Erickson et al., 2002), whereas injection of

KGF in adult wild-type mice induces transient expansion of immature mTECs and promotes their differentiation into mature mTECs (Rossi et al., 2007a). These observations, as well as the reduced numbers of cycling mTECs in *Sin* knock-out mice, prompted us to examine whether *Sin* played a role in KGF-mediated expansion of mTECs. KGF administration resulted in a marked increase in total UEA⁺MHCII⁺ cells in wild-type animals as compared with vehicle injected controls (Fig. 8 B). Strikingly, *Sin* was required for UEA⁺MHCII⁺ mTEC expansion in response to KGF injection (Fig. 8 B). Further analysis of the UEA⁺MHCII^{hi} and UEA⁺MHCII^{lo} mTEC subsets revealed that KGF administration induced the expansion of both subpopulations within the UEA⁺ compartment in wild-type animals (Fig. 8, C and D). This expansion was blocked in *Sin*^{-/-} mice, supporting a role for *Sin* in the expansion of precursor cells and maturation of mTECs.

The lack of mTEC expansion in *Sin*^{-/-} mice was not due to decreased receptor levels, as FGFR2IIb expression was similar or higher in *Sin*^{-/-} mTECs as compared with controls (Fig. 8 F).

It was previously shown that KGF administration promotes expansion of cTECs, presumably by stimulating a minor subset of Keratin-18⁺ cTECs that express FGFR2IIb (Rossi et al., 2007a). Consistent with this, we found a marked increase in the total numbers of UEA⁻MHCII⁺ cTECs in KGF-treated wild-type animals (Fig. 8 E). Surprisingly, given the nearly undetectable expression of *Sin* in the cortex and cTECs (Fig. 1), the normal positive selection of thymocytes in *Sin*^{-/-} mice (Fig. S3), and the lack of an effect of *Sin* deficiency on steady-state cTEC numbers (Fig. 6 A) or cTEC proliferation (Fig. 8 A and Fig. S5), KGF-mediated cTEC expansion was markedly inhibited in the absence of *Sin* expression (Fig. 8 E). This effect could be caused by KGF-mediated expansion of a precursor population that precedes both mTEC and cTEC lineage differentiation, or to transient up-regulation of *Sin* expression during cTEC expansion. Together, these data show that *Sin* is an important mediator of KGF-induced expansion of TEC populations and suggest that

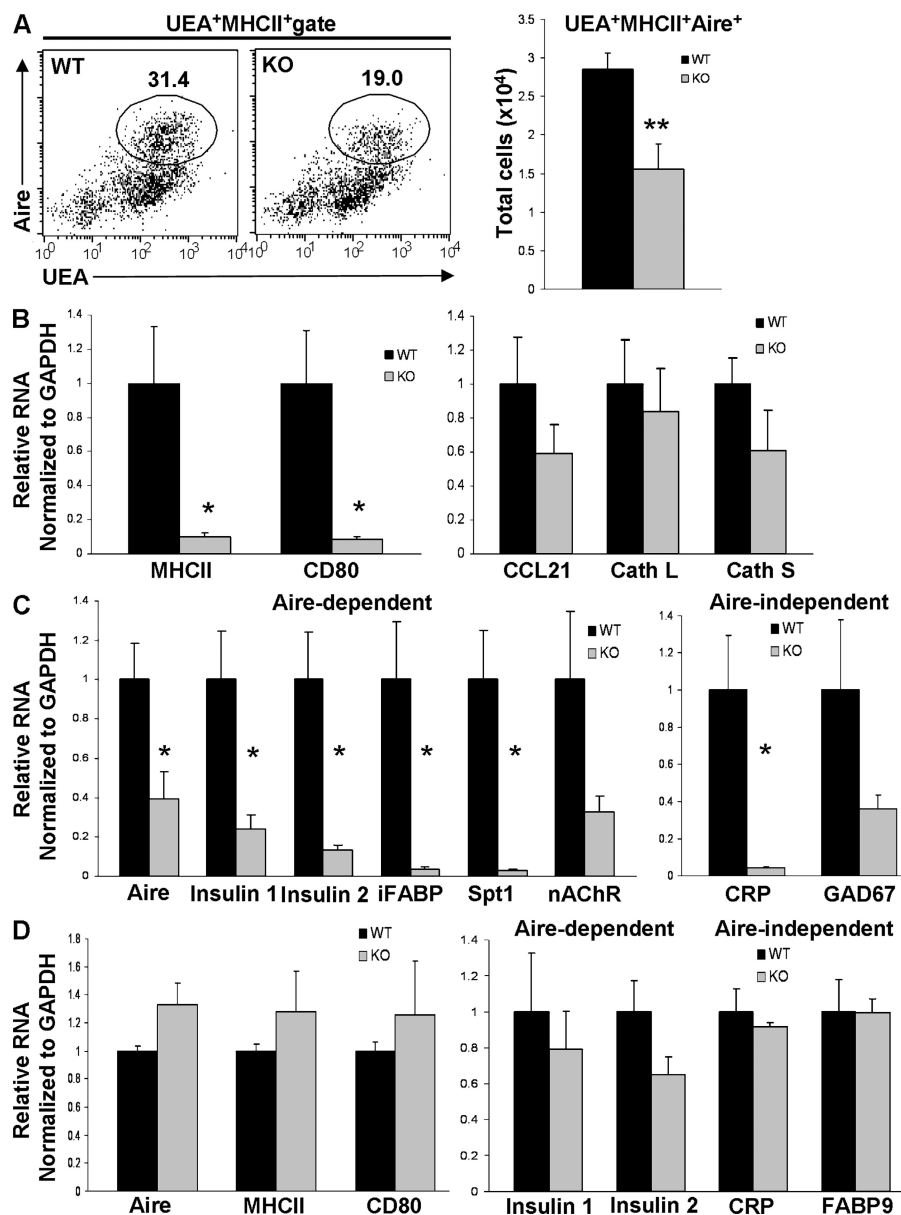


Figure 7. Decreased numbers of UEA⁺MHCII⁺Aire⁺ cells correlate with decreased TRA expression in *Sin*^{-/-} thymus.

(A) Stromal cell suspensions from individual thymi were stained as in Fig. 6, with the addition of an Aire-specific antibody, and UEA⁺MHCII⁺Aire⁺ mTECs were determined by flow cytometry. Bar graph represents mean of at least six mice per genotype from two independent trials + SE. **, $P < 0.00005$. (B and C) Sorted whole TEC-enriched fractions from individual wild-type and *Sin*^{-/-} thymi were analyzed by qPCR for the indicated genes using specific primers. Graphs show the mean of at least two individual experiments, with at least four animals per genotype + SE. (D) UEA⁺MHCII⁺ mTEC populations sorted from pooled wild-type or knockout thymi ($n \geq 5$ each) were analyzed for expression of the indicated genes by qPCR. Graphs show the mean of two independent samples per genotype run in quadruplicates + SE, and are representative of two experiments. Analysis of relative gene expression was normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method, and the wild-type sample set at 1 for each test gene. Gene acronyms are *I-FABP*, intestinal fatty acid binding protein; *Spt1*, salivary protein 1; *nAChR-1*, cholinergic receptor, nicotinic, α polypeptide 1; *CRP*, C-reactive protein; *FABP9*, fatty acid binding protein 9; *GAD67*, glutamic acid decarboxylase. *, $P < 0.05$.

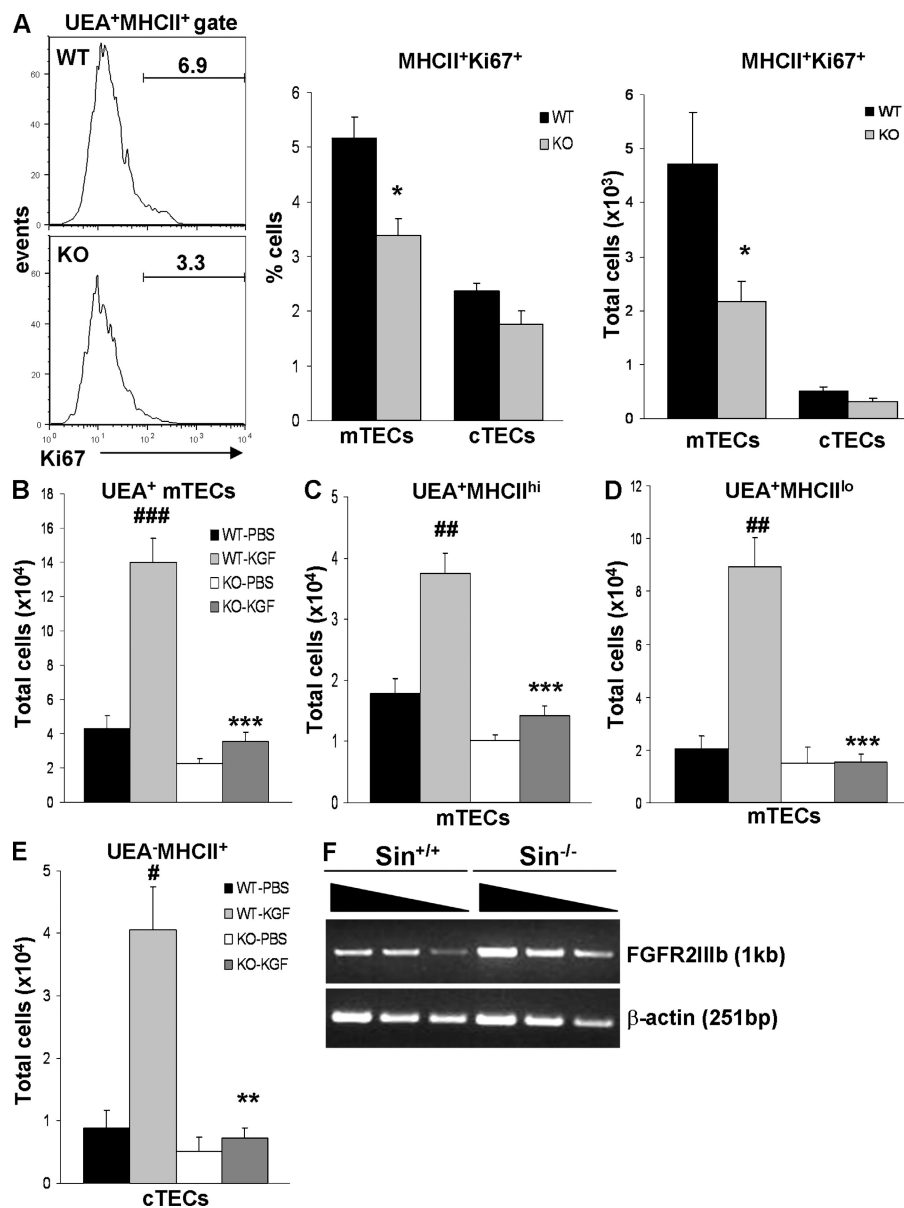


Figure 8. Reduced proliferation rate and block in growth factor-induced expansion of UEA⁺ mTECs in the absence of Sin expression. (A) Percentages and total numbers of CD45⁺ UEA⁺ MHCII⁺ Ki67⁺ cells were determined by flow cytometry. Bar graph represents mean of at least eight mice per genotype in two independent experiments + SE. *, $P < 0.05$. (B–E) TECs purified from individual thymus of PBS- or KGF-injected wild-type or Sin^{-/-} mice were analyzed by flow cytometry for total cell numbers within different gates as shown. Analysis of variance was used to detect an effect by treatment and an effect by genotype in B–E where $P < 0.05$, followed by post hoc Fisher PLSD used for group comparisons. # compares treatment within the same genotype, and * compares treatment between wild-type and knockout genotypes. **, $P < 0.001$; ***, $P \leq 0.0005$; ##, $P < 0.001$; ###, $P < 0.0005$. (E) **, $P < 0.005$; #, $P < 0.005$. Bars represent the mean + SE. 4–7 mice were used per experimental group from two independent experiments. (F) RT-PCR for FGFR2IIIb was performed on sorted purified mTECs. Samples were normalized based on relative β-actin levels. Shown is a representative of two experiments.

Sin regulates growth-factor-mediated maturation of mTECs from functionally immature precursors.

DISCUSSION

We previously showed that Sin deficiency results in development of inflammatory infiltrates against multiple tissues in older animals (Donlin et al., 2005). In this study, we explored the mechanism through which Sin deficiency leads to inflammation and autoimmunity. Our data indicate that Sin functions in the thymic stroma to regulate the maturation of mTECs and, consequently, their ability to establish central tolerance. Sin deficiency resulted in a dispersed medullary architecture and reduced numbers of UEA⁺ MHCII^{hi} Aire⁺ medullary epithelial cells. Aire expression in this cell population coincides with expression of a diverse array of TRAs presented by MHC molecules on the surface of mTECs to

mediate selection of developing T cells. Thus, UEA⁺ MHCII^{hi} Aire⁺ cells are believed to function as terminally differentiated mTECs required for efficient negative selection of thymocytes that express potentially autoreactive TCRs. Our results showing decreased mature mTEC numbers and TRA expression, accompanied by inefficient mTEC-mediated elimination of antigen-specific T cells in Sin^{-/-} mice, reveal Sin as a novel regulator of

mTEC maturation and mTEC-mediated establishment of central tolerance. Reduced total numbers of UEA⁺ MHCII^{hi} Aire⁺ mTECs, reduced TRA expression, and/or defective architecture of the thymic medulla, may all contribute to defective negative selection in Sin^{-/-} mice. Because individual self-antigens are expressed in only a small fraction of total mTECs consisting of CD80^{hi} cells (Derbinski et al., 2008), it has been proposed that extensive interactions between mTECs and thymocytes are required for establishment of central tolerance (Kyewski and Klein, 2006; Mathis and Benoist, 2004). When TRA expression over the entire TEC population was assayed, Sin^{-/-} mice exhibited markedly reduced Aire-dependent and -independent tissue-specific antigen expression caused by a decreased population of mature mTECs. Therefore, the reduced numbers of mTECs in Sin^{-/-} mice accompanied by

markedly reduced Aire-dependent and -independent TRA expression may lead to decreased probability of a TCR/MHC–mTEC interaction, affecting the efficiency of negative selection. The dispersed medullary architecture noted in the $\text{Sin}^{-/-}$ mice may also reduce the efficiency of negative selection, leading to leaky central tolerance. Similar to the $\text{Sin}^{-/-}$ animals, $\text{LT}\beta\text{R}^{-/-}$ mice exhibit a dispersed medullary architecture and reduced total numbers of mTECs. Additionally, TRA expression within the mature mTEC population is normal when either $\text{LT}\beta\text{R}$ or Sin is removed, yet these animals develop age-dependent autoimmunity (Boehm et al., 2003). Thus, the defects in the numbers and/or distribution of mature mTECs documented in the $\text{Sin}^{-/-}$ thymus could significantly perturb the interaction of these cells with maturing thymocytes, leading to defects in central tolerance.

Sin deficiency led to a significant reduction in cycling $\text{UEA}^+\text{MHCII}^+$ cells under steady-state conditions (shown by reduced Ki67 staining and BrdU incorporation), suggesting that Sin functions in signaling pathways that affect the proliferation of these cells. To further investigate the function of Sin in mTEC development, we focused on the growth factor KGF and its receptor FGFR2IIIb , because this ligand/receptor pair has been shown to regulate mitogenesis and differentiation of epithelial cells (Finch and Rubin, 2004). Indeed, *in vivo* administration of KGF led to an expansion of UEA^+ mTECs and UEA^- cTECs in wild-type mice that was completely blocked in the $\text{Sin}^{-/-}$ thymus. The inhibition of cTEC expansion in the $\text{Sin}^{-/-}$ thymus was surprising given the low Sin expression in wild-type cTECs and the observation that Sin deficiency has no discernible effects on cTEC numbers and proliferation under steady-state conditions. It is possible that Sin expression is up-regulated in cTECs in response to KGF treatment or that Sin is expressed in a precursor population that gives rise to both cortical and medullary epithelial cell lineages. In future experiments, it will be important to examine the ontogeny of different TEC populations using additional surface markers in the presence or absence of KGF in the $\text{Sin}^{-/-}$ thymus. This may help us identify bipotent TEC precursors and lead to a better understanding of the development of different thymic epithelial cell lineages and the role of Sin in this process.

It is currently unclear how Sin may regulate signaling downstream of the KGF receptor FGFR2IIIb . The FGF receptor family consists of tyrosine kinases that regulate cell survival and proliferation of epithelial cells (Finch and Rubin, 2004; de Giorgi et al., 2007). Growth factor ligand binding to FGFRs induces receptor dimerization and tyrosine kinase mediated trans-phosphorylation. This leads to activation of phosphoinositide 3 kinase and Akt, as well as activation of the extracellular signal-regulated kinases 1 and 2 (ERK-1/2), two pathways that control cell survival and proliferation, respectively. Src family kinases have also been implicated in signaling downstream of FGF receptors by recruiting and phosphorylating adaptor proteins involved in Akt and ERK-1/2 activation (Bromann et al., 2004; Finch and Rubin, 2004). Because Sin was isolated and identified as an Src family

kinase-interacting protein (Ishino et al., 1995; Alexandropoulos and Baltimore, 1996), we hypothesize that KGF activation of FGFR2IIIb might induce activation of Src kinases, in turn targeting Sin for phosphorylation, and creating binding sites for potential signaling intermediates such as the FRS1a,b and Grb2/Sos. These signaling intermediates have been shown to modulate mitogenic activation through Ras-Erk1/2 signaling cascade (Gotoh, 2008). Therefore, the absence of Sin may cause disruption of signaling complexes and aberrant signaling output, impairing mitogenic signaling and inhibiting proliferation. Attempts to biochemically analyze the role of Sin in KGF-mediated mTEC expansion were hampered by technical limitations caused by the low numbers of mTECs present in each thymus. Additionally, the time required to isolate mTECs proved prohibitive in assaying transient phosphorylation events in response to FGFR2 activation in an intact thymus. Therefore, it will be necessary to develop new biochemical assay methods to investigate the role of Sin downstream of the FGFR2IIIb receptor in mTECs to fully understand the adapter function of Sin in mitogenesis and the ultimate role for Sin in establishment of central tolerance via its effect on mTEC physiology.

Finally, although we showed that Sin deficiency reduced mTEC numbers, the production of mTECs was not completely blocked in $\text{Sin}^{-/-}$ mice, suggesting that multiple signaling pathways contribute to mTEC differentiation. Recently, two members of the TNF superfamily and their receptors, CD40L/CD40 and RANKL/RANK, were implicated in regulating mTEC development in a cooperative, developmental stage-dependent manner (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008). In one of these studies, RANKL was shown to promote expansion of mature mTEC populations through direct cell–cell contact and MHCII–TCR-mediated interactions between positively selected CD4^+ thymocytes and mTECs (Irla et al., 2008). Because precursor mTEC populations express low levels of MHCII, the exact nature of the signals controlling mTEC maturation from precursor MHCII^{lo} populations remains unclear. In our studies, we found that KGF had a robust effect on $\text{UEA}^+\text{MHCII}^{\text{lo}}$ mTEC expansion corresponding to increased $\text{UEA}^+\text{MHCII}^{\text{hi}}$ mTEC numbers. This observation suggests that KGF (and possibly other soluble factors) may exert a mitogenic effect at earlier stages of mTEC development, affecting distinct cell subpopulations independently of autoantigen-dependent interactions and TNF superfamily-mediated signaling. As a consequence, Sin may regulate distinct signaling pathways at specific stages of mTEC differentiation and development, affecting proliferation, survival, and/or differentiation of mTECs. Future studies will focus on the potential role of Sin in other signaling pathways, including those regulated by TNF superfamily members, to better understand the role of Sin in mTEC differentiation.

In summary, our studies provide novel insight for the role of the signaling protein Sin in mTEC physiology and central tolerance and have important implications for therapeutic interventions using KGF. In preclinical studies of allogeneic

BM transplantation, the systemic administration of recombinant human KGF has been shown to exert a beneficial effect by preventing graft versus host disease (GVHD; Rossi et al., 2002; Finch and Rubin, 2004; Bruinsma et al., 2007; Kelly et al., 2008; Krenger and Holländer, 2008). Exposure to exogenous KGF preserves TEC architectural organization and lymphoid cellularity, and results in more efficient reconstitution of the peripheral T cell compartment in preconditioned allogeneic transplant recipients (Rossi et al., 2002; Alpdogan et al., 2006; Seggewiss et al., 2007). In mouse models of acute GVHD, KGF injection suppresses thymic injury regardless of alloreactive donor T cells in the thymus and ongoing GVHD in other target organs such as the skin, liver, and intestinal mucosa (Rossi et al., 2002). Administration of KGF also prevents the emergence of a repertoire of autoreactive T cells that promote the development of experimental chronic GVHD (Chen et al., 2007). The mechanisms through which KGF exerts its beneficial effects are currently unclear. Understanding the role that Sin plays in the molecular mechanisms of mTEC signaling and expansion will provide mechanistic insight in the ability of KGF to boost thymic output. This in turn may help evaluate the potential use of this factor as therapeutic agent for the management of clinical complications associated with BM transplantation and/or autoimmune disease.

MATERIALS AND METHODS

Mice. Sin knockout mice were derived and genotyped as previously described (Donlin et al., 2005). C57BL/6, Rag1^{-/-}, OT-II, CD45.1, and CD45.2 mice were obtained from The Jackson Laboratory. H-Y transgenics and B6.Cg/NTac-Foxn1tm NE9 (Nude) homozygotes were purchased from Taconic Farms. RIP-mOVA mice were provided by R. Clynes (Columbia University, New York, NY) and previously described (Kurts et al., 1996; Harbers et al., 2007). All mice were crossed to Sin^{-/-} mice that were backcrossed for 10 generations to the C57BL/6 background. Animals were housed in specific pathogen-free conditions and were used and maintained in accordance with institutional guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee guidelines of Columbia University and Mount Sinai School of Medicine.

mTEC purification. Thymi from Sin wild-type and knockout animals were dispersed, and single-cell suspensions were enriched using a percoll gradient as described previously (Anderson et al., 2005). TEC fractions were treated with anti-CD16/CD32 (BD) to block Fc receptors, and then incubated with anti-CD45 (30-F11), -MHCII (M5/114.15.2; eBioscience), -Ly-51 (6C3), -CD326 (G8.8), -Ki-67 (B56; BD), -Aire (5H12; provided by H.S. Scott, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), and/or biotinylated UEA-1 (Vector Laboratories) and analyzed by flow cytometry.

BM chimeras and thymic transplants in nude mice. BM from ~3-mo-old wild-type or Sin^{-/-} donors was depleted of lineage-committed cells using a mouse lineage antibody panel (BD) and magnetic separation (Miltenyi Biotec). 2×10^5 hematopoietic-progenitor-enriched cells were injected into the tail vein of lethally irradiated 6–8-wk-old Sin^{+/+} and Sin^{-/-} recipients treated with enrofloxacin (Baytril) for 2 wk after irradiation. Mice were sacrificed ~4 mo after reconstitution, and the percentages of donor-derived cells and cell surface markers were revealed by staining with anti-CD45.1 or CD45.2 (eBioscience) antibodies and flow cytometry.

Thymic lobes from E15 wild-type and knockout embryos were cultured in 1.35 mM 2-deoxyguanosine (2-DOG; Sigma-Aldrich) for 6–8 d to deplete thymic lobes of hematopoietic lineage cells as previously described (Jenkinson et al., 2008). Thymic lobes were surgically transplanted under the kidney capsule of adult nude homozygotes in the Microvascular Surgery Research Facility, Mount Sinai School of Medicine. The presence of T cells in peripheral blood was monitored to verify engraftment at 4 wk after surgery. Peripheral T cells and tissues were collected, and were analyzed by flow cytometry or stained with hematoxylin and eosin (H&E) ~4 mo after transplantation, respectively.

DSS-induced colitis. Colitis was induced as previously described (Okayasu et al., 1990; Huber et al., 2004). In brief, pooled splenocytes from either Sin^{+/+} or Sin^{-/-} mice were stained with anti-CD4 and CD25 antibodies and sorted on a FACS Aria. 7.5×10^5 CD4⁺CD25⁺ suppressor cells or PBS were injected into the peritoneum (i.p.) of 7–9 wk old wild-type animals. Mice were given free access to 3% (wt/vol) 36–40 kD DSS (ICN Biochemicals) in deionized water 12–16 h after T cell injection. Weight loss was monitored daily for 9 d and animals were euthanized when they lost ~20% of their original body weight.

T reg transfer into Rag1^{-/-} mice. CD4⁺ T cells (isolated via CD4 [L3T4] microbeads and AutoMACS separation) from the spleen and lymph nodes of wild-type or Sin^{-/-} donors were stained with anti-CD4 and anti-CD25 antibodies. CD4⁺CD25⁻ and CD4⁺CD25⁺ cell populations were sorted on a FACS Aria. Sorted cells were mixed in the indicated combinations with 4×10^5 CD4⁺CD25⁻: 10^5 CD4⁺CD25⁺ cells or 4×10^5 CD4⁺CD25⁻ cells alone and injected into the tail vein of 5–6 wk old Rag1^{-/-} recipient mice. Weight was monitored weekly for 10 wk, and animals were euthanized when they lost ≥20% of their original body weight (Powrie et al., 1996).

Immunohistochemistry. Medullary area and islet numbers were calculated with the program ImageJ (National Institutes of Health; Abramoff et al., 2004) from 5 H&E-stained thymic sections cut from the center of the thymus over a 200-μm distance, discarding 50 μm between levels, from 5–6-wk-old Sin wild-type and knockout mice.

Acetone-fixed frozen thymic sections were incubated in 1% H₂O₂/PBS for 5 min, blocked in 10% egg white/0.5% BSA in PBS, washed in 0.05% Tween in PBS and incubated in 2 μg/ml UEA-1 conjugated to biotin (Vector Laboratories) or anti-Sin (rabbit polyclonal; Natarajan et al., 2006), -Aire (M-300; Santa Cruz Biotechnology, Inc.), -keratin-5 (AF138 Covance), -keratin-8 (TROMA-1), or MHC class II IA+IE biotin (M5/114.15.2; Abcam) antibodies for 2 h at room temperature. For precipitation reactions, slides were washed twice in 0.05% Tween/PBS and incubated with Vector AP (AK5000) reagent according to the manufacturer's instructions. For fluorescence imaging the anti-rat (H+L) Alexa Fluor 488, anti-rabbit (H+L) Alexa Fluor 488, or Alexa Fluor 594 (Invitrogen), and streptavidin-rhodamine (Jackson ImmunoResearch Laboratories) fluorescent antibodies were used. Images were acquired with an Axioplan II microscope (Carl Zeiss, Inc.).

RT-PCR and qPCR. For RT-PCR, RNA was purified from medullary and cortical TECs sorted on a FACS Aria by the gating strategy shown in Fig. 1 C. mRNA was purified using TRIzol reagent (Invitrogen) and random hexamer primers were used to generate a cDNA library with Superscript III cDNA synthesis kit (Invitrogen). Template cDNA was serially diluted 1:3 for PCR, and amplified fragments were separated on agarose gels for analysis. For qPCR, thymic epithelial cells from 5–6 wk old age-matched wild-type as described above. These percoll enriched fractions from single thymi were used as whole TEC samples (Fig. 7, B and C), whereas TECs enriched from multiple thymi (five or more) were stained for CD45, UEA, and MHCII, and mature CD45⁺UEA⁺MHCII^{hi} mTECs sorted on a FACS Aria (Fig. 7 D). qPCR was performed with QuantiTect SYBR Green PCR kit (QIAGEN) using specific primers (Table S1). Reactions were run on ABI Prism 7900HT for 45 cycles, T_m 55°C. Melting curves and C_t values were assessed with Applied Biosystems

SDS2.2.1 software. Analysis of relative gene expression normalized to GAPDH was performed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

In vivo KGF administration. For in vivo expansion of mTECs, age matched 4–5 wk-old $\text{Sin}^{+/+}$ and $\text{Sin}^{-/-}$ mice were injected i.p. with 2.5 mg/kg recombinant KGF (Amgen) in PBS every other day for 9 d, as previously described (Erickson et al., 2002; Rossi et al., 2007a). Thymic stromal cells from single thymi were isolated and stained as described above in mTEC purification, and mTEC populations were analyzed by FACS.

Statistical analysis. The two-tailed Student's *t* test was used to compare wild-type and knockout samples. For multiple comparisons, the analysis of variance test was used to resolve main effects within and between genotypes and treatment, with $P < 0.05$ considered significant. Post hoc Fisher PLSD was used for individual group comparisons.

Online supplemental material. Fig. S1 shows autoantibody reactivity against several tissues in sera collected from aged $\text{Sin}^{-/-}$ animals. Fig. S2 shows thymic renal transplants and data relating numbers of T cells in the spleen and lymph nodes, as well as inflammatory infiltrates in the kidney and liver. Fig. S3 shows that *Sin* deficiency does not hinder cTEC-mediated elimination of high-affinity T cells in the H-Y TCR transgenic model. Fig. S4 includes additional stains of thymic sections with UEA and keratins 5/8. Fig. S5 shows the analysis of total numbers of mTECs and cTECs using the pan-epithelial marker G8.8 and the cortical marker LY51 to stain thymic stroma cell suspensions. Fig. S6 shows percentage and total number of steady-state proliferating mTECs and cTECs assayed by BrdU incorporation. Primers used for qPCR and genotyping transgenic mice are listed in Table S1. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20092384/DC1>.

The authors would like to thank D. Mathis, and members of the Mathis-Benoit laboratory specifically, E. Venzani, and D. Gray for protocols and advice. We are grateful to Dr. W. Jenkinson for advice on thymic transplants, and K. Gordon (Columbia University FACS Core) for expert sorting. We would also like to thank C. Roman, B. Reizis, and S. Weisberg for critically reading the manuscript.

N.M. Danzl was supported by Columbia University's Immunology Program training grant T32 AI007525-10 and by grant R01 AI068963-01. Work was supported by National Institute of Allergy and Infectious Disease Grants R01 AI49387-01, R56 AI049387-05, and R01 AI068963-01.

The authors have no conflicting financial interests.

Submitted: 5 November 2009

Accepted: 9 March 2010

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