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Thymic Development of Autoreactive T Cells in NOD Mice Is Regulated in an Age-Dependent Manner

Qiuming He,* 1 Y. Maurice Morillon, II,* 1 Nicholas A. Spidale,* Charles J. Kroger,* Bo Liu, † R. Balfour Sartor,*† Bo Wang,* and Roland Tisch*‡

Inefficient thymic negative selection of self-specific T cells is associated with several autoimmune diseases, including type 1 diabetes. The factors that influence the efficacy of thymic negative selection, as well as the kinetics of thymic output of autoreactive T cells remain ill-defined. We investigated thymic production of β cell–specific T cells using a thymus-transplantation model. Thymi from different aged NOD mice, representing distinct stages of type 1 diabetes, were implanted into NOD.scid recipients, and the diabetogenicity of the resulting T cell pool was examined. Strikingly, the development of diabetes-inducing β cell–specific CD4+ and CD8+ T cells was regulated in a manner dependent on thymic age. NOD.scid recipients of newborn NOD thymi developed diabetes. However, recipients of thymi from 7- and 10-d-old NOD donor mice remained diabetes-free and exhibited a progressive decline in islet infiltration and β cell–specific CD4+ and CD8+ T cells. A similar temporal decrease in autoimmune infiltration was detected in some, but not all, tissues of recipient mice implanted with thymus from NOD mice lacking expression of the autoimmune regulator transcription factor, which develop multiorgan T cell–mediated autoimmunity. In contrast, recipients of 10 d or older thymi lacked diabetogenic T cells but developed severe colitis marked by increased effector T cells reactive to intestinal microbiota. These results demonstrate that thymic development of autoreactive T cells is limited to a narrow time window and occurs in a reciprocal manner compared with colonic microbiota–responsive T cells in NOD mice. The Journal of Immunology, 2013, 191: 5858–5866.

Events ongoing in the thymus play a critical role in shaping the repertoire of T cells (1, 2). Positive selection in the thymic cortex generates a pool of T cells restricted to self-MHC molecules. In contrast, negative selection in the medulla of the thymus ensures that thymocytes reactive to self-Ags are purged via induction of apoptosis or anergy (3, 4). Medullary thymic epithelial cells (mTECs) (5–7) and dendritic cells (DCs) (8–10) drive thymocyte negative selection by expressing and/or presenting self-Ag, respectively. A constellation of tissue-specific Ags (TSAs) is expressed by mTECs (5, 11), and expression of many of these TSAs is controlled by the autoimmune regulator (Aire) transcription factor (5, 12, 13). The parameters that influence the efficiency of thymic negative selection are ill-defined but are believed to include the avidity of the interaction of thymocytes with mTECs and DCs, intrinsic responses of thymocytes to apoptosis induction, and/or levels of thymic TSA expression and presentation (14–18).

It is not known whether thymic production of autoreactive T cells in general, and diabetogenic T cells specifically, is a continuous or time-limited process. The appearance of prevalent clones as autoimmunity progresses over time (33, 34) may reflect, for instance, continued thymic production of autoreactive T cell clones, albeit with distinct specificities (35). In contrast, studies using TCR-transgenic mice specific for thymus-expressed neo–self-Ags suggest that the efficiency of negative selection is reduced in younger animals (36, 37). Therefore, a window may exist early in life during which the development of autoreactive clones is enhanced and the pool of anti-self T cells is established. The latter has important implications for understanding the events that regulate thymic negative selection, in addition to establishing strategies to prevent T cell–mediated autoimmunity.
We investigated the ontogeny of autoreactive T cells using a thymus-transplant approach. Immunodeficient NOD.scid recipients were implanted with thymus grafts from different aged NOD donor mice, and the pathogenicity of the resulting T cell pool was assessed. In this study, we demonstrate that thymic production of organ-specific autoreactive Tcells is limited to a 10-d period after birth, indicating that the efficacy of thymic negative selection is regulated in a temporal manner.

**Materials and Methods**

**Mice**

NOD.LtJ, NOD.CB17-Prkdcscid/J (NOD.scid), and NOD.129S2(B6)-Aire<sup>tm1db1</sup>/DoI (NOD.Aire<sup>tm1db1</sup>) mice originally were purchased from The Jackson Laboratory (Bar Harbor, ME). NOD.CTgTcrtcrBDC2.5 D1Oo/DoI (NOD.BDC2.5) mice were described previously (38). NOD. BDC2.5 mice were bred with NOD.129P2(C)-Tcr<sup>tm1Mlo</sup>/DoI (NOD. Ct<sup>tm1Mlo</sup>) mice to generate NOD.BDC2.5.Ct<sup>tm1Mlo</sup> mice. All mice were bred and maintained in specific pathogen–free facilities at the University of North Carolina at Chapel Hill. Mouse experiments were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

**Thymus transplantation and disease assessment**

Thymic lobes from newborn (within 48 h of birth) and various aged female NOD or NOD.Aire<sup>tm1db1</sup> mice were implanted under the kidney capsule of 6-wk-old female NOD.scid mice. NOD.scid recipients were monitored for diabetes by measuring blood glucose weekly; blood glucose levels ≥ 250 mg/dl (Abbott Diabetes Care) for two consecutive measurements were scored as diabetic. The body weight of animals was measured weekly, and the development of weight loss was considered the clinical onset of colitis.

**Immunohistological analyses**

Various tissues were fixed in 10% neutral buffered formalin (Fisher Scientific) and embedded in paraffin, and nonoverlapping sections were prepared and stained with H&E or Alcian blue. Severity of insulitis and colitis was graded as previously described (39, 40).

For thymus immunostaining, thymi were frozen in O.C.T. compound (Sakura Finetek USA) and 7-μM sections were cut. Sections were fixed and permeabilized in ice-cold acetone/methanol for 5 min and then washed in PBS. Thymus sections were stained with UEA-1 biotin (Sigma-Aldrich) and Troma-1 (anti–cytokeratin-8; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), followed by Streptavidin*PE (eBioscience) and Alexa Fluor 488 goat anti-rat IgG (Invitrogen); each step was incubated for 1 h at room temperature. Montage thymus images were taken using a Zeiss Axioplan 2 microscope using a 10× objective and analyzed with Slidebook software (Intelligent Imaging Innovations).

**T cell analyses**

Cells isolated from the spleen, pancreatic lymph nodes (PLNs), mesenteric lymph nodes (MLNs), and colon were stimulated with PMA (50 ng/ml)/ionomycin (1 μg/ml) in complete RPMI 1640 medium at 37°C for 4–5 h, and brefeldin A was included in the culture for the last 2 h of incubation. Cells were washed and stained with Abs specific for CD4 (GK1.5), CD8 (53-67), CD3 (2C11), and TCRδ (H57). After fixation and permeabilization using the Fixation/Permeabilization kit (eBioscience), cells were stained with Abs specific for intracellular IL-17 (TC11-18H10) and IFN-γ (XM21.12). Foxp3-expressing T cells were stained using an anti-mouse Foxp3 staining kit, as per the manufacturer’s instructions (eBioscience). T cells were stained, as previously described (41), with in-house–prepared soluble IA<sup>7</sup> multimers covalently linked to BDC mimetic or hen egg lysozyme peptides or H2K<sup>b</sup> tetramers complexed with in-set–specific glucose-6-phosphatase catalytic subunit–related protein (IGRP) or influenza hemagglutinin peptides (42, 43). Data were acquired with a CyAn flow cytometer (DakoCytomation) and analyzed using FlowJo (TreeStar) or Summit (DakoCytomation) software.

For adoptive-transfer experiments, splenocytes were harvested from NOD.scid thymus recipients 6 wk posttransplantation, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells were purified by negative selection using mouse CD4 or CD8 T Cell Isolation kits (Miltenyi Biotec). At 6 wk of age, female NOD.scid mice were injected i.p. with 2 × 10<sup>6</sup> T cells/mouse and monitored for diabetes, body weight, and rectal prolapse. In some experiments, T cells were labeled prior to transfer with CellTrace Violet (Life Technologies), according to the manufacturer’s instructions.

**Results**

Development of diabetes is restricted to a narrow postnatal thymic age

Thymic structural organization differs with ontogeny; the newborn thymus is characterized by small islands of medullary tissue, whereas the medulla coalesces into a large, well-organized structure with age (Fig. 1A). To assess the ontogeny of β cell–specific T cells, NOD.scid mice were engrafted under the kidney capsule with thymi from newborn and older NOD female mice, which represent different stages of TID. Mature T cells were detected in the blood of thymus recipients as early as 1 wk postimplantation; by 4–6 wk, ~40% of mononuclear cells consisted of CD4<sup>+</sup> T cells in recipients of newborn and adult thymi (Fig. 1B). However, the reconstitution of CD8<sup>+</sup> T cells was delayed in adult- versus newborn-thymus recipients (Fig. 1B).

Overt diabetes was detected in all NOD.scid mice receiving newborn thymi, and islets exhibited significant insulitis, indicating T cell–mediated β cell destruction (Fig. 1C, 1D, Table I). Recipients of 7- and 10-d-old thymi remained diabetes-free; however, insulitis was detected, albeit at a reduced severity, relative to newborn thymus recipients (Fig. 1C, Table I). In contrast, NOD.scid recipients of thymi from 2-wk and older NOD donors failed to develop insulitis or diabetes (Fig. 1C, 1E, Table I). Similarly, the salivary gland and thyroid were infiltrated in recipients of newborn and 7-d thymi (Fig. 1I, 1K, Table I) but not 10-d or older thymi (Fig. 1J, IL, Table I).

Surprisingly, symptoms of colitis, such as weight loss (Fig. 1F), diarrhea, and rectal prolapse, were detected in NOD.scid mice implanted with 10-d and older thymi. Histologic examination of the gastrointestinal tract further revealed severe colitis (Fig. 1I, Table I) based on colonic hyperplasia, inflammation of the mucosal layer, significant infiltration of the lamina propria by mononuclear cells, and the depletion of goblet cells in the crypts (Fig. 1G, 1H). However, NOD.scid mice receiving newborn and 7-d thymi exhibited only limited colitis and no weight loss or rectal prolapse (Fig. 1F, Table I).

Adoptive-transfer experiments confirmed the organ-specific nature of pathogenic T cell populations developing in the thymus recipients. Splenocytes isolated from recipients of newborn and 7-d thymi readily transferred diabetes, but not colitis, to NOD.scid mice (Table II). In contrast, splenocytes from animals receiving 10-d or older thymi developed colitis but not diabetes (Table II). Together, these findings demonstrate that thymic development of diabetogenic and colitogenic T cells are reciprocally regulated in an age-dependent manner.

β cell–specific Th2 cells are increased in the PLNs of newborn-thymus recipients

FACS analyses demonstrated that the number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen, PLNs, and MLNs was increased ~2-fold and...
∼3-fold in recipients of newborn and 7-d-old thymi versus 10-d and older thymi, respectively (Fig. 2A). Furthermore, Teffs in these tissues (Fig. 2B), including the large intestine (Fig. 2C), consisted of IFN-γ+ and/or IL-17+ CD4+ T cells and IFN-γ+ CD8+ T cells, independent of thymus age. However, the frequency of IFN-γ+ and IL-17+ CD4+ T cells and IFN-γ+ CD8+ T cells was considerably

FIGURE 1. Reciprocal development of diabetes and colitis in NOD.scid recipients is dependent on the age of NOD donor thymus. (A) Cytokeratin-8 (green; cortex) and UEA-1 (red; medulla) immunostaining of sections from newborn, 7 d-old, 10 d-old, and 4 wk-old NOD thymi (original magnification ×100). (B) NOD.scid mice were engrafted with thymi from newborn (NB) or 12-wk-old (n = 10) NOD female donors. The frequency of CD4⁺ (left panel) and CD8⁺ (right panel) T cells was measured in peripheral blood. *p < 10⁻⁴. (C) Diabetes (left panel, *p < 10⁻⁴, versus recipients of 7-d or older thymi, Kaplan–Meier log-rank test) and the frequency of insulitis (right panel, *p < 10⁻⁴, Student t test, n = 8) in thymus recipients. Representative H&E staining of pancreatic sections of recipients of newborn (D) and 12-wk-old thymi (E). (F) Body weight (left panel, *p < 10⁻⁴, two-way ANOVA) and colitis scores (right panel, *p < 10⁻⁴, Student t test) of recipients (n = 8) of different-aged thymi and control NOD.scid littermates. Representative Alcian blue–stained colonic sections of recipients of newborn (G) and 12-wk-old (H) thymi. Representative H&E staining of thyroid (I, J) and salivary (K, L) glands from recipients of newborn (I, K) or 12-wk-old (J, L) thymus. Error bars represent SEM. Original magnification ×100 [(D), (E), and (G)–(L)].
CD4+ and CD8+ T cells that recognize a BDC mimetic peptide measured in the spleen, PLNs, and MLNs of newborn or 7- or 10-d recipients, albeit at significantly reduced levels (Fig. 3A), despite increased in cultures from animals receiving thymi from 10-d and 17 and IFN-γ secreted with CBL, and IL-17 and IFN-γ secretion was measured. IL-17 secretion in response to CBL was substantially increased in recipients of 10-d or older thymi versus animals receiving newborn or 7-d-old thymi (Fig. 2B).

The frequency of β cell–specific CD4+ and CD8+ T cells was measured in the spleen, PLNs, and MLNs of newborn or 7- or 10-d thymus recipients. IAβ7 and H2Kd multimers were used to detect CD4+ and CD8+ T cells that recognize a BDC mimic peptide (IAβ7-pBDC) and an IGRP peptide (H2Kd-IGRP), respectively. BDC-specific CD4+ T cells (45) and IGRP-specific CD8+ T cells (24) are prevalent diabetogenic clonotypes in NOD mice. The highest frequencies of CD4+ and CD8+ T cells staining with IAβ7-pBDC and H2Kd-IGRP, respectively, were detected in the PLNs of newborn-thymus recipients (Fig. 2D). Notably, the frequency of IAβ7-pBDC+ CD4+ T cells and H2Kd-IGRP+ CD8+ T cells progressively declined in the PLNs of 7-d and 10-d thymus recipients (Fig. 2D).

To examine temporal changes in Ag reactivity of colitogenic T cells, splenocytes prepared from thymus recipients were stimulated with CBL, and IL-17 and IFN-γ secretion was measured. IL-17 and IFN-γ secretion in response to CBL was substantially increased in cultures from animals receiving thymi from 10-d and older NOD donors (Fig. 3A). CBL also induced IL-17 and IFN-γ secretion in cultures prepared from newborn- and 7-d thymus recipients, albeit at significantly reduced levels (Fig. 3A), despite increased T cell numbers (Fig. 2A). CD4+ T cells alone from adult-thymus recipients were sufficient to transfer diabetes (Fig. 3B). The importance of T cell reactivity to colonic microbiota in the development of colitis was demonstrated further in NOD.scid recipients of thymi from 6-wk-old NOD.BDC2.5 versus NOD.BDC2.5.Cαnull donors. Severe colitis developed in NOD.scid recipients of NOD.BDC2.5 thymus (Fig. 3C), in which T cells expressed both the BDC2.5 clonotypic and endogenous TCR. In contrast, the severity of colitis was markedly reduced in NOD.scid recipients when the specificity of NOD.BDC2.5.Cαnull CD4+ T cells was restricted to the β cell autoantigen chromogranin A (46) (Fig. 3C). However, recipients of NOD.BDC2.5 (or NOD.BDC2.5.Cαnull) thyim developed diabetes (Fig. 3D), indicating that colitis per se did not block β cell autoimmunity. In sum, these results demonstrate that increased thymic development of β cell–specific T cells is restricted to a 7-d window after birth. Furthermore, development of colitogenic T cells specific for microbiota is significantly increased at, and maintained after, 10 d of age in NOD mice.

Thymus age-dependent development of diabetes is not due to changes in Foxp3+ Tregs and immunoregulation in the PLNs of recipients

The above data indicated that thymic development of β cell–specific T cells declines with age, resulting in reduced numbers of Teffs to mediate diabetes. However, lack of β cell autoimmunity in recipients of postnewborn thymi may also be due to a reciprocal increase in Foxp3+ Tregs residing in the PLNs to block expansion of diabetogenic Teffs. To distinguish between these two possibilities, the frequency of Foxp3+ Tregs was assessed in the spleen, PLNs, and MLNs of thymus recipients. Interestingly, PLN Foxp3+CD25+ CD4+ T cells were increased in recipients of newborn or 7-d thymus versus 10-d and older thyms, whereas the frequency of spleen and MLN resident Foxp3+CD25+CD4+ T cells was similar, independent of thymic age (Fig. 4A). To assess the immunoregulatory activity in the PLNs, NOD.BDC2.5 CD4+ T cells were transferred into recipients of newborn and 4-wk thymi, and proliferation was measured. No marked difference was detected in the level of NOD.BDC2.5 CD4+ T cell proliferation between the respective thymus recipients (Fig. 4B). These results indicate that the block in β cell autoimmunity in thymus recipients is not due to an increase in the Foxp3+ Treg pool or immunoregulation in the PLNs but can be attributed to reduced numbers of β cell Ag-specific T cells.

The temporal development of autoreactive T cells occurs in the absence of Aire expression

Regulation of TSA expression by Aire may contribute to the temporal thymic development of autoreactive T precursors. To test this possibility, the pathology of NOD.scid recipients transplanted with thymi from different-aged NOD mice deficient in Aire expression (NOD.Airenull) was investigated. NOD.Airenull mice lack β cell autoimmunity but develop multiorgan T cell–mediated inflammation (47), which includes exocrine pancreatitis. Tissues normally targeted in NOD.Airenull mice were also infiltrated in NOD.scid recipients of newborn NOD.Airenull thyim (Fig. 5A, Table III). Strikingly, a progressive decline in T cell infiltration of the exocrine pancreas and salivary glands was detected in recipients of 7- or 10-d-old NOD.Airenull thyim, whereas no infiltration of these tissues was observed in recipients of 4-wk-old thymus (Fig. 5A, Table III). In contrast, T cell infiltration continued to be detected in the eyes, ovaries, stomach, and lungs of animals implanted with 7-d and older NOD.Airenull thyim (Fig. 5). However, the severity of colitis in the recipients was limited, regardless of NOD.Airenull thyim age (Fig. 5B). These results demonstrate that temporal regulation of thymic development of autoreactive T cells can occur in the absence of Aire expression, albeit for certain tissue specificities and not others.

### Discussion

The dynamics of and the parameters that influence thymic output of autoreactive T cells are poorly understood. To address these issues, a thymus-transplant model system was used. This approach provides a snapshot of the specificities of autoreactive T cells produced in the thymus at a given age.

We demonstrate that thymic production of β cell–specific T cells is regulated in a temporal manner in NOD mice. Insulitis and diabetes developed in recipients of NOD newborn thyim (Fig. 1C,
1D), which corresponded with an increased frequency of PLN-resident pBDC-specific CD4+ and IGRP-specific CD8+ T cells (Fig. 2D), two major clonotypes associated with the progression of β cell autoimmunity in NOD mice. These results are consistent with findings demonstrating that NOD mice develop diabetes with normal kinetics and incidence despite thymectomy 3 d after birth, indicating that a sufficient pool of diabetogenic T cells is established early in ontogeny (48). Interestingly, autoimmunity was reported in immunodeficient children with congenital athymia receiving a human infant thymus transplant (49). Noteworthy was the progressive decline in insulitis (Fig. 1C) and the frequency of β cell–specific T cells in recipients of 7- and 10-d-old NOD thymi (Fig. 2D). This reduction in β cell–specific T cells was not due to a reciprocal increase in the pool of PLN-resident Foxp3+ Tregs or enhanced tissue-specific immunoregulation that would be expected to block the expansion of diabetogenic Teffs (Fig. 4). Furthermore, colitis per se had no suppressive effect on β cell autoimmunity. For instance, both diabetes and colitis were detected in NOD.scid mice receiving NOD.BDC2.5 thymi (Fig. 3C, 3D) or a mixture of splenocytes from colitogenic and diabetic donor animals (Supple-

**FIGURE 2.** Distinct T cell phenotypes and specificities detected in recipients of different-aged NOD donor thymi. The spleen (SPL), PLNs, and MLNs of different-aged NOD thymus recipients at 8 wk postimplantation and control 12-wk-old intact NOD female mice (NOD) were assessed via FACS for CD3+CD4+ and CD3+CD8+ T cell numbers ([A] n = 7, *p < 10−2, Student t test) and the frequency of intracellular IFN-γ– and IL-17–expressing CD4+ and CD8+ T cells ([B] n = 7, *p < 0.005, Student t test). (C) Representative FACS plots (left and middle panels) and frequency of IL-17–producing CD4+ T cells (right panel) isolated from the large intestines of recipients of newborn (NB) and 12-wk-old thymus; gated on CD3+CD4+ T cells (n = 7). *p < 0.05, Student t test. (D) The frequency of IAα7-pBDC–binding CD4+ T cells and H2Kd-IGRP–binding CD8+ T cells with representative FACS plots from PLN-isolated cells gated on CD3+ and CD4+ or CD8+ T cells, respectively (n = 5). Error bars represent SEM. *p < 0.05, Student t test.
NOD.BDC2.5 and NOD.BDC2.5C were used to study the progression of autoimmune diseases in the context of negative selection. The results indicated that the progression of the two pathologies is independent and not mutually exclusive. Together, these findings suggest that the lack of insulitis and diabetes and the reduced frequency of diabetogenic T cells in recipients of postnewborn thymi is due to inefficient thymic negative selection of autoreactive T cells in the neonatal thymus. Indeed, both the frequency of Aire-expressing mTECs and mRNA expression of Aire-dependent TSA genes, such as Ins2, are reduced in thymi from newborn NOD mice compared with older NOD mice. To further investigate the role of Aire, recipients of newborn and older NOD.Aire null thymi developed significant infiltration of the ovaries, stomach, lungs, and eyes (Fig. 5, Table III) supports a role for Aire in the temporal development of these tissue-specific T cells. Strikingly, however, exocrine pancreatitis and sialadenitis failed to develop in recipients of thymi from 10-d or older NOD.Aire null donors. Our observation that recipients implanted with newborn and older NOD.Aire null thymi developed significant infiltration of the ovaries, stomach, lungs, and eyes (Fig. 5, Table III) supports a role for Aire in the temporal development of these tissue-specific T cells. Strikingly, however, exocrine pancreatitis and sialadenitis failed to develop in recipients of thymi from 10-d or older NOD.Aire null donors. Our observation that recipients implanted with newborn and older NOD.Aire null thymi developed significant infiltration of the ovaries, stomach, lungs, and eyes (Fig. 5, Table III) suggests that Aire-dependent TSA expression alone does not account for the observed temporal production of autoreactive T cells specific for these tissues. Age-dependent changes in the stimulatory capacity of the thymic APC pool, due to the number, composition, and maturation status of mTECs and thymic DCs, may contribute to the efficiency of thymic negative selection. Alternatively, the development of autoreactive T cells may reflect intrinsic properties of T cell progenitors residing in the thymus during ontogeny. For example, studies showed that hematopoietic stem cells that seed the thymus at various stages of ontogeny give rise to T cells with distinct properties and Ag specificity. The latter may influence the affinity and/or cross-reactivity or promiscuity of TCRs specific for TSA-derived epitopes that are either Aire dependent or independent. Finally, major changes in the structural organization of the medulla seen during postnatal life may impact the efficiency of negative selection. The rudimentary thymus of newborn animals (Fig. 1A) may limit thymocyte interactions with exclusive possibilities may account for this effect. Reduced expression of Aire due to limiting Aire expression may lead to inefficient thymic negative selection of autoreactive T cells in the neonatal thymus. Indeed, both the frequency of Aire-expressing mTECs and mRNA expression of Aire-dependent TSA genes, such as Ins2, are reduced in thymi from newborn NOD mice compared with older NOD mice. Our observation that recipients implanted with newborn and older NOD.Aire null thymi developed significant infiltration of the ovaries, stomach, lungs, and eyes (Fig. 5, Table III) supports a role for Aire in the temporal development of these tissue-specific T cells. Strikingly, however, exocrine pancreatitis and sialadenitis failed to develop in recipients of thymi from 10-d or older NOD.Aire null donors. 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Alternatively, the development of autoreactive T cells may reflect intrinsic properties of T cell progenitors residing in the thymus during ontogeny. For example, studies showed that hematopoietic stem cells that seed the thymus at various stages of ontogeny give rise to T cells with distinct properties and Ag specificity. The latter may influence the affinity and/or cross-reactivity or promiscuity of TCRs specific for TSA-derived epitopes that are either Aire dependent or independent. Finally, major changes in the structural organization of the medulla seen during postnatal life may impact the efficiency of negative selection. The rudimentary thymus of newborn animals (Fig. 1A) may limit thymocyte interactions with
medullary-resident APCs, thereby reducing the efficiency of negative selection, particularly if given TSAs are expressed and presented at relatively low levels. Efforts are ongoing to delineate what is likely to be a complex interplay between multiple events that regulate the temporal efficiency of thymic negative selection.

An interesting observation made in this study was that thymic development of colitogenic T cells was also temporally regulated. Negligible colitis was detected in recipients of newborn or 7-d NOD thymi (Fig. 1F). However, severe colitis developed in recipients of thymi from NOD donors 10 d of age and older (Fig. 1F), which was marked by an increased number and frequency of IL-17– and IFN-γ–producing CD4+ T cells specific for CBL (Fig. 2B). Unlike β cell–specific T cells, which were selectively increased in the PLNs (Fig. 2D), CBL reactivity was readily detected in all tissues examined (Fig. 3A), likely reflecting systemic trafficking of a relatively large pool of colitogenic Teffs. Recognition of commensal microbiota Ag was necessary for colitis; recipients of thymi from NOD.BDC2.5.Cαnull donors, which express chromogranin A–specific TCR (46), failed to develop significant colitis (Fig. 3B). In contrast, increased colitis was detected in animals receiving thymi from adult NOD.BDC2.5 mice, which coexpress transgenic and endogenous TCR (Fig. 3B). These findings demonstrate that, in addition to autoreactive T cells, the development of T cells specific for exogenous (e.g., microbial) Ags is regulated temporally but in a reciprocal relationship to autoimmune T cells. In this instance, production of T cells specific for microbial Ags is enhanced after postnatal life, suggesting an increase in the efficiency of thymocyte positive selection. In addition, colitis was detected in NOD.scid recipients of thymi from 12-wk-old C57BL/6 mice congenic for H2<sup>b</sup> (Supplemental Fig. 2), suggesting that thymic development of colitogenic T cells is independent of the NOD genotype. These results further support the prevailing concept that chronic immune-mediated colitis is driven by microbial-responsive T cells rather than autoimmune responses (57).

Because T cell reconstitution occurred under identical conditions in NOD.scid recipients, lymphopenic expansion cannot explain the temporal development of autoreactive and colitogenic T cell repertoires. However, it is likely that lymphopenia favored the differentiation of pathogenic Teffs driving autoimmunity and colitis. Development of autoreactive T cells, which is largely restricted to early ontogeny, further underscores the role of peripheral mechanisms in maintaining life-long self-tolerance. Our findings also may explain, in part, the long-lasting and robust tolerance typically induced by administration of self-Ag to neonates (58–60). In this case, deletion early in ontogeny would be expected to permanently purge the corresponding autoreactive clonotype(s) from the immune system.

In conclusion, our results demonstrate that thymic development of T cells specific for self- and foreign Ags is tightly regulated over a short ontogenic window. These findings also indicate that the pool of β cell (and other tissue)-specific T cells is, to a large extent, established early in ontogeny. A number of coordinated events within the thymus is likely to contribute to the temporal development of autoreactive and bacterial Ag–responsive T cells in a reciprocal manner. Exploiting the use of the thymus-transplant model provides a novel approach to better define these events.

Table III. Relative T cell infiltration of organs in NOD.scid recipients of different-aged NOD.Aire<sup>null</sup> thymi

<table>
<thead>
<tr>
<th>Organ</th>
<th>NB Aire&lt;sup&gt;null&lt;/sup&gt;</th>
<th>7-d Aire&lt;sup&gt;null&lt;/sup&gt;</th>
<th>10-d Aire&lt;sup&gt;null&lt;/sup&gt;</th>
<th>4-wk Aire&lt;sup&gt;null&lt;/sup&gt;</th>
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<tr>
<td>Pancreas</td>
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<td>+</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>Ovary</td>
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<tr>
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<td>Lung</td>
<td>+</td>
<td>Not observed</td>
<td>Not observed</td>
<td>+</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>++</td>
<td>+</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Small intestines</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Eyes</td>
<td>+++</td>
<td>Not observed</td>
<td>Not observed</td>
<td>+++</td>
</tr>
</tbody>
</table>

+++ Heavy/complete T cell infiltration; ++, moderate T cell infiltration; +, mild T cell infiltration.

FIGURE 5. Temporal development of autoreactive T cells is independent of Aire in some organs. NOD.scid mice received thymi from different-aged NOD.Aire<sup>null</sup> donors. (A) Representative H&E-stained sections of organs; arrowheads highlight areas of infiltration. Original magnification ×40. (B) Colitis scores of different-aged NOD.Aire<sup>null</sup> thymus recipients (n = 5). Error bars represent SEM.
Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figures

Fig. S1. Diabetes development is unaffected by colitogenic T cells.

Diabetes incidence (left) and colitis scores (right) for NOD.scid recipients of splenocytes (10^7) from diabetic NOD donors alone (n=3), or mixtures of diabetic splenocytes (10^7) plus splenocytes (10^7) from newborn (NB; n=5) or 4 wk (n=4) thymus transplant recipients (*p<0.02, 4wk thymus+diabetic splenocytes versus newborn thymus+diabetic splenocytes and diabetic splenocytes alone; Student’s t test). Error bars represent SEM.

Fig. S2. Organ infiltration in NOD.scid recipients of 12 wk-old B6g7 thymi.

Representative H&E stained sections from 12 wk-old WT B6g7 (right column) and NOD.scid recipients of 12 wk-old B6g7 thymi (left column) 6 wks post engraftment. Arrows highlight areas of infiltration.
Fig. S2: