THYMIC DEVELOPMENT OF AUTOREACTIVE T CELLS IN NOD MICE, AND THE ALTERED TRAFFICKING OF T CELLS FOLLOWING ANTIBODY MEDIATED CROSSLINKING OF THE CD4 CORECEPTOR

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ABSTRACT

Yves Maurice Morillon II: Thymic development of autoreactive T cells in NOD mice, and the altered trafficking of T cells following antibody mediated crosslinking of the CD4 coreceptor
(Under the direction of Roland Tisch)

Two key processes in driving type 1 diabetes are the development and trafficking of β cell-specific T cells into the pancreas. The first part of this dissertation examines thymic development of autoreactive T cells, whereas the second part explores novel T cell trafficking pathways upon antibody binding to CD4.

The production of autoreactive T cells occurs through a failure of negative selection in the thymus. We utilized a thymic transplant approach to investigate possible ontogenic changes in autoreactive T cell production. Various aged NOD thymi were transplanted into NOD.*scid* recipients, which were then assessed for pathology. The development of diabetes was restricted to recipients of newborn thymi, insulitis was observed in recipients of 7 and 10 day, but not older thymi. Increased diabetes and insulitis was associated with increased diabetogenic T effectors and not with altered regulatory T cell frequency or activity. In contrast, recipients of older thymi developed colitis, characterized by IFNγ and IL-17 producing T cells reactive to gut microbiota. These findings demonstrate that thymic development of autoreactive T cells is restricted to a narrow window, and that the efficacy of thymic negative and positive selection increases with age in mice.

T cell migration is a highly controlled process of adaptive immunity. Defects in trafficking can result in inappropriate immune responses, including autoimmunity. We

recently reported diabetes remission in NOD mice following administration of anti-CD4 and anti-CD8 antibodies. Remission was associated with T cell purging from the pancreas and pancreatic lymph nodes. In this study, we investigated the mechanisms of purging following anti-CD4 treatment. Shortly after anti-CD4 treatment, the activity of the GTPases Rac1 and Rac2 were markedly increased. Notably, Rac1 and Rac2 function are associated with T cell migration. In addition, T cell polarization was increased, and expression of lymph node homing adhesion molecules were decreased, concomitant with T cell egress from the pancreatic lymph nodes. Furthermore pancreatic lymph node but not splenic T cells isolated from anti-CD4 treated mice exhibited increased *in vitro* chemotaxis to tested chemokines. These findings demonstrate that crosslinking of CD4 induces a pro-migratory phenotype via a novel Rac associated pathway in T cells.

DEDICATION

This dissertation is dedicated to my wife, Dr. Elizabeth Lessey-Morillon, and my parents, Yves and Fay Morillon. Without the love and support of my family, none of this work would have been possible.

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ABBREVIATIONS

AIRE	autoimmune regulator
APC	antigen presenting cells
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
CBL	cecal bacterial lysate
CKR	chemokine receptor
cTEC	cortical thymic epithelial cell
DC	dendritic cell
DN	double negative
DP	double positive
$Foxp3^+$ Treg	Foxp3-expressing immunoregulatory $CD4^+$ T cell
GEF	guanine nucleotide exchange factor
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
ICAM-1	inter-cellular adhesion molecule-1
IGRP	islet-specific glucose-6-phosphatase catalytic subunit–related protein
IS	immunological synapse
Lck	lymphocyte-specific protein tyrosine kinase
LFA-1	leukocyte function-associated molecule-1
Mac-1	macrophage-1 antigen
MHC	major histocompatibility complex
MLN	mesenteric lymph node
mTEC	medullary thymic epithelial cells

NOD	nonobese diabetic
PI3K	Phosphoinositide 3-kinase
PLN	pancreatic lymph node
RAG	recombinase activating gene
SP	single positive
T1D	type 1 diabetes
TCR	T cell receptor
Teff	effector T cell
TSA	tissue-specific antigen
VCAM-1	vascular adhesion molecule-1
WASP	Wiskott-Aldrich syndrome protein

Chapter 1: Background and Significance

Etiology of Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin producing β cells located in the islets of Langerhans of the pancreas (1, 2). Clinical disease, as defined by an inability to regulate blood glucose levels, develops once approximately 85-90% of β cell mass has been destroyed or rendered nonfunctional. The initiating events in the development of T1D are unknown; however, both genetic and epigenetic/environmental factors play a role. Susceptibility to T1D is associated with a number of genes (3) with greatest risk in humans linked to genes encoding specific alleles of human leukocyte antigen class II molecules (4). Ill-defined environmental factors also play a role, which is most popularly described using the "Hygiene hypothesis", where "cleaner" environments and better hygiene are associated with increased incidence of T1D, as well as a reduction in disease in less developed and less hygienic regions (5).

T1D is mediated by several immune effector cell types (6-8). Experimentally, nonobese diabetic (NOD) mice lacking either CD4+ or CD8+ T cells fail to develop spontaneous T1D (6). Indirect evidence also suggests a key role for CD4+ and CD8+ T cells in human T1D; for instance both cell types are found infiltrating the islets of pancreata from T1D patients, with CD8+ T cells being the most abundant (7).

In order for autoreactive T cells to mediate pathogenic activity, they must first be primed via their interaction with antigen presenting cells (APC), such as B cells,

macrophages, and dendritic cells (DC). Macrophages, DC, and B cells are among the first adaptive immune cells to enter the islets (8). The importance of B cells in T1D pathogenesis is evident in NOD μMT-/- mice, which lack B cells, and do not develop diabetes (9). Certain B cell subsets are suggested to be important in disease development, although studies have correlated increased subset presence with onset of disease (10), no studies report an absolute requirement for a particular subset. Although β cell-specific autoantibodies are detected and remain an indicator of T1D in both mice and humans, the necessity of autoantibodies in disease progression has yet to be demonstrated. It is therefore believed that B cells serve as a critical APC (11), directing autoantigen presentation through recognition of a self-reactive monoclonal B cell receptor. This idea is supported by depleting B cells in NOD mice using an anti-CD20 therapy (12). Transiently depleting B cells significantly delays or protects from diabetes onset.

Ultimately, it is autoreactive T cells, which have escaped mechanisms of both central and peripheral tolerance which carry out the final destruction of β cells. Investigations have described a genetic defect in negative selection of autoreactive T cells in NOD animals (13, 14). Thymocytes in NOD versus non-autoimmune strains of mice have been shown to be more resistant to apoptotic signals during negative selection (13, 15). Although we will provide a more in depth description below, briefly, thymic selection is comprised of positive and negative selection events (16). Positive selection insures that mature T cells are able to interact with self major histocompatibility complex (MHC) molecules, whereas negative selection results in the elimination of autoreactive T cells but can also lead to the generation of Foxp3 expressing CD4+ regulatory T cells (Foxp3+Treg). In a non-autoimmune prone environment, some degree of autoreactive T cell escape occurs, however, mechanisms of

peripheral tolerance, in part mediated by CD4+ Treg, prevent the development of a pathological autoimmune response. A reduction in Treg frequency and/or function is believed to result in preferential differentiation and expansion of pathogenic β cell-specific CD4+ and CD8+ T cells, typically exhibiting a type 1 phenotype marked by IFNγ secretion (17).

In the event that autoimmune T cells escape mechanisms of central and peripheral tolerance, they must be primed by autoantigen expressing APC. Once activated, T cells exit the lymph nodes and enter into the target tissue. In the case of T1D, the draining pancreatic lymph nodes (PLN) are the key site for β cell-specific T cell activation, expansion and differentiation prior to trafficking into the pancreas (18). Once in the islets, established T effectors encounter cognate antigen to drive β cell destruction.

This thesis will concentrate on two distinct aspects of T1D. In Chapter 2, we focus on central tolerance and provide evidence that the efficacy of thymic negative selection is regulated in a temporal manner. In Chapter 3, we investigate the mechanisms by which an antibody-mediated T cell coreceptor-based immunotherapy affects the trafficking properties of β cell-specific T cells.

Thymic Selection and Central Tolerance

Thymic development of thymocytes entails a number of selection events that results in the production of mature T cells that mediate protection against pathogens, and which do not elicit a pathological response to self-antigens. Hematopoietic-derived T cell precursors arrive in the thymus lacking expression of the T cell receptor (TCR) and the CD4 and CD8 coreceptor molecules (19). These double negative (DN) thymocytes interact with cortical thymic epithelial cells (cTEC) resulting in upregulation of the recombinase activating gene

(RAG) and expression of a functional in frame TCR variable (V)β chain (20). Only DN thymocytes that successfully produce a pre-TCR complex, a Vβ chain with an invariant surrogate Vα chain, continue on to the double positive (DP) thymocyte stage (21, 22). DP thymocytes must first rearrange a functional Vα chain in order to express an αβ TCR. DP thymocytes also characteristically express both CD4 and CD8. It is at this stage, that DP thymocytes undergo positive selection in which survival relies on the successful TCR recognition of MHC class I and II expressed by cTEC. In the absence of TCR signals generated upon appropriate MHC class I and II binding, DP thymocytes undergo apoptosis by "neglect". Attrition is high since only 3-5% of thymocytes successfully progress past the DP stage (23).

Successful completion of positive selection results in migration from the thymic cortex to the medulla, where DP thymocytes mature to become single positive (SP) thymocytes, expressing either the CD4 or CD8 receptor. SP thymocytes undergo negative selection via interactions with medullary thymic epithelial cells (mTEC) and thymic DC. Notably, mTEC via the autoimmune regulator (AIRE) transcription factor express a multitude of self-antigens normally expressed in the periphery (24). A pivotal role for AIRE was demonstrated in mice deficient of the transcription factor. $AIRE^{null}$ mice develop a T cell mediated systemic multi-organ autoimmune disease. Notably, similar pathology is seen in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (25, 26) which is marked by mutations in the AIRE gene. Not all self-antigens are presented by mTEC in an AIRE-dependent manner. For instance "migratory" DC trafficking into the thymic medulla can present self-antigens that had earlier been endocytosed in the periphery (27, 28). Thymic DC may also capture blood borne self-antigens.

Thymic negative selection relies on the affinity/avidity of TCR recognition of the self-antigen peptide-MHC complex; increased binding promotes apoptosis and purging of the TCR repertoire (29). Alternatively, interactions with increased affinity/avidity may promote development of Foxp3+Treg (30). Thymocytes continually interact with self-expressing APC over a period of up to 12 days (31), which ensures efficient elimination of autoreactive T cells prior to entering the periphery. The process of thymic selection is estimated to take 16-19 days. As efficient and stringent as thymic selection is, some autoreactive T cells escape, and in the absence of sufficient peripheral tolerance mechanisms, autoimmune disease can result.

Thymocytes require interactions with both cTEC and mTEC in order to survive and develop into T cells, however, the thymic stroma also requires thymocyte interactions in order to mature and develop (32-34). Mice deficient in the ability of thymocytes to progress through selection also exhibit reduced thymic cortical or medullary mass.

Age Dependent Changes in Central Tolerance

During development of both humans and mice, thymus maturation is associated with structural reorganization of the cortex and medulla coupled with an increase in size due to an expanding pool of thymic stroma, APC and thymocytes. In humans, the thymus is estimated to continue to develop until the teen aged years, at which time thymic involution begins (35). A similar phenomenon is observed in rodents, where development continues until 4 weeks of age, after which involution is observed (36). Ontogenic changes in thymic structure may impact the efficacy of positive and negative selection events.

Other age related events may also play a role in the efficiency of thymic selection. A temporal role for AIRE was described, where AIRE is required to prevent autoimmunity in

NOD mice early in ontogeny, after 3 weeks of age AIRE was dispensable in preventing the development of systemic autoimmunity (37).

Another ontogenically regulated key event that may influence the efficacy of thymic selection is the origin of thymocyte precursors. During embryonic development in mice, T cell precursors are derived from the liver up until embryonic day 13 (38, 39) followed by a switch to bone marrow derived precursors by embryonic day 15. Key phenotypic differences exist between thymocytes derived from embryonic liver versus adult bone marrow. Thymocytes derived during embryonic development are hyperproliferative (40-42) whereas adult thymocytes exhibit little proliferative ability (43). Embryonic precursors give rise to longer lasting thymocytes when compared to those derived from bone marrow (42, 44). In addition, embryonic precursors have been shown to utilize Vβ6 as well as Vβ11 in much higher frequency than their adult counterparts (45, 46). Although an investigation into particular specificities has not been completed, disparate usage of particular Vβ chains based on precursor origin is suggestive of differences in specificity. Taken together, it is plausible that a functional difference exists in thymic selection early versus later in ontogeny.

Onset of Type 1 Diabetes Primarily Occurs in Younger Individuals

T1D has been commonly referred to as "juvenile diabetes", implying that disease onset primarily occurs in young individuals. Evidence for a skewing towards early onset was provided in an epidemiologic study of European populations which described disease onset in humans primarily occurring prior to 14 years of age (47). Early development of disease is mirrored in the NOD mouse, where insulitis typically begins at 3 weeks of age, progressing to clinical onset between 15 and 25 weeks of age. Evidence of early production of autoreactive T cells can also be observed by the minimal impact on disease incidence in

NOD mice thymectomized at day 3 post birth (48, 49). Suggesting a sufficient number of autoreactive T cells have been produced by 3 days of age for disease to develop.

Early disease development provides support that autoreactive T cells are produced early in ontogeny. However, the question remains if autoreactive T cells are continuously produced at the same frequency throughout life. In view of the various age-dependent changes in the thymus and thymocyte precursors, an intriguing hypothesis is that early in ontogeny, autoreactive T cells more readily escape negative selection. The implications of a narrow developmental window of autoreactive T cell production would provide a temporal target to intervene in the prevention of T1D.

Insight into Basic T Cell Biology By Manipulating T1D Via Immunotherapy

Identifying therapeutic targets is paramount in preventing and treating T1D. The body of knowledge currently available provides us with a large source of potential targets, however, basic knowledge in the mechanisms behind T cell production and activity is still lacking. One of the defining characteristics of T cells is their ability to rapidly circulate through the body, searching for cognate antigen and to respond as needed. However, regulation of T cell circulation in the absence of an inflammatory environment is still not fully understood. A novel approach to combating autoimmune disease would be one where pathogenic T cells are rendered unresponsive to inflammatory cues to block tissue pathology.

We recently reported that nondepleting anti-CD4 (YTS177) and anti-CD8 (YTS105) antibodies induce long-term remission in newly diabetic NOD mice (50). Notably, distinct mechanisms of induction and maintenance of remission are established by coreceptor therapy. The non-depleting antibody immunotherapy is cleared from recipients within 45 days post administration; however, β cell tolerance is maintained. We have demonstrated that

Foxp3+ Treg isolated from maintenance phase long term remission animals are able to suppress the adoptive transfer of diabetes. *In vitro* models have also demonstrated an increased suppressive ability of Foxp3+Treg isolated from long term (>100 days) treated vs untreated NOD mice. For the purpose of this thesis, we will concentrate on the effect of only the anti-CD4 (YTS177) immunotherapy during the induction phase of remission, which is characterized by T cell purging from the pancreas and PLN. While YTS177 alone is not capable of inducing diabetes remission, it is capable of causing CD4+ T cell migration from the pancreas and peripheral lymph nodes.

T Cell Migration From Blood to Lymphoid Tissue

T cell migration is a dynamic process where environmental cues signal T cells to phenotypically change to promote directed and purposeful movement. T cells are among the most migratory cells in the body (51), continually circulating from blood to the lymphatics (52), termed homing. Circulating T cells have a circular morphology with even distribution of chemokine receptors (CKR) and adhesion molecules. In response to chemokines or vascular expressed adhesion molecules, T cells begin to slow, roll, and tether to endothelial surfaces (53). This is primarily accomplished through the interactions of CD62L binding to its respective ligand glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) (or other sialylated ligands) (54). Firm adhesion ultimately halts circulating T cells via the interaction of leukocyte function-associated molecule-1 (LFA-1) or $\alpha 4\beta 1$ with their respective ligands inter-cellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) (55). T cell migration is accomplished via polarization, which is characterized by the formation of a leading edge (lamellipodium) and a trailing uropod (56). The leading edge becomes enriched with CKR which assist in providing directional cues for

the T cell. The leading edge also becomes enriched with polymerizing F-actin, creating cellular protrusions which serve to pull the cell forward, allowing for transendothelial migration. The uropod becomes enriched with a variety of adhesion molecules such as CD43, CD44, ICAM-1 and others (57). The function of the uropod is still unclear, however it has been suggested that it may sequester adhesion molecules not required for transendothelial migration (58).

T cells enter lymphoid organs by crossing high endothelial venules (53). Following lymph node entry, T cells "sample" or "scan" MHC presented antigen by APC searching for recognition of their cognate antigen, this process is regulated by the interaction of LFA-1 on T cells and ICAM-1 on APC (59). When antigen recognition occurs, "sampling" turns to firm adhesion via the generation of the immunological synapse (IS) (60). In the absence of antigen recognition, T cells disengage from their initial contact and either continue to sample MHC presented antigen or egress from the lymphoid tissue to return to circulation. The molecular events leading to lymph node egress are not fully understood; however, T cells must again polarize and cross endothelial barriers in order to return to circulation (61).

Intracellular Events Leading to T Cell Migration

Migration of T cells through circulation and lymphatics is controlled by signaling which originates from environmental cues, such as activation, chemokine, or adhesion signals. The most widely studied stimulus has been that derived from the activation of the TCR signaling cascade. Upon TCR recognition of cognate antigen, T cells form an IS with APC (60, 62). The association of the T cell:APC interaction is regulated by adhesion molecules, primarily LFA-1 and ICAM-1, on T cells and APC, respectively (59). CD4 or CD8 coreceptor engagement is important in regulating the strength and duration of the TCR

signal. Upon binding of CD4 to conserved regions of the MHC class II molecule expressed by APC, the Src family tyrosine kinase, lymphocyte-specific protein tyrosine kinase (Lck), becomes active via phosphorylation at tyrosine 394 (63). Having adopted kinase potential, p394 Lck is then able to phosphorylate ZAP-70 (64) which in turn acts as a kinase in the phosphorylation of the guanine nucleotide exchange factor (GEF), Vav (65). GEFs have the ability to activate small Rho GTPases, such as Rac, by facilitating the exchange of GDP for GTP. A limited number of GEFs have been described in the context of T cell activation and migration including: Vav, Tiam1, Dock2, and Sos (66, 67). The utilization of different GEFs in the activation of Rac has been associated with distinct phenotypic activities (66). Rac activation through the activity of Dock2 or Tiam1 has been associated with lymph node egress (68-70). Vav1 activation of Rac has been linked to T cell activation (71), although evidence also exists showing Vav regulation of cytoskeletal reorganization, suggesting a role in polarization and migration (72, 73). A role for Sos has been more difficult to determine, as Sos1 knockout mice exhibit embryonic lethality (74).

Although three isoforms of Rac have been described: Rac1, Rac2, and Rac3; only the first two have been described in the context of T cell activity. While Rac1 is ubiquitously expressed in most cell types, Rac2 is restricted to mainly T, B, and myeloid hemopoietic cells (75-77). Each isoform has been suggested to control different aspects of T cell function. Rac1 is known to control lamellipodium formation, a branch actin network used to create forward motility (78). Rac1 is also implicated in cytoskeletal rearrangements leading to lipid raft formation and clustering (79). Most Rac1 functional studies have been performed either in cell culture or in the context of overexpression or constitutive activation, due to the fact that Rac1 knockout mice are not viable (80); Rac2 knockout mice have, however, been

established (81). T cells from Rac2 knockout animals exhibit defects in polarization and actin polymerization, as well defects in downstream TCR signaling (82). The common theme of both isoforms of Rac is control of the cytoskeleton and as such, polarization and migration. In short, Rac is the molecular switch, toggling T cells between a stationary and migratory state.

Downstream effector molecules of Rac activation can vary, however. Control of the cytoskeleton is accomplished mainly through an association with the Wiskott-Aldrich syndrome protein (WASP) (83). WASP knockout mice exhibit a phenotype very similar to those of certain GEF knockouts, where T cells have a defect in activation and polarization (84). Activation defects can be traced back to an inability to modulate the cytoskeleton in response to activation cues, for instance, blocking upstream Rac activation results in a failure of PKC-φ to localize to the IS during TCR priming, and as such prevents downstream TCR signaling (73).

In this dissertation, we examine two processes involved in T1D pathogensis: 1) the identification of a narrow age window in which autoreactive T cells are produced, which ultimately leads to the development of autoimmune disease, and 2) the identification of a novel TCR independent signaling pathway resulting in T cell migration away from the pancreas and pancreatic lymph nodes, which is activated following antibody mediated crosslinking of the CD4 molecule.

Chapter 2: Thymic Development Of Autoreactive T Cells In NOD Mice Is Regulated In An Age-Dependent Manner[1]

Introduction

Events ongoing in the thymus play a critical role in shaping the repertoire of T cells (85, 86). Positive selection in the thymic cortex generates a pool of T cells restricted to self-MHC molecules. On the other hand, negative selection in the medulla of the thymus ensures that thymocytes reactive to self-antigens are purged via induction of apoptosis or anergy (14, 87). mTEC (88-90) and dendritic cells (DC) (27, 28, 91) drive thymocyte negative selection by expressing and/or presenting self-antigens, respectively. A constellation of tissue-specific antigens (TSA) is expressed by mTEC (88, 92) and expression of many of these TSA is controlled by the Aire transcription factor (88, 93, 94). 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 mTEC and DC, intrinsic responses of thymocytes to apoptosis induction, and/or levels of thymic TSA expression and presentation (24, 95-98).

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Inefficient thymic negative selection has been associated with various T cell-mediated autoimmune diseases such as T1D (13, 14, 99). T1D in humans and rodent models, such as the NOD mouse, is characterized by the $CD4^+$ and $CD8^+$ T cell-mediated destruction of the insulin-producing β cells residing in the pancreatic islets of Langerhans (2). In NOD mice the diabetogenic response involves progressive insulitis in which T cells and other immune effectors infiltrate the islets over time. Insulitis is first detected at 3-4 wk of age and relatively few β cell autoantigens and epitopes are targeted by $CD4^+$ and $CD8^+$ T cells (100-103). By 12 wk of age, a late preclinical stage of T1D, the islets in NOD mice are heavily infiltrated, marked by effector T cells (Teff) targeting numerous β cell autoantigens and epitopes. Aberrant survival of islet resident $Foxp3^+$Treg is then believed to promote a wave of robust β cell destruction and the onset of overt diabetes (17, 104). NOD mice also exhibit T cell autoimmunity to other tissues such as the thyroid (105, 106) and salivary gland (107), and low levels of colitis (108, 109) are detected suggesting general defects in mechanisms regulating autoimmune and inflammatory responses, respectively.

Currently, it is not known whether thymic production of autoreactive T cells in general, and diabetogenic T cells specifically, is a continuous versus time-limited process. The appearance of prevalent clones as autoimmunity progresses over time (110, 111) may for instance, reflect continued thymic production of autoreactive T cell clones albeit with distinct specificities (112). On the other hand, studies employing TCR transgenic mice specific for thymus-expressed neo-self antigens suggest that the efficiency of negative selection is reduced in younger animals (113, 114). A "window" may therefore exist early in life during which the development of autoreactive clones is enhanced, and the pool of anti-self T cells 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 assessed. Here we demonstrate that thymic production of organ-specific autoreactive Teff 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-Prkdc^{scid}/J (NOD.*scid*), and NOD.129S2(B6)-Aire$^{tm1.1Doi}$/DoiJ (NOD.Aire^{null}) were originally purchased from The Jackson Laboratory (Bar Harbor). NOD.Cg-Tg(TcrαTcrβBDC2.5)1Doi/DoiJ (NOD.BDC2.5) mice have been previously described (115). NOD.BDC2.5 mice were bred with NOD.129P2(C)-Tcra^{tm1Mjo}/DoiJ (NOD.Cα^{null}) mice to generate NOD.BDC2.5.Cα^{null} 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 hr of birth) and various aged female NOD or NOD.Aire^{null} 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 $\geq$250 mg/dl (Abbott Diabetes Care Inc) for 2 consecutive measurements

were scored as diabetic. The body weight of animals was measured weekly, and the development of weight loss was considered as the clinical onset of colitis.

Immunohistological analyses.

Various tissues were fixed in 10% neutral buffered formalin (Fisher Scientific), paraffin embedded, and non-overlapping sections prepared and stained with hematoxylin and eosin (H&E) or Alcian blue. Severity of insulitis and colitis were graded as previously described (116, 117).

For thymus immunostaining, thymi were frozen in O.C.T. compound (Sakura Finetek USA) and 7μM sections cut. Sections were fixed and permeabilized in ice cold acetone/methanol for 5 min, 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) followed by Streptavidin*PE (eBiosciences) and AlexaFluor*488 goat anti-rat IgG (Invitrogen); each step was incubated 1 hr at room temperature. Montage thymus images were taken using a Zeiss Axioplan 2 microscope 10x objective, and analyzed with Slidebook software (Intelligent Imaging Innovations).

T cell analyses.

Cells isolated from the spleen, PLN, MLN and colon were stimulated with PMA (50ng/ml)/ionomycin (1μg/ml) in complete RPMI 1640 medium at 37^0C for 4 to 5 hr and Brefeldin A was included in the culture for the last 2 hr of incubation. Cells were washed, stained with Abs specific for CD4 (GK1.5), CD8 (53-6.7), CD3 (2C11) and TCRβ (H57). After fixation and permeabilization using the Fixation/Permeabilization kit (eBioscience), cells were stained with Abs specific for intracellular IL-17 (TC11-18H10) and IFNγ (XMG1.2). 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 (50) with in house prepared soluble IA^{g7} multimers covalently linked to BDC mimetic or HEL peptides or $H2K^{d}$ tetramers complexed with IGRP or HA peptides (118, 119). Data were acquired with CyAn flow cytometer (DakoCytomation) and analyzed using FlowJo (Tree Star Inc.) or Summit (DakoCytomation) software.

Single cell suspensions were prepared from NOD.*scid* thymus recipients 6 wks post-transplantation and 5-10x10^5 cells/well cultured in triplicate in complete RPMI1640 and 100μg/ml cecal bacterial lysate (CBL) (120) prepared from 6 wk-old NOD mice in 96-well round bottom plates at 37°C for 48 hr. The supernatants were harvested, and IFNγ and IL-17 measured using ELISA kits (eBiosciences) according to the manufacturer's instructions.

For adoptive transfer experiments, splenocytes were harvested from NOD.*scid* thymus recipients 6 wk post-transplantation and $CD4^+$ and $CD8^+$ T cells purified by negative selection using mouse CD4 or CD8 T Cell Isolation Kits (Miltenyi Biotec). Female NOD.*scid* mice 6 wk of age were injected i.p. with 2x10^6 T cells per mouse, and then 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.

Statistical analysis.

Statistical tests were performed using prism 4.0 software (GraphPad). Body weight data were analyzed using the Two-way ANOVA. Kaplan-Meier Long-Rank Test was used to analyze the incidence of diabetes and colitis. Student's *t* test and ANOVA were used for all other data.

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 (Figure 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 T1D. Mature T cells were detected in the blood of thymus recipients as early as 1 wk post-implantation; by 4-6 wk ~40% of mononuclear cells consisted of $CD4^+$ T cells in recipients of newborn and adult thymi (Figure 1B). The reconstitution of $CD8^+$ T cells, however, was delayed in adult versus newborn thymus recipients (Figure 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 (Figure 1C,D, Table I) Recipients of 7 and 10 d-old thymi remained diabetes-free; insulitis, however, was detected albeit at a reduced severity relative to newborn thymus recipients (Figure 1C, Table I). In contrast, NOD.*scid* recipients of thymi from 2 wk and older NOD donors failed to develop both insulitis and diabetes (Figure 1C,E, Table I). Similarly, the salivary gland and thyroid were infiltrated in recipients of newborn and 7 d thymi (Figure 1I,K) but not 10 d or older thymi (Figure 1J,L, Table I).

Surprisingly, symptoms of colitis such as weight loss (Figure 1F, Table I), 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 (Figure 1F, 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 (Figure 1G,H). NOD.*scid* mice receiving newborn and 7 d thymi, however, exhibited only limited colitis and no weight loss or rectal prolapse (Figure 1F, Table I).

Adoptive transfer experiments confirmed the organ-specific nature of pathogenic T cells 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 Teff are increased in the PLN of newborn thymus recipients.

FACS analyses demonstrated that the number of $CD4^+$ and $CD8^+$ T cells was increased ~2 and ~3-fold, respectively, in the spleen, PLN, and MLN of recipients of newborn and 7 d old thymi versus 10 d and older thymi (Figure 2B).

The frequency of β cell-specific $CD4^+$ and $CD8^+$ T cells were measured in the spleen, PLN and MLN of newborn, 7 and 10 d thymus recipients. IA^{g7} and $H2K^d$ multimers were used to detect $CD4^+$ and $CD8^+$ T cells that recognize a BDC mimetic peptide (IA^{g7}-pBDC), and an islet-specific glucose-6-phosphatase catalytic subunit-related protein peptide (IGRP; $H2K^d$-IGRP), respectively. pBDC-specific $CD4^+$ T cells (121) and IGRP-specific $CD8^+$ T cells (102) are prevalent diabetogenic clonotypes in NOD mice. The highest frequency of $CD4^+$ and $CD8^+$ T cells staining with IA^{g7}-pBDC and $H2K^d$-IGRP, respectively, were detected in the PLN of newborn thymus recipients (Figure 2D). Notably, the frequency of IA^{g7}-$pBDC^+$ $CD4^+$ T cells and $H2K^d$-$IGRP^+$ $CD8^+$ T cells progressively declined in the PLN of 7 d and 10 d thymus recipients (Figure 2D).

To examine temporal changes in antigen reactivity of colitogenic T cells, splenocytes prepared from thymus recipients were stimulated with CBL and IL-17 and IFNγ secretion measured. IL-17 and IFNγ secretion in response to CBL was substantially increased in cultures from animals receiving thymi from 10 d and older NOD donors (Figure 3A). CBL also induced IL-17 and IFNγ secretion in cultures prepared from newborn and 7 d thymus recipients albeit at significantly reduced levels (Figure 3A). $CD4^+$ T cells alone from adult thymus recipients were sufficient to transfer diabetes (Figure 3B). The importance of T cell reactivity to colonic microbiota in the development of colitis was further demonstrated in NOD.*scid* recipients of thymi from 6 wk-old NOD.BDC2.5 versus NOD.BDC2.5.$C\alpha^{null}$ donors. Severe colitis developed in NOD.*scid* recipients of NOD.BDC2.5 thymus (Figure 3C), in which T cells expressed both the BDC2.5 clonotypic and endogenous TCR. On the other hand, severity of colitis was markedly reduced in NOD.*scid* recipients when the specificity of NOD.BDC2.5.$C\alpha^{null}$ $CD4^+$ T cells was restricted to the β cell autoantigen chromogranin A (122) (Figure 3C). Recipients of NOD.BDC2.5 (or NOD.BDC2.5.$C\alpha^{null}$) thymi, however, developed diabetes (Figure 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+Treg and immunoregulation in the PLN of recipients.

The above data indicated that thymic development of β cell-specific T cells declines with age resulting in reduced numbers of Teff to mediate diabetes. However, lack of β cell

autoimmunity in recipients of post-newborn thymi may also be due to a reciprocal increase in Foxp3^{+}Treg residing in the PLN to block expansion of diabetogenic Teff. To distinguish between these two possibilities, the frequency of Foxp3^{+}Treg was assessed in the spleen, PLN and MLN of thymus recipients. Interestingly, PLN Foxp3^{+}CD25^{+}CD4^{+} T cells were increased in newborn and 7 d versus 10 d and older thymus recipients, whereas the frequency of spleen and MLN resident Foxp3^{+}CD25^{+}CD4^{+} T cells was similar independent of thymic age (Figure 4A).To assess the immunoregulatory activity in the PLN, NOD.BDC2.5 CD4^{+} T cells were transferred into recipients of newborn and 4 wk thymi, and proliferation measured. No marked difference was detected in the level of NOD.BDC2.5 CD4^{+} T cell proliferation between the respective thymus recipients (Figure 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 PLN, but attributed to reduced numbers of β cell antigen-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.Aire^{null}) was investigated. NOD.Aire^{null} mice lack β cell autoimmunity but develop multi-organ T cell-mediated inflammation (123), which includes exocrine pancreatitis. Tissues normally targeted in NOD.Aire^{null} mice were also infiltrated in NOD.*scid* recipients of newborn NOD.Aire^{null} thymi (Figure 5A, Table III. Relative T cell infiltration of organs in NOD.*scid* recipients of different-aged NOD.Aire^{null} thymi.). Strikingly, a progressive decline in T cell infiltration of the exocrine pancreas and salivary

glands was detected in recipients of 7 and 10 d old NOD.Aire^{null} thymi, and no infiltration of these tissues was observed in 4 wk-old thymus recipients (Figure 5ATable III). On the other hand, T cell infiltration continued to be detected in the eyes, ovaries, stomach and lungs of animals implanted with day 7 and older NOD.Aire^{null} thymi (Figure 5A,B). The severity of colitis in the recipients, however, was limited regardless of NOD.Aire^{null} thymi age (Figure 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 employed. 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 thymi (Figure 1C,D), which corresponded with an increased frequency of PLN-resident pBDC-specific CD4$^+$ and IGRP-specific CD8$^+$ T cells (Figure 2D), 2 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 (49). Interestingly, autoimmunity has been reported in immunodeficient children with congenital athymia receiving a human infant thymus transplant (124). Noteworthy was the progressive decline in

insulitis (Figure 1C) and the frequency of β cell-specific T cells in recipients of 7 and 10 d old NOD thymi (Figure 2D). This reduction in β cell-specific T cells was not due to a reciprocal increase in the pool of PLN-resident Foxp3^{+}Treg or enhanced tissue-specific immunoregulation that would be expected to block the expansion of diabetogenic Teff (Figure 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 (Figure 3C,D) or a mixture of splenocytes from colitogenic and diabetic donor animals (Appendix 1), showing that the progression of the 2 pathologies is independent and not mutually exclusive. Together these findings indicate that the lack of insulitis and diabetes, and reduced frequency of diabetogenic T cells in recipients of post-newborn thymi is the result of diminished thymic production of β cell-specific T cells. Importantly, thyroiditis and sialitis detected in newborn thymus recipients were also reduced in recipients of 7 and 10 d-old thymi (Figure 1I-L, Table I) demonstrating that thymic production of autoreactive T cells in general is regulated in an age-dependent manner.

To explain the temporal decline in autoreactive T cell production we favor a model in which the efficacy of thymic negative selection increases during postnatal life. Several mutually nonexclusive possibilities may account for this effect. Reduced expression of TSA due to limiting Aire expression may lead to inefficient thymic negative selection of autoreactive T cells in the neonatal thymus (93, 96, 98). Indeed, both the frequency of AIRE-expressing mTEC, and mRNA expression of AIRE-dependent TSA genes such as *Ins2* are reduced in thymi from newborn versus older NOD mice (R.T. & C.J.K; unpublished data). Furthermore, Guerau-de-Arellano and colleagues reported that induced expression of AIRE and corresponding TSA by mTEC during embryonic life and up to 21 d after birth was

critical to block the multi-organ autoimmunity typical of NOD.Aire^{null} mice (37). Our observation that recipients developed significant infiltration of the ovaries, stomach, lungs and eyes implanted with newborn and older NOD.Aire^{null} thymi (Figure 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 (Figure 5, Table III) suggesting 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/or maturation status of mTEC and thymic DC may contribute to the efficiency of thymic negative selection (37, 98, 125, 126). Alternatively, the development of autoreactive T cells may reflect intrinsic properties of T cell progenitors residing in the thymus during ontogeny. For example, studies have shown that hematopoietic stem cells that seed the thymus at various stages of ontogeny give rise to T cells with distinct properties and antigen specificity (127-130). The latter may influence the affinity and/or cross-reactivity or promiscuity of TCR 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 (Figure 1A) may limit thymocyte interactions with medullary resident APC thereby reducing the efficiency of negative selection, particularly if given TSA 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 and 7 d NOD thymi (Figure 1F). However, severe colitis developed in recipients of thymi from NOD donors 10 d of age and older (Figure 1F) which was marked by an increased number and frequency of IL-17 and IFNγ producing $CD4^+$ T cells specific for CBL (Figure 2B). Unlike β cell-specific T cells, which were selectively increased in the PLN (Figure 2D), CBL reactivity was readily detected in all tissues examined (Figure 3A), likely reflecting systemic trafficking of a relatively large pool of colitogenic Teff. Recognition of commensal microbiota antigen was necessary for colitis; recipients of thymi from NOD.BDC2.5.$C\alpha^{null}$ donors which express chromogranin A-specific TCR (122) failed to develop significant colitis (Figure 3B). In contrast, increased colitis was detected in animals receiving thymi from adult NOD.BDC2.5 mice, which co-express transgenic and endogenous TCR (Figure 3B). These findings demonstrate that in addition to autoreactive T cells, the development of T cells specific for exogenous (e.g. microbial) antigens is regulated temporally, but in a reciprocal relationship to autoimmune T cells. In this instance, production of T cells specific for microbial antigens 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 $H2^{g7}$ (Appendix 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 (131).

Since 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. It is likely, however, that lymphopenia favored the differentiation of pathogenic Teff driving autoimmunity and colitis. Development of autoreactive T cells that is largely restricted to early ontogeny further underscores the role of peripheral mechanisms in maintaining life-long self-tolerance. Our findings may also in part explain the long-lasting and robust tolerance typically induced by administration of self-antigen to neonates (132-134). Here, 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 antigens is tightly regulated over a short ontogenic time 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 are likely to contribute to the temporal development of autoreactive and bacterial antigen-responsive T cells in a reciprocal manner. Exploiting the use of the thymus transplant model provides a novel approach to better define these events.

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-, 10 d-, and 4 wk-old NOD thymi; images were taken at 100X magnification. (B) NOD.scid mice were engrafted with thymi from newborn (NB) or 12 wk-old (n=10) NOD female donors and the frequency of $CD4^+$ (left panel) and $CD8^+$ (right panel; *$p<10^{-4}$) T cells measured in peripheral blood. (C) Diabetes (left panel; *$p<10^{-4}$ versus recipients of 7 d or older thymi; Kaplan-Meier Log Rank) and the frequency of insulitis (right panel; n=8; *$p<10^{-4}$; Student's t test) 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; *$p<10^{-4}$; 2-way ANOVA) and colitis scores (right panel; *$p<10^{-4}$; Student's 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 newborn (I,K) and 12 wk-old (J,L) thymus recipients. Error bars represent SEM.*

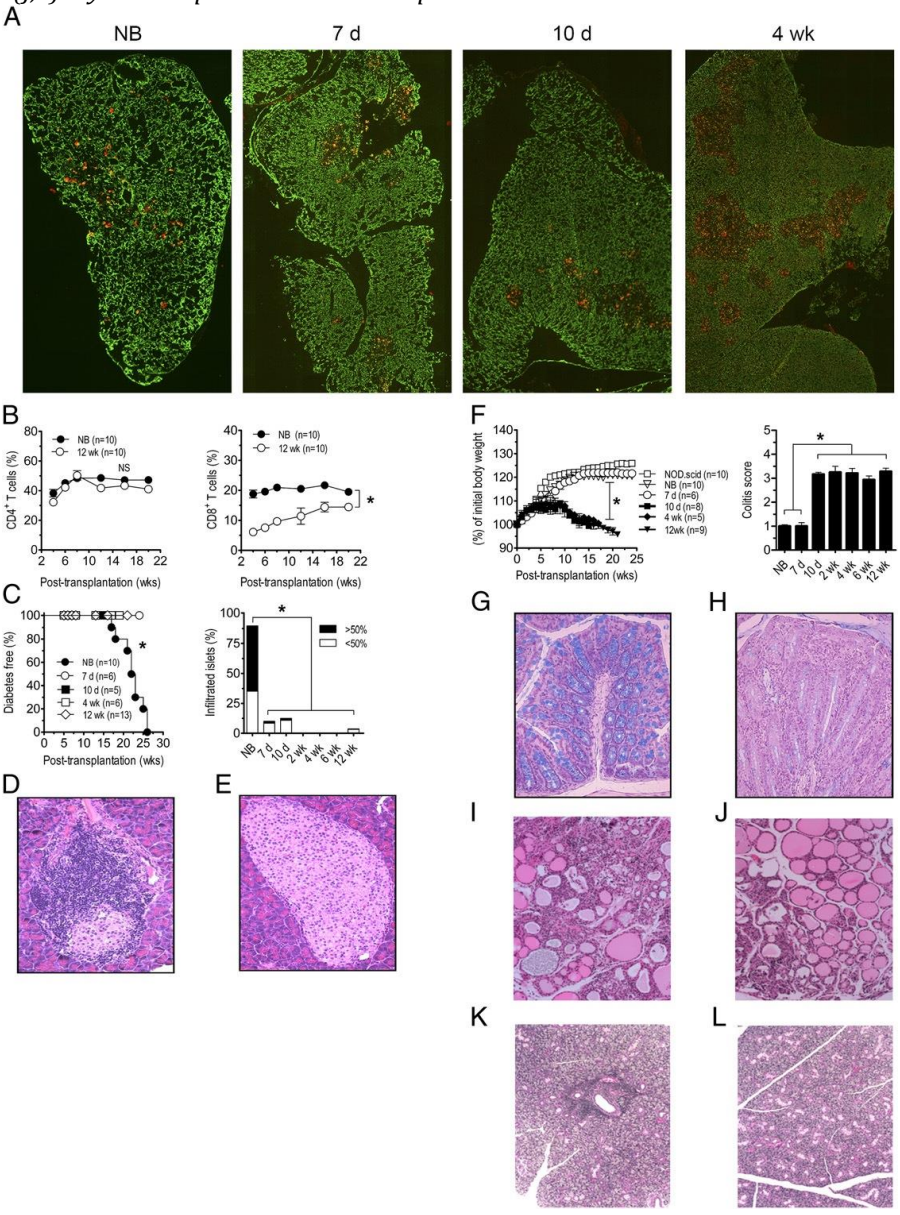


Table I. T cell infiltration of organs in NOD.*scid* recipients of different-aged NOD thymi.
Thymi from various-aged female NOD mice were transplanted under the kidney capsule of 6-wk-old NOD.scid recipients.

	Pancreas	Large Intestine	Salivary Gland	Thyroid
Newborn	5/5	0/5	5/5	5/5
7 d	5/6	0/6	6/6	6/6
10 d	0/7	7/7	0/7	4/7
2 wk	0/6	6/6	0/6	0/7
4 wk	0/7	7/7	0/7	0/7
6 wk	0/6	6/6	0/6	0/6
12 wk	0/13	13/13	0/13	0/13

Table II. Disease incidence in NOD.*scid* mice adoptively transferred with splenocytes from different-aged NOD-thymus recipients.

	Diabetes	Colitis
Newborn	13/13	0/8
7 d	5/6	0/6
10 d	0/7	7/7
2 wk	0/6	6/6
4 wk	0/7	7/7
6 wk	0/6	6/6
12 wk	0/13	13/13

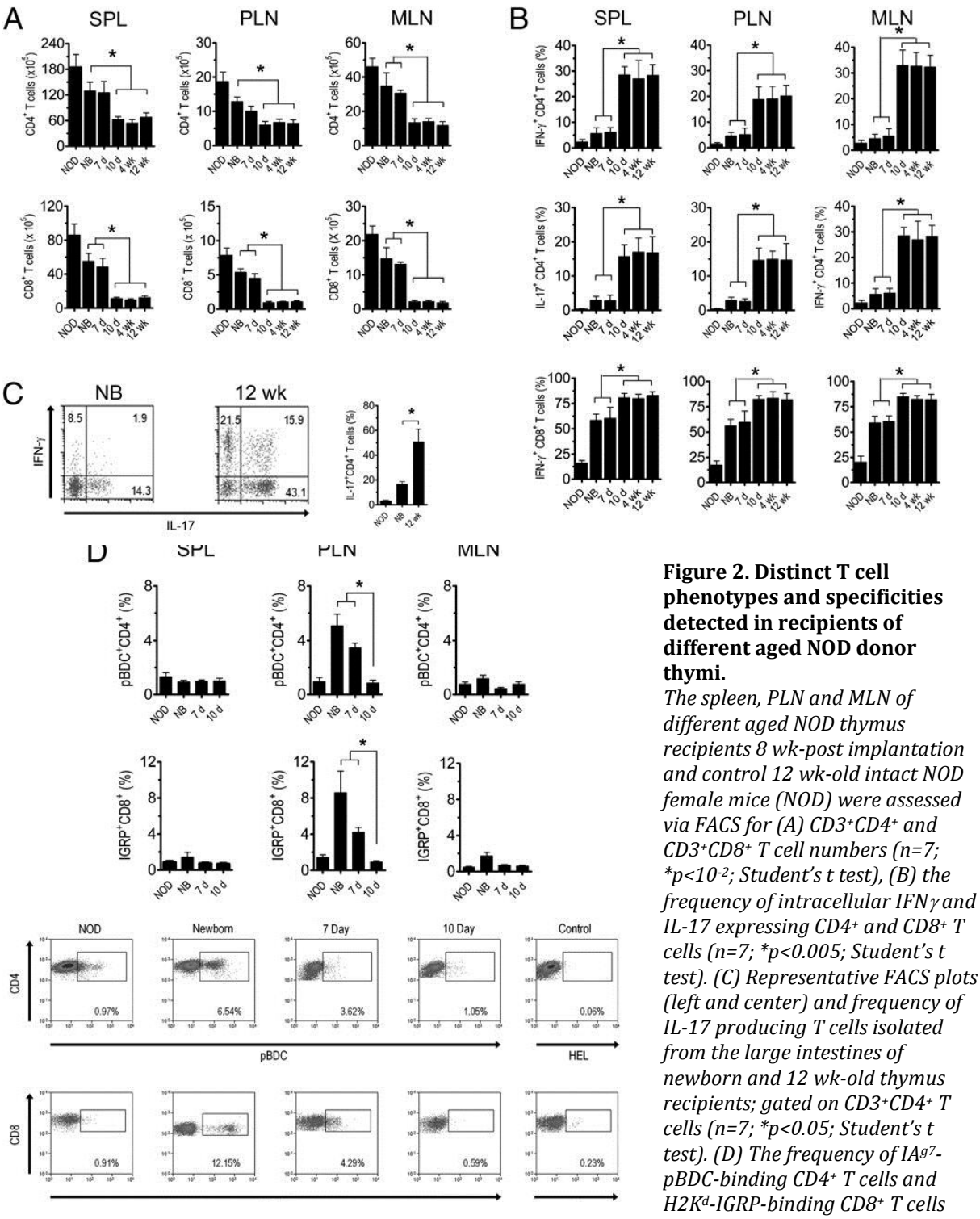


Figure 2. Distinct T cell phenotypes and specificities detected in recipients of different aged NOD donor thymi.

*The spleen, PLN and MLN of different aged NOD thymus recipients 8 wk-post implantation and control 12 wk-old intact NOD female mice (NOD) were assessed via FACS for (A) $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell numbers (n=7; *$p<10^{-2}$; Student's t test), (B) the frequency of intracellular IFNγ and IL-17 expressing $CD4^+$ and $CD8^+$ T cells (n=7; *p<0.005; Student's t test). (C) Representative FACS plots (left and center) and frequency of IL-17 producing T cells isolated from the large intestines of newborn and 12 wk-old thymus recipients; gated on $CD3^+CD4^+$ T cells (n=7; *p<0.05; Student's t test). (D) The frequency of IA^{g7}-pBDC-binding $CD4^+$ T cells and $H2K^d$-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; *p<0.05; Student's t test). Error bars represent SEM.*

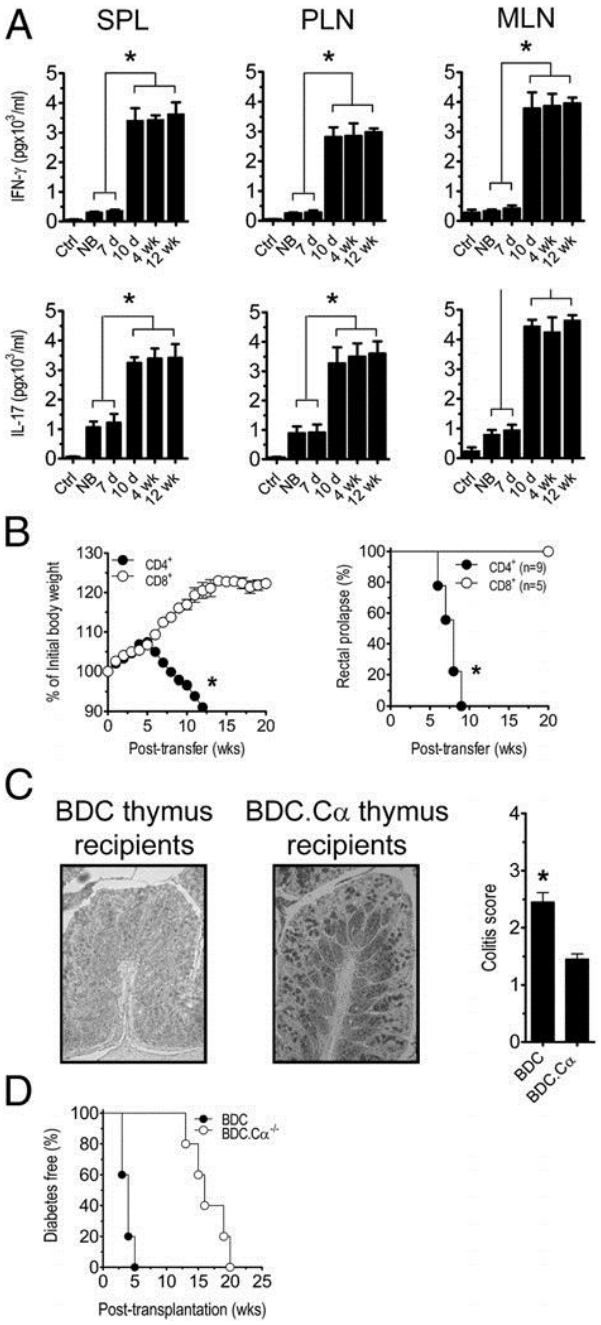


Figure 3. Colitogenic T cells respond to intestinal microbial antigens.

*(A) Secretion of IFNγ and IL-17 by isolated T cells stimulated with CBL, as measured by ELISA, in the spleen, PLN, and MLN of recipients of different aged thymi 8 wk post-implantation (n=7; *p<10^{-4}; Student's t test). (B) Splenic $CD4^+$ and $CD8^+$ T cells isolated from NOD.scid thymus recipients 8 wk post-implantation, and adoptively transferred into NOD.scid mice, which were monitored for body weight loss (left panel; *p<0.05, 2-way ANOVA) and development of rectal prolapse (right panel; *p<0.05, 2-way ANOVA). (C) Representative colonic sections stained with Alcian blue, and colitis scores (*p<0.05; Student's t test) and (D) diabetes incidence for recipients (n=5) of thymi from 6 wk-old NOD.BDC2.5 and NOD.BDC2.5Cα^{null} donors. Error bars represent SEM.*

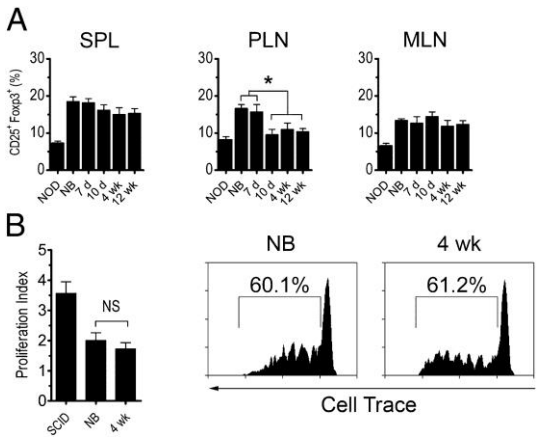


Figure 4. Development of diabetes is not due to increased Foxp3⁺Treg frequency or suppression. *(A) Frequency of Foxp3⁺CD25⁺CD4⁺ T cells in recipients of different aged NOD thymi (n=7) 6 wk-post implantation or 12 wk-old intact NOD female mice (NOD). (B) In vivo proliferation of cell trace-labeled NOD.BDC2.5 CD4⁺ T cells 4 d post-transfer in the PLN of recipients of newborn and 4 wk-old NOD thymus 6 wk post-implantation or unmanipulated NOD.scid controls. Data is reported as proliferation index (left) with representative FACS histograms gated on CD3⁺CD4⁺cell trace⁺ T cells (right) (n=5). Error bars represent SEM.*

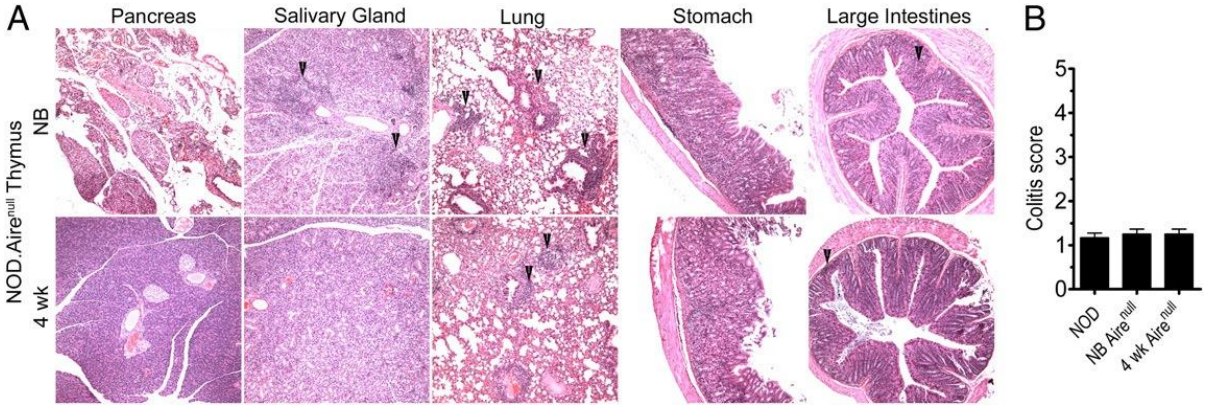


Figure 5. Temporal development of autoreactive T cells is independent of AIRE in some organs. *NOD.scid mice received thymi from different aged NOD.Aire^{null} donors. (A) Representative H&E stained sections of organs; arrows highlight areas of infiltration. (B) colitis scores of different aged NOD.Aire^{null} thymus recipients (n=5). Error bars represent SEM.*

Table III. Relative T cell infiltration of organs in NOD.*scid* recipients of different-aged NOD.Aire^{null} thymi.

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

Organ	NB Aire^{null}	7-d Aire^{null}	10-d Aire^{null}	4-wk Aire^{null}
Pancreas	+++	++	+	None
Ovary	+++	+++	+++	++
Large intestines	+	+	+	+
Stomach	++	++	++	++
Lung	++	Not observed	Not observed	++
Salivary gland	++	++	+	None
Cecum	+	+	+	+
Small intestines	+	+	+	+
Eyes	+++	Not observed	Not observed	+++

Chapter 3: Altered T Cell Trafficking Following Antibody Mediated Crosslinking of the CD4 Coreceptor.

Introduction

T cell trafficking from lymph nodes into tissues or blood is a highly regulated process. Secondary lymphatics serve as a primary site for adaptive immunity. A distinct chemokine *milieu* and integrin expression profile help to maintain a non-inflammatory "standby" state (135, 136), such that T cells can probe for productive antigen interactions, and when absent, continue to traffic to additional lymph nodes. Dysregulation of appropriate trafficking cues and mechanisms can result in a failure to mount an efficient protective immune response, or in the development of a pathological response, associated for instance with autoimmunity. We recently reported that non-depleting antibodies specific for the CD4 (YTS177) and CD8 (YTS105) T cell coreceptor molecules induce remission in new onset diabetic NOD mice (50). Remission was accompanied by a reduction of T cell cellularity in both the pancreatic islets, and draining PLN. This "purging" event was independent of apoptosis induction and was attributed to altered trafficking properties of the pancreas and PLN resident CD4+ and CD8+ T cells. The mechanisms involved in T cell purging from the PLN induced by co-receptor therapy remain unclear.

T cells continually cycle between lymphatics and blood (52), scanning for productive and/or activating interactions with APCs (136, 137). Crossing between lymphatics and blood is an active process requiring polarization of, and interactions with, the vascular or lymphatic

endothelial cells by T cells (135, 138). Steady state T cells have a rounded morphology with evenly distributed CKRs and adhesion molecules (135, 139). Upon a productive encounter in lymph nodes, T cells and APCs form an immunological synapse, where T cells recognize cognate antigen via interactions of the TCR and the peptide-MHC complex (62, 137). Additional costimulatory signals, and engagement of the CD4 or CD8 coreceptors, are required for full T cell activation (140). Phosphorylation of LCK at tyrosine 394 (63) located at the intracellular region of CD4 or CD8 is an early event contributing to downstream TCR signaling. Once activated the cell membrane is polarized and T cells egress from lymphatics (138). In the absence of a productive APC:T cell interaction, T cells exit the lymph nodes and enter circulation in response to elevated blood levels of S1P (141), followed by re-entry into lymphatics.

Like other migrating cells, T cell polarization is necessary to cross endothelial barriers, characterized by an actin cytoskeleton rearrangement to form a leading edge in the direction of migration with a retracting tail at the rear of the cell (61). Small Rho GTPases are regulators of the cytoskeleton and function by cycling though an active GTP bound and an inactive GDP bound state (142). Rac1 regulates migration by inducing a branch actin network termed the lamellipodia, at the leading edge (78). T cells also undergo a specific Rac dependent polarization resulting in a more oblong morphology with localization of CKRs and adhesion molecules to different poles of the cell (66). The leading edge pole becomes a clustering of specific CKRs, including: CCR2, CCR5, and CXCR4 (56, 143). Also found in the leading edge are adhesion molecules such as LFA-1 (144, 145) which serve to slow circulating T cells and facilitate transendothelial migration from blood to lymphatics and vice versa. Polymerizing F-actin is detected at highest concentrations in the leading edge of a

polarized T cell, which allows extension of the leading edge, ultimately pulling the T cell forward or allowing for T cell scanning of APC (61). The trailing pole of a T cell is comprised of the "uropod", where adhesion molecules such as ICAM-1, CD43, and CD44 localize (144, 146).

The T cell specific pleomorphic effects of Rac activation include: lymphoid egress or entrance, circulation in blood and lymphatics, or entry into target tissues (61, 147, 148). The different outcomes of Rac activation are contextual, but can at least partially be attributed to the activating GEF. For instance, Rac activation as a result of the GEF Dock2, has been reported to promote T cell homing to lymph nodes (149), whereas Tiam1 mediated Rac activation is implicated more specifically in T cell adhesion and transendothelial migration (70). While over 70 GEFs have been identified (150), those identified in the context of T cell activity belong to the Sos, Vav, Tiam, and Dock families (66, 68-70, 72, 73).

Activation of Rac resulting in altered T cell migration has been characterized as downstream of T cell activation (82, 151). The signaling upstream and downstream of Rac activation in the absence of TCR stimulation is not well understood. We provide the first evidence that CD4 coreceptor crosslinking results in downstream effects independent of the TCR signaling cascade, leading to a T cell migratory phenotype through activation of Rac. The identification of this novel mechanism of T cell migration not only further clarifies the mechanisms of T cell trafficking in the absence of activation but also provides an additional target for modulating T cell activity during a pathological response.

Materials and Methods

Preparation of recombinant proteins

pGEX GST-PBD and pGEX GST-RhoAG17A fusion proteins were prepared from lysates from Bl21 *Escherichia coli* cells induced with 100 µM IPTG for 16 hours at RT. For GST-PBD, bacterial cells were lysed in 20 mM Tris pH 7.8, 1% Triton X-100, 10 mM $MgCl_2$ 1 mM DTT, 1mM PMSF, and 10 µg/ml aprotinin and leupeptin. For GST-RhoAG17A bacterial cells were lysed in 20 mM HEPES pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 1mM PMSF, and 10 µg/ml aprotinin and leupeptin. The recombinant proteins were isolated from the bacterial lysates by incubating with glutathione-Sepharose 4B beads (GE Healthcare) at 4^oC for 4 hours. The beads were sedimented and washed 3 times in 20 mM HEPES, pH 7.5; 150 mM NaCl, 1 mM DTT.

GST-PBD and GST-RhoAG17A Pull-down Assay

Rac activation assays were performed as described (152). T cells were lysed in 300 µL of 10 mM $MgCl_2$, 500 mM NaCl, 50 mM Tris, pH 7.8, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1mM PMSF, and 10 µg/ml aprotinin and leupeptin, cleared at 14,000g at 4^oC for 3 minutes and incubated with at least 20 µg of GST-PBD for 20 minutes at 4^oC. Beads were then washed 3 times in 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100, 1mM PMSF, and 10 µg/ml aprotinin and leupeptin. Active GEFs were assayed by binding to GST-RhoAG17A as described (153). In short it was performed as the RhoA activation assays with the following changes. T cells were lysed in 150 mM NaCl, 20 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 1% Triton X-100 1mM PMSF, and 10 µg/ml aprotinin and leupeptin, and incubated with GST-RhoAG17A beads for 60 minutes at 4^oC and washed in the same lysis buffer. Samples were then analyzed by western blotting.

Western Blotting

Samples were run on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked and incubated with the specified primary antibodies followed by species-specific secondary antibodies conjugated with horseradish peroxidase. Blots were developed with a chemiluminescent HRP substrate, and visualized on x-ray film or ChemiDoc™ MP System (BioRad) and corresponding software. For quantification, blots were scanned and the intensity values determined using Image J software (NIH) and protein levels were normalized to control protein levels. All quantification graphs include ≥3 independent experiments. Error bars represent SEM.

Immunoprecipitation

Cells were lysed in preheated gel sample buffer containing 200 mM Tris pH 6.8, 20% glycerol, 4% SDS and 5% 2-ME, and boiled for 5 minutes and sheared with needle. Samples were then diluted with 20 volumes of 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100 and 1% DOC, 10 µg/ml othrovanidate 1mM PMSF, and 10 µg/ml aprotinin and leupeptin, 2 µg of PY-20 mAb anti-phospho-tyrosine antibody for 2 hours at 4^{o}C. Protein G-Sepharose was then added and samples were rotated for 2 hours at 4^{o}C. Beads were then washed in dilution buffer and analyzed by western blotting. All quantification graphs include ≥3 independent experiments. Error bars represent SEM.

Transendothelial migration assay

Cell migration assays were performed in transwell plates (Corning) of 6.5-mm diameter with 3 or 5-µm pore filters. The upper transwell plate was either uncoated, coated with 10 µg/ml collagen IV or a primary mouse endothelial cell monolayer. Cells were added to the upper chamber and allowed to migrate into the lower chamber containing either 10 µM

S1P (Sigma) or 100 μM CXCL12 (Sigma) at 37°C and 5% CO_2. An input control was taken. Cells in the lower chamber for each condition were collected and counted by flow cytometery.

Mice

NOD/LtJ mice were bred and maintained in specific pathogen-free facilities at the University of North Carolina at Chapel Hill (UNC-CH). All animal experiments were approved by the UNC-CH Institutional Animal Care and Use Committee.

Antibody preparation and treatment

The YTS177.9 hybridoma was a gift from Dr. Waldmann (Oxford, U.K.). Antibody was purified via ammonium sulfate precipitation from culture supernatants.
12 week old prediabetic female NOD mice were given intraperitoneal injection (IP) with 600 μg YTS177 or the 2A3 rat IgG2a isotype control (BioXCell).

Treatment with inhibitors

Ly294002 (LC Laboratories): Mice were injected IP with 75mg/kg 6 hours prior to YTS177 or isotype treatment. An additional dose of Ly294002 was given daily until mice were euthanized. NSC237666 (Santa Cruz Biotechnology): Mice were injected IP with 3mg/kg 2 hours prior to YTS177 or isotype treatment. An additional dose of NSC237666 was given every 12 hours until mice were euthanized.

Primary cell isolation

Spleen and PLN suspensions were made by mechanical dissociation using frosted glass slides. Single cell suspensions were made by filtering suspensions through a 40 μM nylon cell strainer (Corning). Isolation of murine peripheral blood lymphocytes was performed by removing 25 μl blood from the tail vein, followed by isolation using

Lympholyte M (Cedarlane). Primary mouse pulmonary endothelial cells (EC) were isolated from 1 week old NOD mice as described in Sobczak *et al.* (154) and grown in EGM-2 Bullet Kit (Lonza).

Flow cytometry

Cells were stained with anti-CD3 (145-2C11), anti-CD62L (MEL-14), anti-CD4 (RM-4-5), anti-LFA-1 (M17/4). When appropriate, cells were fixed with 1.5% paraformaldehyde diluted in FACS buffer. Flow cytometry data was acquired at the UNC-Flow Cytometry Facility using the Cyan (DakoCytomation) cytometer and analyzed with Summit (DakoCytomation).

Microscopy

Tissues were homogenized as previously described directly in 1.5% paraformaldehyde diluted in 2% FBS containing PBS. Cells were stained with anti-CD3 (145-2C11), anti-LFA-1 (M17/4), Phalloidin Alexa 488 (Life Technologies). Confocal microscopy data was acquired at the UNC-Microscopy Services Laboratory using the Olympus FV500 Confocal Laser Scanning microscope. Image analysis was completed using Image J software (NIH).

Statistics

Data represent mean $\pm$ SEM. Statistical comparisons of differences between sample means used the two-tailed Student *t* test and was calculated using Prism (Version 6.0; GraphPad).

Results

Antibody mediated CD4 binding results in T cell egress from the pancreatic lymph nodes.

We have previously reported that treating new onset diabetic NOD mice with non-depleting anti-CD4 (YTS177) and anti-CD8 (YTS105) antibodies results in diabetes remission (50). Reversal of diabetes is mediated in part by purging of CD4+ and CD8+ T cells from the pancreas and PLN. To define the short-term effects of coreceptor crosslinking, 12 week old pre-diabetic NOD mice were administered 600 μg of either YTS177 or 2A3 (an isotype control), and 1, 3, or 6 days later, T cells in the spleen and PLN analyzed by flow cytometry (Figure 6A). Following YTS177 treatment, CD4+ T cells migrated from the PLN between days 1 and 3 post-treatment, reaching maximum egress by day 6 (Figure 6B). No reduction was observed in CD4+ T cell numbers in the spleen within 6 days post treatment. The YTS177-mediated decrease in PLN was concomitant with a temporal increase in CD4+ T cells in blood (Figure 6C).

Migration of T cells from lymph nodes into blood is controlled by S1P levels in circulation interacting with the S1P receptor (S1Pr) on T cells (141). To determine if YTS177-bound CD4+ T cells have increased reactivity to S1P, an *in vitro* transwell approach was utilized. Chemotaxis in response to S1P across a 5 μm transwell was increased in a dose dependent manner up to 3-fold by CD4+ T cells isolated from the PLN of YTS177 compared to 2A3-treated NOD mice (Figure 7A,B). To establish a more stringent barrier for T cells to migrate through, collagen coated transwells were employed. Under these conditions, an ~2-fold increase in S1P-mediated chemotaxis was observed for CD4+ T cells prepared from YTS177 versus 2A3 treated PLN (Figure 7C). To further increase stringency and establish a more physiologically relevant set of conditions, a monolayer of endothelial cells was cultured

on the surface of the transwells. Endothelial cells provide receptor-ligand interactions, as well as junctions for T cells to traffic through. Albeit reduced, under these more stringent conditions, the frequency of YTS177 treated CD4+ T cells migrating across the endothelial coated transwells was increased ~3-fold relative to CD4+ T cells from 2A3-treated animals (Figure 7D). Increased migration of YTS177 treated T cells towards S1P was not due to upregulated S1Pr expression, as no difference in receptor surface expression was observed following YTS177 treatment (Figure 7F). Increased chemotaxis by YTS177-treated T cells was also observed in response to CXCL12 (SDF-1) (Figure 7E). Notably, under all of the experimental conditions tested, CD8+ T cell chemotaxis was unaltered demonstrating the specificity of the effects of YTS177 binding to CD4 (Figure 7). Taken together, these results suggest that CD4+ T cells exhibit an enhanced migratory phenotype after YTS177 treatment, which is not specific to the chemotactic cues of just one chemokine.

Increased migration is a function of T cell polarization.

T cell polarization results in clustering of CKRs to the leading edge, which in the absence of CKR upregulation allows for greater sensitivity to chemotactic cues. T cell polarization was examined via confocal microscopy. Consistent with characteristic polarization, increased localization of F-actin and LFA-1 to the leading edge, as well as an altered, oblong morphology was observed 6 hours post YTS177 treatment (Figure 8A). Forty eight hours post YTS177 treatment, immediately prior to lymph node egress, T cell polarization was increased ~2-fold compared to steady state T cell polarization (Figure 8B,C). The finding of a phenotypic promigatory change in T cells following CD4 coreceptor engagement is consistent with the observed increase in transwell migration (Figure 7A-E) and provides a mechanism for lymph node egress in the absence of CKR upregulation.

Retention and entry of T cells in lymph nodes is adhesion molecule dependent, specifically the expression of the integrin LFA-1 and the selectin CD62L. Six hours post YTS177 treatment, a reduction in LFA-1 expression was detected (Figure 8D) suggesting a reduction in lymph node retention (155). A reduction in CD62L expression was also seen by 6 hours post YTS177 treatment (Figure 8E), suggesting reduced potential for entry into lymph nodes. Together these data suggest a phenotypic change in T cells resulting in a polarized and migratory phenotype directed out from lymphatics and into circulation.

Signaling downstream of CD4 engagement results in a pro-migratory phenotype.

T cell polarization is controlled by activation of the small RhoGTPase Rac1 and Rac2. To investigate the mechanism of T cell polarization and migration as a result of CD4 engagement, activation of Rac1 and Rac2 in T cells isolated from the PLN of YTS177 or 2A3 treated NOD mice was assessed. A temporal increase, reaching a maximum of ~3 and ~8-fold for Rac1 and Rac2 activation, respectively, was detected by 6 hours post treatment with YTS177 versus 2A3 (Figure 9A).

T cell egress from lymph nodes and migration into circulation requires T cell polarization; circulating cells do not require polarization, as such, should not exhibit Rac activation. To determine if the effect is specific to lymph nodes, we compared Rac activation of lymph node and spleen derived cells from YTS177 and 2A3 treated mice. No significant difference in the activation of either Rac1 or Rac2 was observed in the spleen of YTS177 versus 2A3 treated animals (Figure 9) consistent with the lack of T cell purging in the spleen (Figure 6B).

Classically, Rac activation has been described downstream of TCR signaling (82, 151, 156). The Src family tyrosine kinase LCK is the immediate downstream signaling

molecule of the CD4 coreceptor. LCK can be found in an active state, characterized by its phosphorylation at tyrosine 394, or an inactive state exhibiting phosphorylation at tyrosine 505 (157). Six hours post YTS177 treatment, active LCK was reduced with a concomitant increase in the inactive form (Figure 10A). LCK has been shown to initiate the TCR signaling cascade; therefore reduced kinase activity by LCK would result in a reduction of TCR signaling. Reduced LCK activity suggests the classical model of Rac activation through TCR signaling would not apply, as such, an alternate pathway must be responsible.

To determine the upstream GEF responsible for Rac activation after YTS177 treatment, a Rac.G15A pulldown was performed, which serves to isolate all activated Rac GEFs. No increase following YTS177 treatment was detected in the activity of Vav1, Vav2, or Dock2, which are GEFs normally associated with T cell Rac activation (Figure 10B). To identify the Rac GEF mediating YTS177-induced Rac activation, the Rac GEF inhibitor NSC237666 was utilized. This inhibitor blocks activation of Rac1 by a specific family of GEFs that includes Tiam1 and Trio. NSC237666 had no marked effect on YTS177-induced purging of CD4+ T cells in the PLN (Figure 10C). These data indicate that the class of GEFs inhibited by NSC237666, including Tiam1 and Trio, play no role in Rac activation and the downstream migratory phenotype we observe after YTS177 treatment.

Phosphoinositide 3-kinase (PI3K) signaling in T cells is associated with a number of events including T cell migration and activation of T cell-associated GEFs such as Vav, Sos, Dock, and Tiam. Accordingly, the effect of the PI3K inhibitor Ly294002 on YTS177-mediated purging of the PLN was examined. At day 3 post treatment, PI3K inhibition utilizing a dose and regimen shown to effect *in vivo* leukocyte migration (158) had no effect on YTS177-mediated PLN egress (Figure 10D). Although this was again a negative result, it

provided us with evidence that the observed migratory phenotype following YTS177 treatment was independent of PI3K activation and that the involved GEF was not reliant on PI3K activity.

Discussion

We provide evidence that CD4 crosslinking can have marked effects on the trafficking properties of T cells by: 1) promoting Rac1/2 activation and membrane polarization, 2) downregulating surface adhesion molecules, and 3) enhancing reactivity to chemokines such as S1P and CXCL12.

Sequestration of adhesion molecules and CKR to the poles of T cells is characteristic of T cell polarization and is typically associated with enhanced cellular migration. Although receptor clustering was not investigated, we observed clustering of adhesion molecules and cytoskeletal changes consistent with T cell polarization (Figure 8A,B). Literature describes a role for both Rac1 and Rac2 in the polarization and migration of T cells in response to activation cues (61, 147). Our demonstration of increased Rac activation within hours after antibody mediated CD4 crosslinking would suggest an increased migratory capacity. The mechanisms of Rac mediated migration are known to be via cytoskeletal changes and T cell polarization (61). Consistent with this, we observed increased Rac activation and polarization within 6 hours post YTS177 treatment, which in the case of polarization becomes statistically different from isotype control treated T cells within 48 hours post treatment. T cell polarization results in increased sensitivity and efficiency in responding to chemokines. Normally, activation of T cells, and as a result Rac activation, promotes an increase in the kinetics of lymphoid egress. We find it likely that the delayed kinetics of T cell egress in our model are most likely due to a lack of sufficient chemotactic cues at the time of treatment,

where in the case of T cell activation, an inflammatory environment would be present to immediately direct T cells to target tissue. In the case of antibody mediated crosslinking of CD4, we hypothesize that only by 2 days post treatment has a sufficient shift in the chemokine gradient occurred towards circulation to warrant lymph node egress. In short, T cells are primed to migrate early after treatment, but in the absence of a sufficient directional cue, remain in the lymphatics.

In addition to polarization, Rac is associated with expression of the integrin LFA-1, which controls T cell:DC and T cell:endothelial interactions via association with its ligand ICAM-1 (55). T cells from LFA-1 knockout mice exhibit accelerated egress from lymph nodes, and as such have reduced numbers in peripheral lymph nodes (155). LFA-1 blockade results in populations of T cells exiting the lymph nodes and entering blood (159). Following YTS177 treatment, we observed a systemic CD4 specific downregulation of LFA-1. Although the mechanism of LFA-1 blockade mediated egress is unclear, the importance of further investigation is highlighted by the fact that this approach is currently being investigated for clinical use in the context of islet transplantation tolerance (160). While it has been described that LFA-1 plays a central role in lymph node entry and egress (155), mechanisms remain ill defined. Our model provides insight into the potential upstream and downstream mediators of LFA-1 activity.

The pro-migratory phenotype observed after YTS177 treatment may serve to accelerate lymph node egress, however trafficking out of lymph nodes into circulation is a normal phenomenon. Under static conditions, following egress, T cells circulate followed by re-entry into the lymphatics. Extravasation from circulation and into lymphatics is initiated by tethering then rolling along the vascular endothelium, which is mediated by the adhesion

molecules LFA-1 and CD62L interacting with their respective ligands ICAM-1 and GlyCAM-1 (161), among others. The importance of CD62L in the entry of T cells into lymphatics is highlighted by the fact that CD62L deficient mice exhibit deficiencies in homing to lymph nodes (162). The observed systemic decrease in CD62L expression following YTS177 treatment may play a role in preventing T cell extravasation and re-entry into lymph nodes. Together, these data would support a model where T cells exit the lymphatics and are then unable to return and therefore remain in circulation.

The current model of Rac-mediated T cell polarization and migration is dependent on TCR activation. The inactivation of LCK (Figure 10A) suggested an alternative signaling pathway induced by CD4 upon YTS177 binding. A similar result was reported following HIV gp120 binding to CD4 (163), where increased Rac activation was observed; other studies have also shown that gp120 binding inactivates the TCR signaling cascade (164). While we cannot conclude that the downstream signaling resulting from antibody and gp120 binding are identical, it provides support of an inactivating TCR signal resulting in Rac activation. Additionally, we have demonstrated Rac activation independent of the GEFs Vav1, Vav2, Trio, Dock2, and Tiam1, those normally implicated in T cell activation dependent migration (66, 68-70, 72, 73). Taken together, these data provide evidence of an alternate pathway to T cell polarization and migration.

In short, T cell Rac activation results in downstream phenotypic changes normally associated with polarization and migration out of lymphatics. The classical pathway of T cell polarization has been one centered around TCR activation signals, our data suggests a model where migration occurs in the absence of TCR signaling. We provide evidence that LCK, the molecule associated with CD4 and the initiation of TCR signaling, exhibits an inactive state

within 6 hours after CD4 crosslinking. Although we were unable to identify the GEFs activating Rac, we are able to rule out those normally associated with Rac activation in the context of positive/activating TCR signaling. Taken together, our data supports a model where antibody mediated CD4 engagement causes downstream signaling independent of the TCR, resulting in Rac activation. Rac acts as a molecular switch, ultimately turning on a migratory program in T cells, resulting in their egress from lymph nodes and into circulation, and that a loss of adhesion molecules results in their inability to re-enter lymphatics (Figure 12).

T cells searching for activation through cognate antigen recognition continually migrate between circulation and lymph nodes. Activation of CD4+ T cells typically occurs in the lymph nodes and results in T cell polarization. Likewise, a lack of TCR activation also results in T cell polarization and mobilization. The ultimate action of T cells is context dependent, in the case of an immune response, T cells enter target tissues, in the absence of an immune response, T cells enter circulation. The primary difference between the two scenarios, outside of T cell activation, is the surrounding cytokine environment and chemokine gradient. Regardless of inflammatory state, T cells are primed for directed movement. The ability to control T cell polarization and migration provides a unique approach to control a pathological inflammatory response associated with autoimmunity for instance. Indeed, co-treatment with non-depleting anti-CD4 and -CD8 antibody is highly effective at suppressing ongoing β cell autoimmunity and reversing diabetes in NOD mice.

A role for Rac1 and Rac2 mediating the T cell polarization and egress induced by CD4 crosslinking is likely, however, experiments are still needed to directly confirm this role. Future studies will investigate the *in vitro* migratory potential of YTS177 treated T cells

across transwells following the knockdown of Rac via lentiviral expression of shRNA. We will also further investigate the TCR independent nature of polarization and migration by utilizing the upstream TCR signaling inhibitor Desatineb, which inhibits LCK activation, as well as the downstream TCR signaling inhibitor FK506, which inhibits calcineurin. We are confident that future data will continue to support our model of alternative pathway activation of Rac resulting in T cell polarization and lymph node egress.

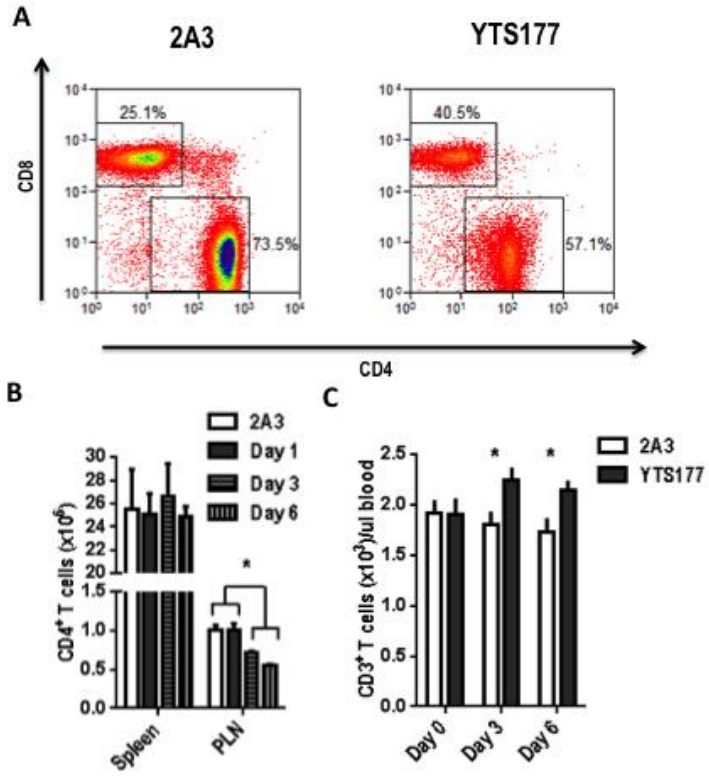


Figure 6. T cells migrate from lymph nodes and enter circulation following administration of YTS177.

(A) Representative flow cytometric plots of CD3+ T cells isolated from the PLN of mice 6 days post treatment of 600 μg of isotype control 2A3 (left) or YTS177 (right). (B) The number of CD4+ T cells isolated from the spleen and PLN of 2A3 or YTS177 treated mice at day 1, 3, or 6 post treatment (n=3; p≤0.05, Student t test) (C) The number of CD3+ T cells per μl of blood in mice treated with either 2A3 or YTS177 at day 0, 3, or 6 post treatment (n=10; p≤0.05, Student t test).

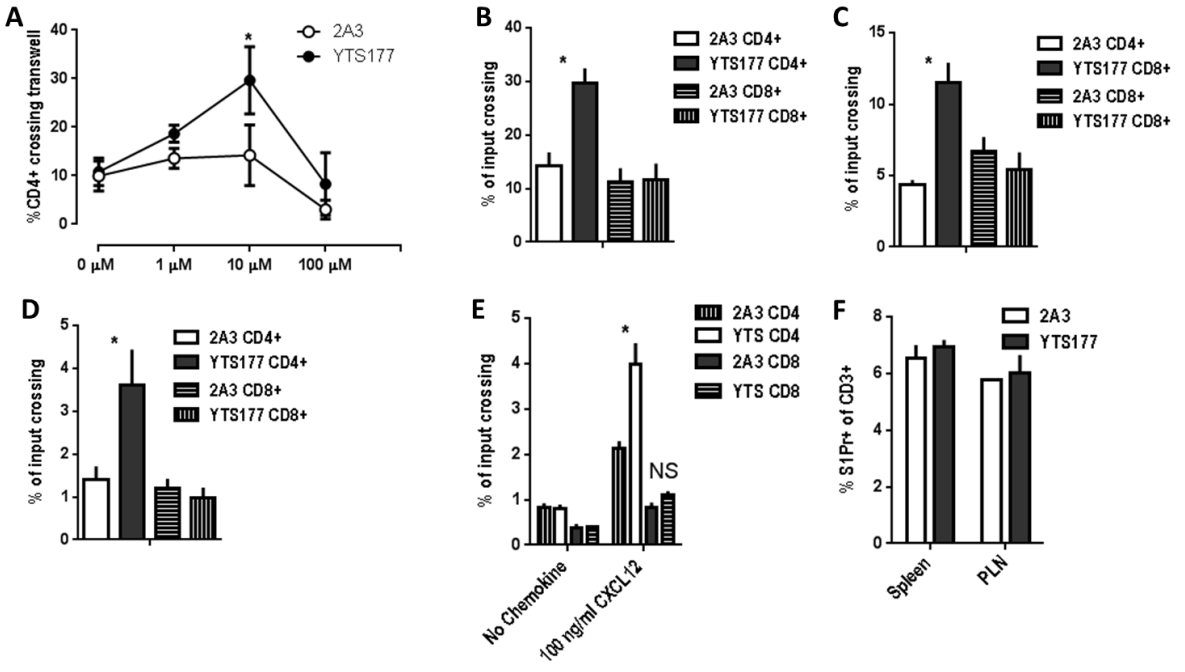


Figure 7. CD4+ T cells exhibit increased sensitivity to chemokines after YTS177 treatment.
(A) Percent chemotaxis of input CD4+ T cells in response to varying concentrations of S1P (n=3; p<0.05; Student t test). Percentage of input CD4+ or CD8+ T cells migrating across a 5 μM (B) unmanipulated (n=6), (C) collagen (n=4), or (D) endothelial cell (n=6) coated transwell in response to 10 μM S1P (p<0.05, Student t test). (E) Percentage of input CD4+ T cells migrating across a transwell in response to 100 μM CXCL12 (n=6; p≤0.01, Student t test). (F) Flow cytometric analysis of S1Pr stained PLN derived CD4+ T cells 48 hours post YTS177 treatment (n=3).

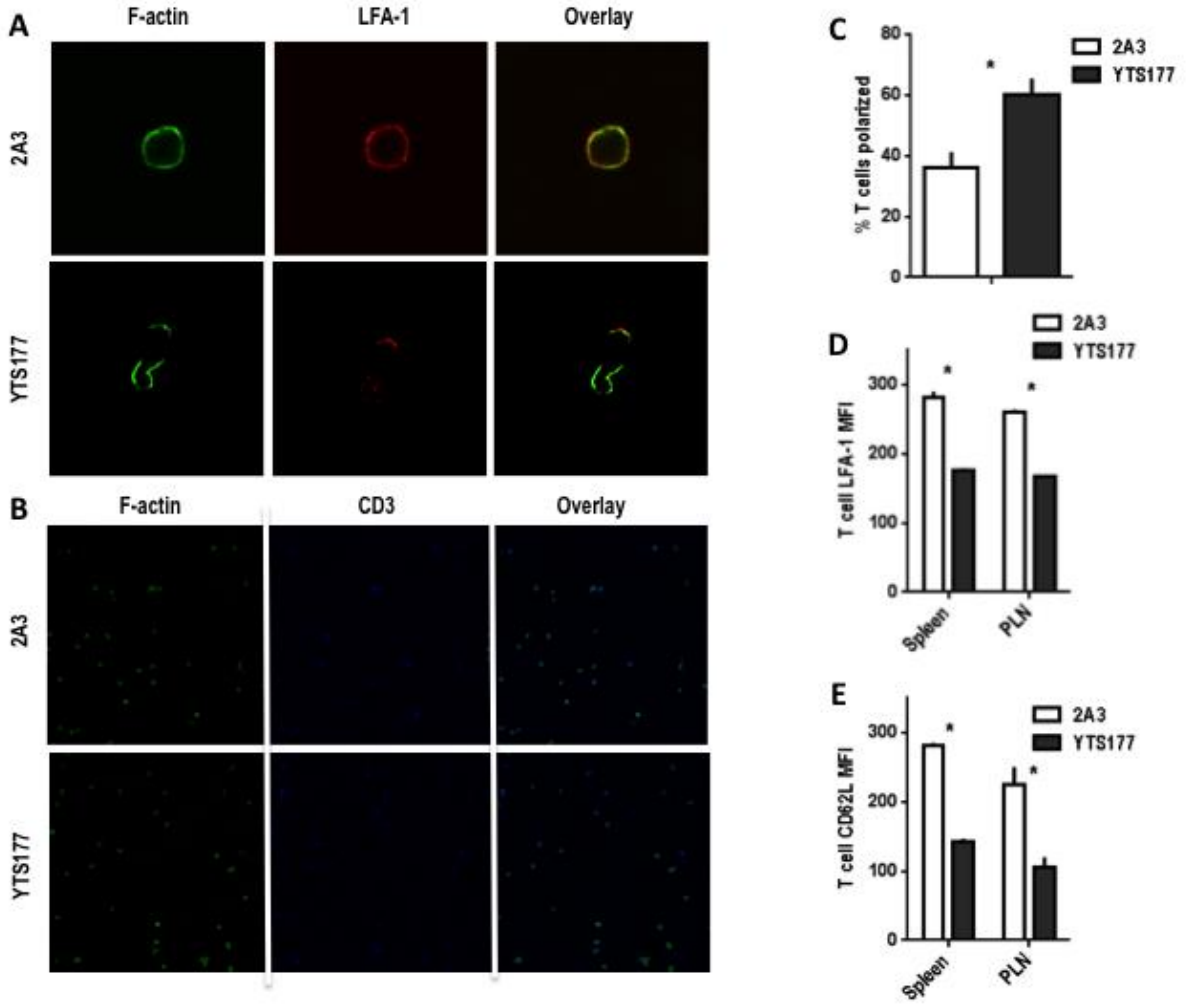


Figure 8. PLN derived T cells rapidly adopt a polarized phenotype following YTS177 treatment. *(A) Representative images of YTS177 treated/polarized and isotype control 2A3 treated/unpolarized T cells exhibiting localization of F-actin (green) and LFA-1 (red) (400X magnification with digitial zoom). (B) Representative images used for quantification of polarization stained for F-actin (green) and CD3 (blue). (C) Quantification of polarization of PLN-derived T cells harvested 48 hours post-YTS177 or 2A3 treatment, at least 500 T cells were scored from each mouse (n=5; $p \leq 0.05$, Student t test). LFA-1 (D) and CD62L (E) expression was assessed for CD4+ T cells isolated from YTS177 or 2A3 treated animals 6 hours post treatment (n=6; $p \leq 0.005$, Student t test).*

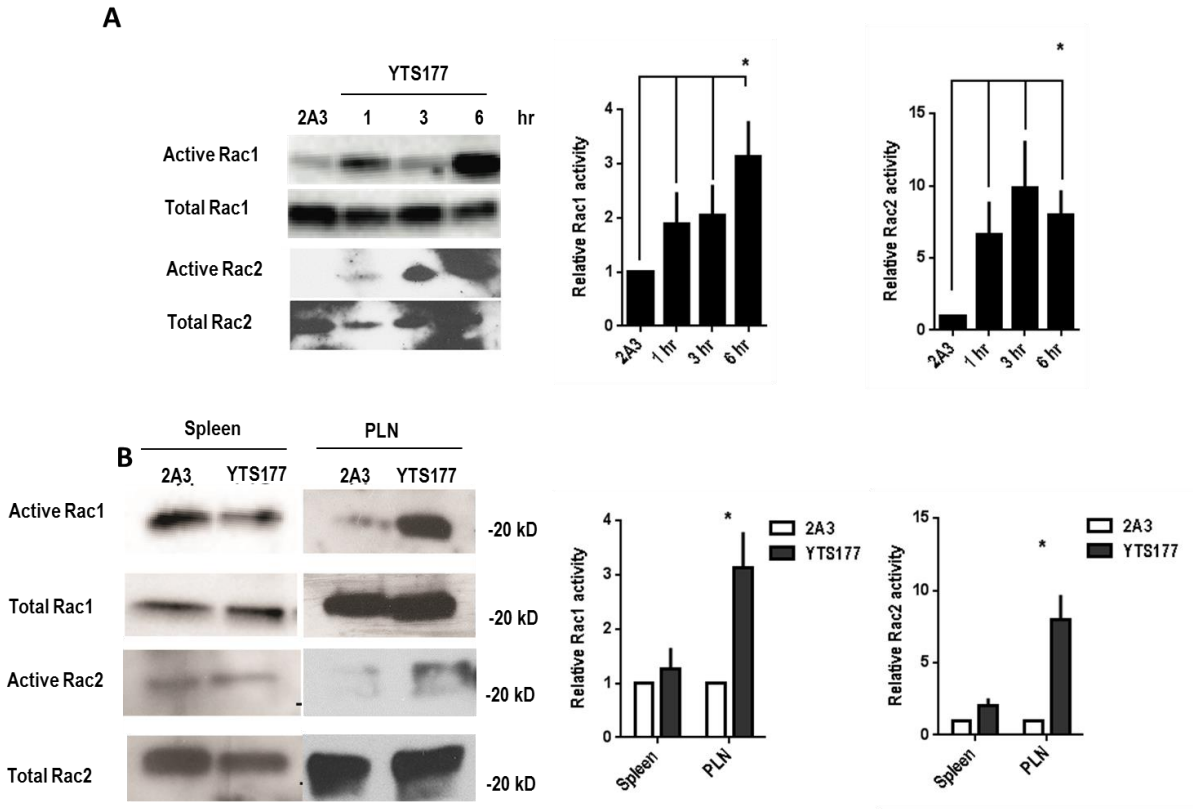


Figure 9. Increased Rac1 and Rac2 activity in PLN T cells after YTS177 treatment.
(A) Active Rac1 was examined in T cells isolated from NOD mice at 1 (n=3), 3 (n=3), and 6 hours (n=7) post-2A3 or -YTS177 treatment and measured via immunoblot (left); relative Rac activity is presented as a fold increase in densitometric readings (right) comparing YTS177 vs 2A3 treated ($p \leq 0.05$, Student t test). (B) Similarly, active Rac1 and Rac2 was examined for T cells isolated from the spleen (n=5) and PLN (n=7) of 2A3 or YTS177 treated NOD mice 6 hours post-treatment; graphs report a densitometric fold increase in YTS177 versus 2A3 treated ($P \leq 0.05$, Student t test).

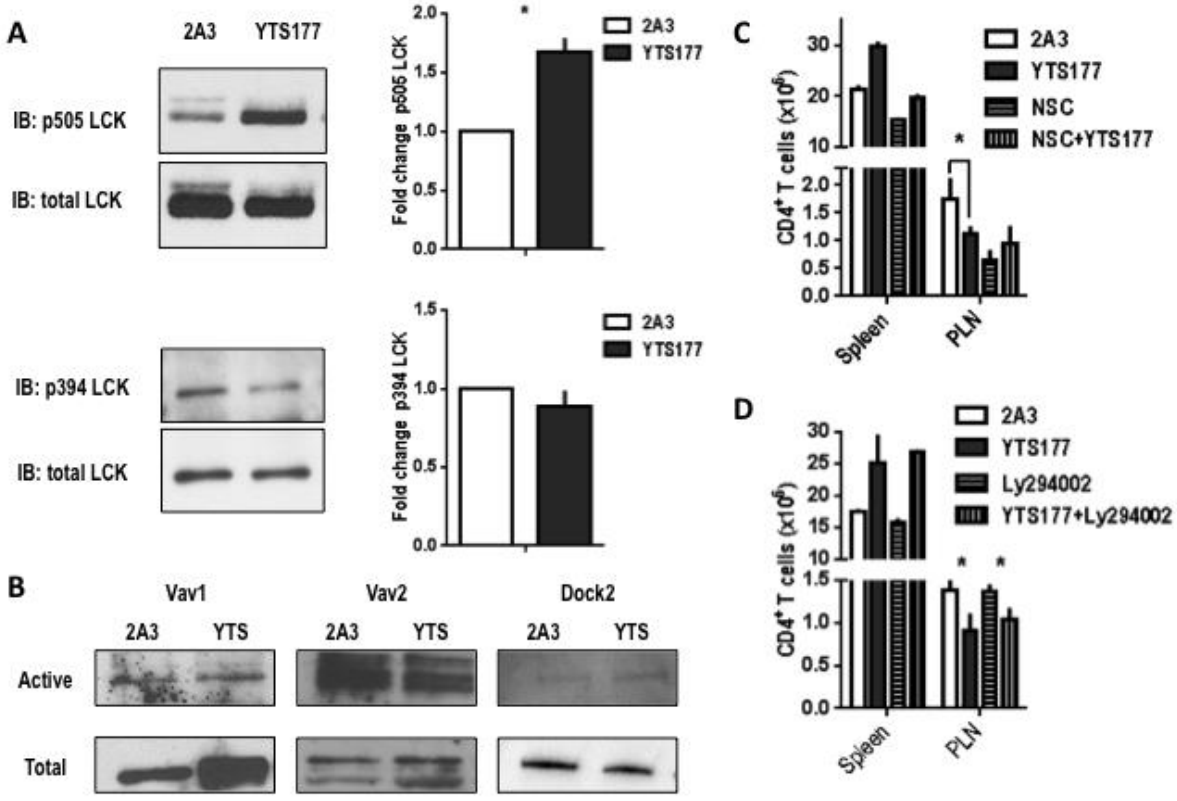


Figure 10. Altered activity upstream of Rac activation in T cells following YTS177 treatment.
(A) Representative immunoblots (left) for p505.LCK (top) and p394.LCK (bottom) with corresponding densitometric measurements (right) (n=3; p≤0.05, Student t test). (B) Representative GEF immunoblots following Rac.G15A pulldown of cells isolated from the PLN of YTS177 or isotype treated mice 6 hours post treatment. (C) CD4+ T cell numbers in the spleen and PLN of NOD mice 5 days post-YTS177 or 2A3 treatment ± Rac.GEF inhibitor NSC237666 injected every 12 hours (n=5; p≤0.05, Student t test). (D) CD4+ T cell numbers in the spleen and PLN of NOD mice 3 days post-YTS177 or 2A3 treatment ± PI3K inhibitor Ly294002 or PBS injected daily (n=3; p≤0.05, Student t test).

Chapter 4: Discussion

Discussion

Two major themes have been discussed in this dissertation: 1) the definition of a narrow age window during which thymic development of autoreactive T cells is increased, and 2) the description of a novel signaling cascade transduced by antibody binding to CD4 that in turn establishes a pro-migratory phenotype in T cells.

The key events driving thymic development of autoreactive T cells are ill-defined. Our thymus transplant approach described in Chapter 2 provides a "snapshot" of thymic production of autoreactive T cells as a function of age. Notably, development of autoreactive T cells was largely restricted to a period within 10 days post-birth. We argue that this is the consequence of reduced efficiency in thymic negative selection, which may be due to a number of mutually nonexclusive scenarios involving temporal changes in: thymic stroma maturation, and the source of thymocyte precursors and related intrinsic properties (Figure 11). Since, thymic epithelial cells (TEC) require interactions with thymocytes to fully mature, it is possible that due to limited interactions early in ontogeny, TEC mostly exhibit an immature phenotype which would favor inefficient T cell selection. As mice age and the thymus undergoes marked structural reorganization of the cortex and medulla, interactions between thymocytes and TEC are more readily facilitated to enhance maturation and in turn the function of TECs.

The source of T cell precursors may also play a role in the thymic development of autoreactive T cells. The liver is the primary source of thymocyte precursors up until day 13 of embryonic development, after which, precursors are derived from bone marrow. Consequently, early after birth the majority of thymocytes will have been derived from "liver" thymocyte precursors. Preliminary experiments comparing embryonic liver versus adult bone marrow in the development of β cell autoimmunity are consistent with this model. Various aged NOD.*scid* thymi were transplanted under the kidney capsule of NOD.*scid* recipients, which then received embryonic liver- or adult bone marrow-derived stem cells. Development of insulitis was only observed in recipients of both newborn thymi and embryonic liver-derived thymocyte precursors. These data suggest that embryonic precursors promote the development of a pool of thymocytes sufficient to drive autoimmunity. The latter may reflect for instance reduced sensitivity of embryonic liver-derived thymocytes to apoptosis induction. Notably, induction of apoptosis has been shown by some to be limited in NOD thymocytes. Interestingly, various studies have reported differential Vβ chain usage between embryonic and adult thymocyte precursors (45, 46) suggesting development of T cells with distinct specificities. With this in mind, it is possible that the TCR repertoire of thymocytes derived from embryonic liver may exhibit a bias towards self-reactivity, which combined with inefficient thymic selection readily leads to the development of autoreactive T cells early after birth. The TCR repertoire is also likely to be further influenced by the peptide binding properties of MHC class I and II molecules. Susceptibility to type 1 diabetes in NOD mice and humans is strongly associated with specific alleles of MHC class II molecules. In NOD mice for instance, IA^{g7} (165) has been reported to exhibit poor peptide

binding properties, which is expected to limit the efficacy of negative (166) and positive (167) selection.

We have provided data supporting a model where a defect in central tolerance early in ontogeny results in the escape of autoreactive T cells, and in NOD mice, the development of autoimmunity. We have also provided evidence that the development of T cell mediated disease in some autoimmune prone organs is independent of the transcription factor AIRE. Although important observations, further elucidation of the mechanisms defining the temporal production of autoimmune T cells would prove beneficial from both a basic immunology standpoint, as well as providing additional targets with which to combat autoimmune disease. We have provided a number of hypotheses which could result in the age dependent development of autoimmunity, including incomplete thymic development early in ontogeny, as well as the source of thymocyte precursors changing during development.

Investigating the role of thymocyte precursors in the temporal development of autoimmunity could be accomplished *in vitro* utilizing a reaggregate thymic organ culture approach, where thymocyte depleted thymic stroma from different aged NOD mice are cultured with double negative thymocytes from donors of various ages, followed by IAg7-peptide multimer staining and FACS analysis for diabetogenic specificities. Development of autoreactive T cells in only those cultures seeded with embryonic precursor would suggest a role for precursor origin in the temporal production of autoreactive T cells. In contrast, increased production of autoreactive T cells in cultures seeded with only newborn thymic stroma, regardless of precursor source, would suggest a role for thymic age in the observed temporal development of autoreactive T cells. An *in vivo* approach to determining precursor

involvement would be similar to those described earlier in this chapter, where newborn NOD.*scid* mice would be given T cell precursors isolated from various aged NOD mice. Development of autoimmunity in only those recipients of embryonic precursors would again suggest precursor involvement in the age dependent development of autoimmunity.

In order for the development of type 1 diabetes, T cells must first traffic to the PLN, remain in the lymph node and interact with APCs presenting self-peptide-MHC complexes to become activated, followed by migration into the pancreas to mediate β cell destruction. Preventing any step of T cell migration, retention, or activation has been shown by our studies and those of others to have marked effects on the disease process.

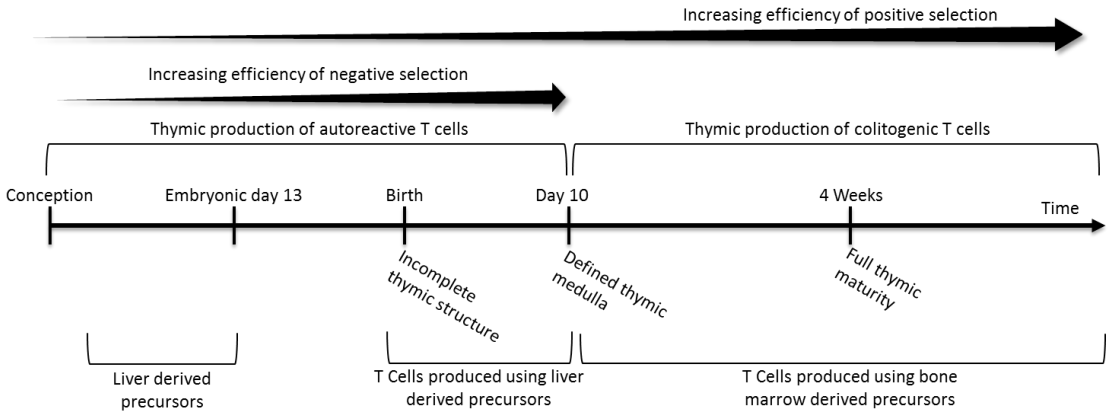
Our studies described in Chapter 3 define a unique pathway that promotes a T cell pro-migratory program via engagement of the CD4 coreceptor (Figure 12). This effect is independent of TCR signaling since purging of the PLN and pancreas is seen for all T cells, including naïve T cells. The observed transient T cell polarization, reduction in retention cues, and increased migration in response to Rac activation, results in lymph node egress; downregulation of adhesion molecules is then believed to prevent T cells from returning to the lymphatics.

We were unable to identify the upstream GEF resulting in Rac activation, or the direct downstream effector of CD4. Known activating GEFs were ruled out by either immunoblotting or utilizing GEF inhibitors. The ultimate cellular phenotype arising from small RhoGTPase activation is reliant on the activating GEF as well as its localization. There are many described Rac GEFs (150), however, in the context of T cells, there are few. The dependence of Rac activation on migration, in addition to the highly motile nature of T cells, make it surprising that additional GEFs have not been described. A mass spectrometry

approach can be used to identify either a novel GEF, or a GEF previously not described in the context of T cell migration. Identification of a Rac GEF in our system would provide insight into T cell migration and circulation outside of an inflammatory environment, as well as novel targets for modulating T cell activity.

Following antibody mediated crosslinking of CD4, we have identified the activation of an Lck independent pathway. We were unable to identify the direct downstream effector which results in activation of the signaling cascade. One method to determining the initiator of the signaling cascade would be utilizing a pulldown approach. Briefly, pulldown of the CD4 molecule in T cells isolated from YTS177 or isotype treated mice, followed by gel electrophoresis and silver staining could determine a unique protein band in CD4 crosslinked T cells. Identification of the unique molecule would be accomplished utilizing a mass spectrometry approach. Determining the direct downstream effector of CD4, as well as the GEF resulting in Rac activation would complete the identification of the signaling cascade, which provides a cellular mechanism for T cell migration in the absence of TCR activation.

Figure 11: A model for age dependent thymic production of autoreactive and colitogenic T cells. *Incomplete thymic medullary structure and utilization of T cell precursors derived from embryonic liver result in reduced efficiency of negative selection and increased escape of autoreactive T cells up until day 10 post birth. Better defined medulla and utilization of bone marrow derived thymocyte precursors in animals older than 10 days, results in increased efficiency of both negative and positive selection, resulting in reduced production of autoreactive T cells and increased production of T cells reactive to gut microbiota (colitogenic).*



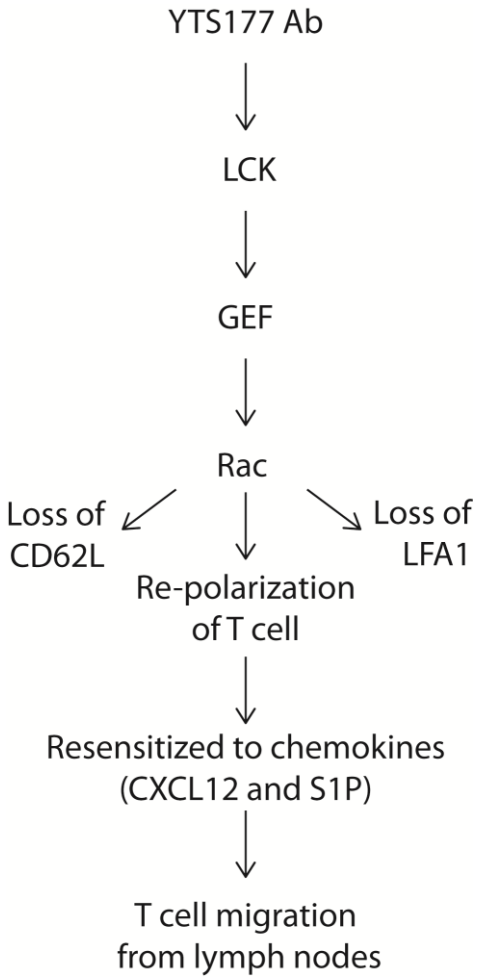


Figure 12. Model for altered T cell trafficking following YTS177 mediated crosslinking of CD4. *CD4 crosslinking in lymph nodes results in downstream Rac1 and Rac2 activation independent of LCK, PI3K, and the Rac GEFs Tiam1, Dock2, Vav1, Vav2, and Trio. T cells then become polarized and exhibit a reduction in the lymph node retention molecule LFA-1 as well as the cell adhesion molecule CD62L. Increased sensitivity to S1P and chemokines results in lymph node egress where the reduction of cell adhesion molecules prevents re-entry into the lymphatics.*

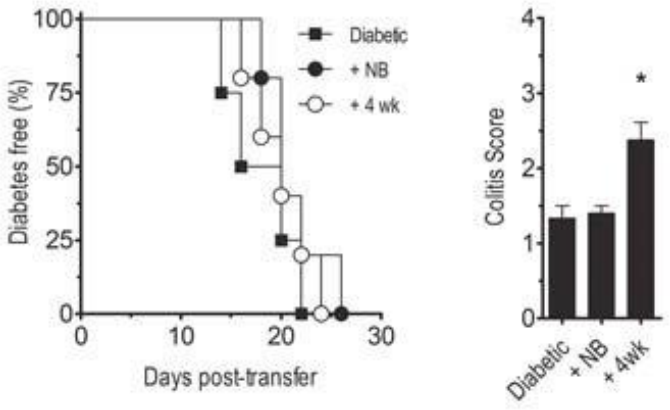
Conclusion

In summary, these studies provide new insight into events: i) that influence thymic development of autoreactive T cells, and ii) that regulate T cell migratory properties during an inflammatory response. These findings may be further exploited in the future to prevent and treat type 1 diabetes. Prevention of type 1 diabetes may be the most effective when

intervening with a given strategy in neonates found to be at high risk based on genotyping for instance. In addition, the signaling pathway(s) engaged by CD4 crosslinking may provide new targets to selectively modify the migratory properties and block the pathogenicity of T cells mediating unwanted inflammatory responses.

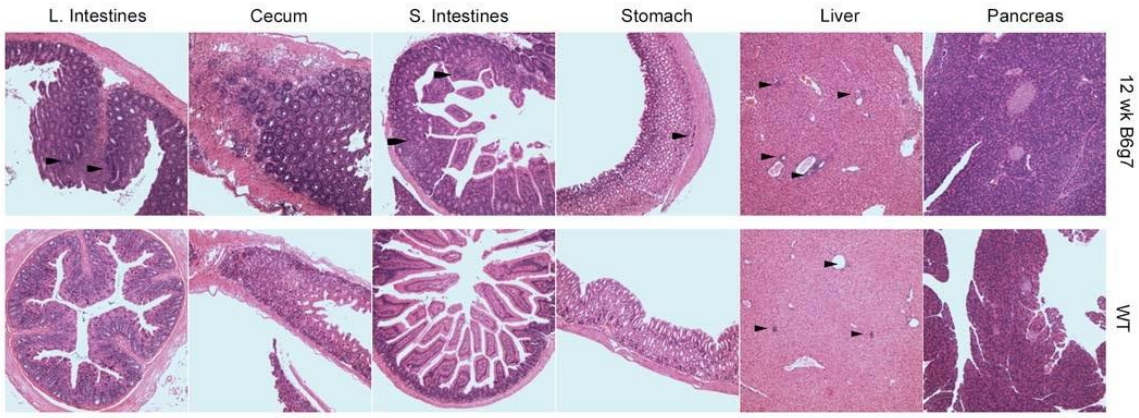
Appendix

Appendix 1. Diabetes development is unaffected by colitogenic T cells.
*Diabetes incidence (left) and colitis scores (right) for NOD.scid recipients of splenocytes (107) from diabetic NOD donors alone (n=3), or mixtures of diabetic splenocytes (107) plus splenocytes (107) 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.*



Appendix 2. Organ infiltration in NOD.scid recipients of 12 wk-old B6^{g7} 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.

.



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