Tolerance Induction in Recent Thymic Emigrants

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Abstract

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Recent thymic emigrants (RTEs) comprise the subset of T cells that have most recently undergone thymic maturation and egress to the lymphoid periphery. RTEs are an important lymphocyte population to study because they represent the majority of T cells in neonates and contribute to T cell diversity throughout life. Using RAG2p-GFP transgenic mice to identify RTEs, our lab has discovered that T cell development, once thought to be completed within the thymic microenvironment, continues for several weeks after cells have left the thymus and entered the lymphoid periphery. While it is now appreciated that post-thymic maturation is the norm, it remains unclear what advantages are provided to the individual by the export of T cells that interpret and respond to their immunological environment in a manner so distinct from that of their mature, but still naïve counterparts. We hypothesized that post-thymic maturation entails a T cell developmental period that facilitates tolerance induction to peripheral antigens, both self and commensal-derived, not previously encountered during thymic development. Using a well-established mouse model of peripheral self-antigen, we show here that the first few weeks of T cell residence in the lymphoid periphery define a period of heightened susceptibility to tolerance induction to tissue-restricted antigens (TRAs), the outcome of which depends on the context in which RTEs encounter antigen. Following encounter with TRAs in the absence of inflammation, RTEs exhibited defects in proliferation, diminished cytokine production, elevated expression of anergy-associated genes, and diminished ability to cause diabetes. These properties were mirrored *in vitro* by enhanced RTE susceptibility to regulatory T cell-mediated suppression. In the presence of inflammation, RTEs and mature T cells were, in contrast, equally capable of inducing diabetes, proliferating, and producing cytokines. Thus, recirculating RTEs encounter TRAs during a transitional developmental stage that facilitates tolerance induction, but inflammation converts antigen-exposed, tolerance-prone RTEs into competent effector cells.

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List of Abbreviations

Abx – antibiotics

AIRE – autoimmune regulator

APC – antigen presenting cell

B6 – C57BL/6

BrdU – bromodeoxyuridine

Cbl-b – casitas B-cell lymphoma-b

cDNA – complementary DNA

CDR – complementarity determining region

CFA – complete Freund’s adjuvant

CFSE – carboxyfluorescein succinimidyl ester

D – diversity gene segment

DC – dendritic cell

Divs – divisions

DGK – diacylglycerol kinase

DN – double negative

Egr-2 – early growth response-2

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

Foxp3 – forkhead box P3

FR4 – folate receptor-4

GALT – gut-associated lymphoid tissue

GFP – green fluorescent protein

GRAIL – gene related to anergy in lymphocytes

HBSS – Hank’s balanced salt solution

HPRT – hypoxanthine guanine phosphoribosyltransferase

IBD – inflammatory bowel disease

IEL – intraepithelial lymphocyte

IFNγ – interferon gamma

IL – interleukin

iLN – inguinal lymph node

IRES – internal ribosome entry site

J – joining gene segment

LAG-3 – lymphocyte-activation gene-3

LPL – lamina propria lymphocyte

MFI – mean fluorescence intensity

MHC – major histocompatibility complex

mLN – mesenteric lymph node

MN – mature naïve

NP – nitrophenylacetyl

NS – not significant

OT-I – MHC class I restricted OVA-specific TCR Tg

OT-II – MHC class II restricted OVA-specific TCR Tg

OVA – ovalbumin

pancLN – pancreatic lymph node

PCR – polymerase chain reaction

PFA – paraformaldehyde

pLN – peripheral lymph node

PMA – phorbol 12-myristate 13-acetate

PP – Peyer’s patch

PTK7 – protein tyrosine kinase 7

RAG – recombination activating gene

RAG2p-GFP Tg – mice that express GFP under control of the RAG2 promoter

RFP – red fluorescent protein

RIP-mOVA Tg – mice that express membrane bound OVA under control of the rat insulin promoter

RPMI – Roswell Park Memorial Institute

RTE – recent thymic emigrant

SD – standard deviation

SEM – standard error of the mean

SP – single positive

spl – spleen

Tconv – conventional T cell

TCR – T cell receptor

Tg – transgenic

Th – T helper

TRA – tissue-restricted antigen

TREC – TCR rearrangement excision circle

Treg – regulatory T

Unstim – unstimulated

V – variable gene segment

VLA-4 – very late antigen-4

WT – wild-type

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Chapter One: Introduction

The goal of this introduction is to describe the field at the time this thesis work was initiated and to lay the foundation for the experiments that constitute this thesis.

T cells and immunity

In order to ensure host survival upon infection, the immune system must respond to the constantly evolving world of microbial pathogens with specificity and rapidity. While the innate immune system activates rapidly following infection, its response relies on the recognition of evolutionarily conserved patterns, and is therefore nonspecific. The adaptive immune response, on the other hand, is delayed, but possesses the ability to recognize antigen-specific traits that are unique to individual pathogens. This specific immunity is provided by B and T cells, whose antigen receptors recognize and trigger a cellular response to a diverse array of pathogens. It is the coordinated response of both the innate and adaptive immune systems that ensures effective pathogen clearance following infection (1).

B cells, which develop in the bone marrow, provide humoral, or antibody-mediated immunity. T cells, on the other hand, develop in the thymus and provide cell-mediated immunity. T cells can be divided into two broad subsets based on their expression of the heterodimeric T cell antigen receptor (TCR). γδ T cells express TCRs that are comprised of a γ- and δ-chain. Because their TCRs are limited in diversity, they exhibit more innate-like characteristics by responding to conserved antigens (2). Alternatively, conventional T cells (Tconv) express TCRs that are comprised of an α- and β-chain. αβ T cells predominate in the lymphoid periphery and comprise the major subset of T cells involved in the adaptive immune response; these cells are the principal research subjects of this thesis work.

Based on their expression of the co-receptors CD4$^+$ and CD8$^+$, αβ T cells can be further divided into two main groups. CD4$^+$ T cells, otherwise known as T helper (Th) cells, are necessary for the elimination of extracellular pathogens and the regulation of immune responses (3). These cells recognize antigen bound to Class II major histocompatibility complex (MHC) molecules on the surface of professional antigen presenting cells (APCs) such as dendritic cells (DCs) and B cells (4, 5). Full activation of the T cell, however, requires both a productive TCR:MHC interaction and a second signal through a costimulatory molecule such as CD28, which interacts with CD80/86 on the surface of a DC or B cell (6). Once the two-signal activation is complete, Th cells proliferate and provide help in the form of secreted cytokines to other cell types, including B cells, CD8$^+$ T cells and macrophages. A third signal is required to amplify the T cell response and is determined by the inflammatory milieu, often provided by innate immune cells (7). CD8$^+$ T cells recognize antigen bound to Class I MHC molecules, which are expressed on the surface of all nucleated cells (4, 5). Following infection with a virus or intracellular pathogen, Class I MHC-expressing cells process foreign antigen into peptide fragments to present on the cell surface. Upon peptide:MHC recognition in the presence of costimulation, CD8$^+$ T cells differentiate into cytotoxic T lymphocytes. Once activated, these cells secrete effector proteins such as perforin and granzyme B to kill infected or otherwise damaged cells. Cytotoxic T lymphocytes are thus important for the elimination of viruses and intracellular pathogens (8).

αβ T cell development

Pluripotent hematopoietic stem cells located in the adult bone marrow give rise to T cell progenitors that travel through the blood to the thymus where they differentiate and undergo extensive selection that results in a diverse and functional, but self-tolerant T cell pool. This process begins at the cortico-medullary junction of the thymus where the earliest T cell progenitors to arrive are $CD4^-CD8^-$ double negative (DN) cells. Development proceeds as thymocytes move through the cortex and back to the medulla, as the cells traverse a series of DN stages (DN1-4) defined on the basis of their expression of the activation markers CD44 and CD25.

Rearrangement of the TCR β-chain gene (*Tcrb*) occurs first and begins in DN2, following expression of the recombination activating gene-1 (*Rag1*) and -2 (*Rag2*) encoded proteins. β-chain gene rearrangement is completed in DN3 and requires first the successful recombination of Diversity (D) and Joining (J) gene segments, followed by Variable (V) to DJ gene segment recombination (9). Following successful rearrangement, the expressed β-chain is paired with an invariant pre-TCR α-chain in association with CD3 to form a complete pre-TCR. This assembly triggers signaling through the pre-TCR which drives β selection, a process that triggers cell proliferation, preventing further β-chain rearrangement through allelic exclusion, and promotes progression to the $CD4^+CD8^+$ double positive (DP) stage (10).

Once proliferation subsides, *Rag1* and *Rag2* are reexpressed, and TCR α-chain (*Tcra*) rearrangement is completed following successful V to J recombination. The presence of multiple V and J gene segments at the *Tcra* locus allows T cells that initially fail to make a productive α-chain rearrangement to undergo successive recombination attempts in order to generate a complete αβ TCR (9, 11).

The ability to pair a large number of β-chain V, D, and J gene segments together with α-chain V and J gene segments allows for the generation of a population of T cells expressing a diverse array of TCRs. This combinatorial diversity is further amplified by the addition of nontemplated P- and N-nucleotides between the junctions of these gene segments (12). Within each TCR β- and α-chain, three hypervariable regions exist. These regions, known as complementarity determining regions (CDRs) 1-3, correspond to loops that extend from the TCR and come into close contact with peptide:MHC complexes. CDR3 is encoded in part by the areas of P- and N-nucleotide additions and is the most variable of these regions (13).

Following successful rearrangement of α-chain genes and surface expression of the αβ TCR, DP thymocytes migrate through the thymic cortex where they undergo positive selection. Positive selection ensures that newly generated αβ TCRs expressed by developing thymocytes are functional and capable of recognizing antigen in the context of self-MHC molecules (14-16). During this process, DP thymocytes must bind self-peptide:self-MHC complexes expressed by specialized cells in the thymus called thymic cortical epithelial cells. Only those cells that bind self-peptide:MHC with low affinity will survive positive selection, while those that do not bind will undergo apoptotic “death by neglect.” Positive selection also determines which co-receptor a DP thymocyte will maintain, either CD4 or CD8, depending on whether the thymocyte interacts with MHC Class II or Class I, respectively.

Following positive selection, single positive (SP) thymocytes migrate back to the thymic medulla, where they undergo negative selection, otherwise known as central tolerance. While positive selection ensures the generation of a functional αβ TCR, negative selection removes SP thymocytes expressing potentially autoreactive TCRs (16). During this process, SP thymocytes bind “tissue-restricted” antigen (TRA) whose expression is regulated in the thymus by the autoimmune regulator (AIRE) transcription factor in medullary thymic epithelial cells (17). While SP thymocytes that bind self-peptide:MHC with high affinity undergo clonal deletion, thymocytes that pass negative selection enter the lymphoid periphery as naïve CD4$^+$ or CD8$^+$ T cells that are tolerant to self, but capable of recognizing foreign antigen presented on self-MHC. As a result of death by neglect and β positive and negative selection, greater than 95% of thymocytes die, highlighting the importance of selection processes for the generation of a functional and self-tolerant peripheral T cell pool (17).

Recent Thymic Emigrants

Recent thymic emigrants (RTEs) comprise the subset of T cells that have most recently undergone thymic development and egress to the lymphoid periphery. Along with homeostatic proliferation, thymic export of RTEs helps maintain the peripheral T cell pool in healthy individuals. RTEs comprise the majority of T cells in neonates and help maintain TCR repertoire diversity throughout life, despite reduced thymic output in the aged (18-20). The role of RTEs in maintaining TCR repertoire diversity is illustrated in female mice, who gradually lose reactivity to the male H-Y antigen following thymectomy (21). Moreover, RTEs help reconstitute the peripheral T cell pool following lymphoablative viral infections or therapies, such as HIV infection and chemotherapy (22). Thus, RTEs constitute a clinically important lymphocyte population to study.

Our current understanding of RTE biology has benefited from novel immunological tools that helped improve the identification and isolation of RTEs from their more mature, but still naïve (MN) T cell counterparts. One early roadblock to the study of the RTE compartment was their lack of a unique cell surface marker. Various methods have been employed in order to label RTEs, including direct injection of fluorescein isothiocyanate (FITC) into the mouse thymus. Isolation of FITC$^+$ peripheral T cells 18-24 hours later accurately identifies RTEs, but also inadvertently labels mature T cells recirculating through the thymus (23). RTEs can also be identified in mice using bromodeoxyuridine (BrdU) treatment. BrdU is a synthetic nucleoside that is incorporated into the deoxyribonucleic acid (DNA) of dividing cells. Rapidly dividing thymocytes take up BrdU, and following thymic egress, they remain BrdU^{lo} in the periphery (24). However, because cells must be permeabilized in order to identify the BrdU label, functional studies in BrdU^{lo} RTEs are precluded. In humans, RTEs can be identified by the appearance of TCR rearrangement excision circles (TRECs), which are nonreplicating pieces of extrachromosomal DNA left over from TCR gene rearrangement. Although TRECs are enriched in RTEs, not all TREC$^+$ cells are RTEs. Furthermore, cells are killed in the process of TREC extraction, preventing the phenotypic and functional analysis of the cell (25). RTEs can also be identified as CD31$^+$ (26) and protein tyrosine kinase 7$^+$ (PTK7$^+$) T cells (27) in humans. The problem with these markers is that they only accurately identify CD4$^+$ and not CD8$^+$ RTEs. Clearly, better methods are required for the study of RTE biology.

The development of a transgenic (Tg) mouse model in which live RTEs can be accurately identified and isolated from their MN counterparts has allowed for a more thorough analysis of RTE biology. In these mice (RAG2p-GFP Tg), a transgene encoding green fluorescent protein (GFP) is driven under control of the *Rag2* gene promoter. Although *Rag2* expression is turned off during the DP stage of T cell development, residual GFP signal lingers in thymocytes as they move out into the lymphoid periphery, marking RTEs as GFP$^+$ peripheral T cells (28). Importantly, the GFP signal strength correlates inversely with the time since loss of *Rag2* expression, such that the brightest cells for GFP in the periphery are the youngest RTEs (29). Analyses of RTEs isolated as GFP$^+$ peripheral T cells from RAG2p-GFP mice have revealed that T cell maturation, a process once thought to be completed within the thymic microenvironment, continues for several weeks after cells have left the thymus and entered the lymphoid periphery (28).

Phenotypic maturation of RTEs includes downregulation of TCR/CD3 and CD24 and upregulation of Qa2, CD28, IL-7Rα, and CD45RB surface antigens (18, 23, 28, 30, 31). These changes signal a progressive maturation process that requires both T cell exit from the thymus and access to secondary lymphoid organs, but does not result in selective RTE survival or proliferation (32), nor is it driven by molecules known to influence T cell homeostasis (33).

Echoing these phenotypic differences, RTEs also exhibit functional differences relative to their mature counterparts. Compared to mature T cells, *in vitro* stimulated $CD4^+$ RTEs exhibit diminished proliferation, IL-2Rα (CD25) expression, and IL-2, TNFα, and IFNγ production (28, 34-36). Under Th2 polarizing conditions both *in vitro* and *in vivo*, neonatal and adult $CD4^+$ RTEs produce more IL-4, IL-5, and IL-13 than their mature counterparts, skewing them away from the Th1 and toward the Th2 lineage (19, 35, 37). $CD8^+$ RTE and mature T cell functions also differ. Compared to mature T cells, *in vitro* stimulated $CD8^+$ RTEs exhibit diminished proliferation and CD25 upregulation (28). *In vivo*, antigen-specific $CD8^+$ RTEs are deficient in their production of IL-2 and IFNγ following infection with bacteria expressing a model antigen (28, 38). Moreover, relative to their mature counterparts, $CD8^+$ RTEs exhibit increased responses to low affinity ligands and improved access to peripheral sites of inflammation due to their enhanced expression of the tissue homing receptor very late antigen-4 (VLA-4) (39). While the mechanistic causes of these differences remain unclear, hypermethylation of cytokine promoter regions, as well as increased expression of DNA modifying enzymes by $CD4^+$ RTEs, suggest that RTEs are uniquely poised to rapidly alter their DNA methylation by both adding and removing repressive methyl groups (40). Together, these data argue that RTE function is not universally defective, but is modulated by an interpretation of the antigenic milieu that is distinct from that of mature T cells.

T cell peripheral tolerance

To ensure the generation of functional, self-tolerant T cells during thymic development, self-reactive thymocytes are negatively selected during the process of central tolerance. This process is imperfect, however, in part because not all peripheral self-antigens are expressed in the thymus (41), including those expressed by commensal bacteria of the gut (42). As a result, T cells recognizing self- and commensal-derived antigens escape deletion and enter the lymphoid periphery.

To avoid damage to the host, the immune system employs several peripheral tolerance mechanisms to prevent aberrant activation of these cells. One such mechanism is deletion, or programmed cell death. During this process, one of two apoptotic pathways is typically induced. In the cell intrinsic pathway, activation of the pro-apoptotic molecule BIM results in permeabilization of the outer mitochondrial membrane and subsequent activation of the executioner enzyme caspase 9 (43). In the cell extrinsic pathway, apoptosis can be induced through engagement of cell-surface death receptors such as Fas. Stimulation of these receptors activates caspase 8, which induces cell death (43).

Another peripheral tolerance mechanism is the induction of a functionally unresponsive state, or anergy. During a protective immune response, T cell activation requires stimulation of TCR through the recognition of peptide:MHC complexes, as well as engagement of co-stimulatory molecules such as CD28. Conversely, anergy is induced upon TCR stimulation in the presence of weak costimulatory and IL-2R signals (44). The upregulation of multiple genes is associated with the anergic state, including early growth response-2 and -3 (Egr2 and Egr3, respectively), diacylglycerol kinase-α and -ζ (DGKα and DGKζ, respectively), casitas B-cell lymphoma-b (Cbl-b), Itch, Ikaros, lymphocyte-activation gene-3 (LAG-3), and gene related to anergy in lymphoyctes (GRAIL) (44). Defects in proliferation and diminished IL-2 production characterize anergic T cells (44).

The activation of self-reactive T cells in the periphery can also be actively prevented by the activity of CD4$^+$ regulatory T (Treg) cells expressing the transcription factor forkhead box p3 (Foxp3). These immunosuppressive cells dampen immune responses to both self- and foreign-antigens through their actions on a number of immune cells, including effector T cells, B cells, DCs, natural killer cells, and macrophages (45). Similar to conventional CD4$^+$ T cells, most CD4$^+$Foxp3$^+$ regulatory T cells are produced in the thymus, although some Treg cells arise in the periphery and are referred to as "induced" Treg cells (46). Treg cells have been shown to play an essential role in maintaining peripheral tolerance, as evidenced by the widespread autoimmunity that develops in Foxp3-deficient immunodysregulation polyendocrinopathy enteropathy X-linked syndrome patients (47) and *scurfy* mice (48). In fact, it was recently shown that CD4$^+$ T cell tolerance to TRA is often mediated by Treg cells, rather than by deletion (49). Decreased Treg cell activity has also been implicated in the development of a number of more common autoimmune and inflammatory diseases, including type-1 diabetes, rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (50, 51). Treg cells are thought to function through a variety of actions, including both antigen-specific and -nonspecific mechanisms. The TCR repertoire of Treg cells is skewed toward the high-affinity recognition of self-peptides, allowing them to compete with auto-reactive T cells for binding to self-peptide:MHC complexes, and thereby limiting the activation of self-reactive T cells (52). Alternatively, Treg cells can function in an antigen-independent manner by either their secretion of anti-inflammatory cytokines such as IL-10 and TGFβ, or through the consumption of the pro-survival cytokine IL-2, thereby limiting its availability to other T cells (53, 54). Thus, while the activity of Treg cells is essential for peripheral self-tolerance and preventing autoimmunity, uncontrolled Treg cell activity can interfere with protective immune responses directed toward harmful antigens.

Goals of this thesis

While it is now appreciated that post-thymic maturation is the norm, one key question remains unanswered. What is the purpose of this transitional period? One consequence of RTE immaturity is the dampened secretion of potentially harmful inflammatory cytokines, which may serve to limit the immune response directed against both self- and commensal-derived antigens that are not expressed intrathymically. This may be of particular benefit to neonates, who are rapidly colonized with commensal microbes soon after birth (55) and whose entire peripheral T cell pool consists of RTEs (18, 56, 57). That this process may be associated with tolerance induction was first suggested by the fact that, compared to their mature counterparts, RTEs express TCRs with longer average CDR3 lengths (33), reminiscent of the CDR3s of TCRs expressed by pre-negative selection thymocytes (58). These observations led us to hypothesize that the first few weeks after thymic egress comprise a transitional period during which RTEs are prone to tolerance induction upon encountering peripheral antigen not expressed within the confines of the thymus. The thesis work described below was conducted to test this overarching hypothesis. We show here that both $CD4^+$ and $CD8^+$ RTEs, when compared to their MN counterparts, are more prone to tolerance induction upon encountering peripheral self-antigen, and that this response depends upon the inflammatory environment in which RTEs encounter antigen.

Chapter Two: Materials and Methods

Mice

Wild-type (WT) C57BL/6 (B6), B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (B6.CD45.1^{+}), B6xB6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (B6xB6.CD45.1^{+}) and B6.129P2-*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom}/J (TCRβ/δ$^{-/-}$) mice were purchased from The Jackson Laboratory or bred in-house at the University of Washington. RAG2p-GFP Tg mice (59) were backcrossed in our laboratory at least 12 generations onto the B6 background to express one or both of the CD45.1 and CD45.2 congenic markers. These mice were further crossed to OT-I [C57BL/6-Tg(TcraTcrb)1100Mjb/J] (60) and OT-II [C57BL/6-Tg(TcraTcrb)425Cbn/J] (61) mice carrying a transgene-encoded TCR that recognizes chicken ovalbumin (OVA)$_{257\text{-}264}$ plus K^{b} or OVA$_{323\text{-}339}$ plus I-A^{b}, respectively. C57BL/6-*Foxp3*^{tm1Flv}/J (Foxp3-IRES-RFP) (62) and B6.129S7-*Rag1*^{tm1Mom}Tg(TcraTcrb)425Cbn (OT-II RAG1$^{-/-}$) mice were originally gifts from Drs. D. Campbell and S. Ziegler, respectively (Benaroya Research Institute, Seattle, WA), and were bred in-house. B6.129S7-*Rag1*^{tm1Mom}/J (RAG1$^{-/-}$) (63) mice were a gift from Dr. D. Stetson (University of Washington) and were bred in-house. RIP-mOVA [C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ] Tg mice express membrane-bound OVA under the control of the rat insulin promoter in the pancreatic β cells (64). All mice were housed under specific pathogen-free conditions and used in accordance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Cell preparation, staining, and sorting

Single-cell suspensions for adoptive transfer were prepared from water-lysed splenocytes and axial, cervical, brachial, inguinal and mesenteric LNs. T cells from RAG2p-GFP Tg TCR Tg mice were enriched using EasySep Negative Selection Mouse CD4^{+} or CD8^{+} T cell Enrichment

Kits (Stem Cell Technologies), and Fc receptors were blocked with anti-CD16/32 (2.4G2; BD Biosciences). Cells were stained with fluorochrome-conjugated antibodies against B220 (RA3-6B2), CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), NK1.1 (PK136), Ter119 (Ly-76), Vα2 (B20.1), and Vβ5 (MR9-4), all from BD Biosciences, BioLegend, or eBioscience. Following surface staining for 20 minutes at 4°C, cells were sorted to >98% purity as $\mathrm{CD44^{lo}CD62L^{hi}CD4^{-}B220^{-}CD11b^{-}NK1.1^{-}Ter119^{-}}$ (OT-I) or $\mathrm{CD44^{lo}CD62L^{hi}CD8^{-}B220^{-}CD11b^{-}NK1.1^{-}Ter119^{-}}$ (OT-II) cells that were either $\mathrm{GFP^{+}}$ (RTEs) or $\mathrm{GFP^{-}}$ mature naïve (MN) T cells using a FACSAria II (Becton Dickinson). Where indicated, cells were labeled in the presence of 2.5-5µM carboxyfluorescein succinimidyl ester (CFSE) for 10 min at 37°C to facilitate quantification of cell proliferation. The relatively dim GFP signal from RTEs did not interfere with the substantially brighter CFSE (35). CD8 SP thymocytes were isolated RAG2p-GFP Tg mice as $\mathrm{CD8^{+}CD62L^{hi}TCR^{hi}GFP^{+}}$ to exclude immature SP thymocytes and mature recirculating T cells.

Adoptive transfers and immunizations

The indicated numbers of congenically-marked sort-purified naïve TCR Tg RTEs and MN T cells were mixed 1:1, or kept separate as indicated, and transferred intravenously into adoptive recipients. Where indicated, recipient mice were immunized subcutaneously at the base of the tail 1d post adoptive transfer with a total of 100µL containing 100µg 4-hydroxy-3-nitrophenylacetyl (NP)-OVA (Sigma-Aldrich) in a 1:1 solution with Imject Alum (Thermo Scientific). Donor cell function was measured 7 days later in draining iLNs.

Intracellular cytokine staining

Seven days following adoptive transfer or immunization, *in vivo* differentiated donor T cells were isolated from the draining pancLNs or iLNs of adoptive recipients and restimulated *in vitro* using either 50ng/mL phorbol 12-myristate 13-acetate (PMA) and 0.7µM ionomycin (both from Sigma-Aldrich) for OT-II T cells, or 10nM $OVA_{257\text{-}264}$ peptide for OT-I T cells, all in the presence of either brefeldin A (GolgiPlug) or monensin (GolgiStop; both from BD Biosciences). Cells were surface stained as indicated and fixed using either 2% paraformaldehyde (PFA; Electron Microscopy Sciences) for OT-II T cells, or Cytofix (BD Biosciences) for OT-I T cells, and permeabilized using Cytoperm (BD Biosciences). Cells were stained intracellularly with fluorochrome-conjugated antibodies against IL-2 (JES6-5H4), IL-4 (11B11), or IFNγ (XMG1.2) from either BD Biosciences or eBioscience. Samples were run on a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star), using unstimulated controls to define gates.

Diabetes induction

Five days following adoptive transfer of the indicated numbers of OVA-specific T cells, the blood glucose of RIP-mOVA Tg recipients was monitored daily using a OneTouch Ultra2 (LifeScan) blood glucose meter along with OneTouch Ultra (LifeScan) test strips. Mice with blood glucose readings over 300mg/dL for 2 consecutive days were considered diabetic and were sacrificed. Although high numbers (~$1x10^6$) of OT-I T cells alone have been reported to induce diabetes in 50% of RIP-mOVA Tg adoptive recipients (65), transfer of even $2x10^6$ OT-I T cells into otherwise unmanipulated RIP-mOVA Tg hosts in our vivarium failed to induce autoimmune diabetes. This is likely a consequence of the altered gut microbiome of mice housed in SPF facilities, as perturbations in the microbiota can influence autoimmune diabetes incidence (66). For this reason, we provided CD4$^+$ T cell help in the form of co-transferred OT-II T cells, a scenario known to lower the number of OT-I T cells required to induce autoimmune diabetes (65).

Quantitative polymerase chain reaction (PCR)

Seven days following adoptive cell transfer, antigen-exposed CD44^{hi} OT-II T cells were sort-purified from the draining pancLNs and total RNA was extracted using the RNeasy Micro kit (Qiagen). Complementary DNA (cDNA) synthesis was performed with a mix of oligo(dT) and random primers using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Quantitative PCR was performed on the resulting cDNA using a ViiA^{TM} 7 Real Time PCR System with TaqMan® Universal Master Mix II, no UNG (both from Applied Biosystems) and with TaqMan® primers/probes as indicated (Life Technologies). Data were normalized to the expression of hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) in each sample, and the relative expression was calculated using $2^{-\Delta CT}$.

Treg suppression assays and in vitro cytokine stimulation

$1x10^5$ sort-purified naïve polyclonal CFSE-labeled CD4$^+$ RTEs or MN T cells were co-cultured *in vitro* at the indicated ratios with sort-purified Foxp3$^+$ Treg cells from Foxp3-IRES-RFP Tg mice and $1x10^5$ TCRβ/δ$^{-/-}$ splenocytes irradiated with 3,000 Rad. Cells were stimulated for 72 hours at 37°C in 7% CO_2 with 50ng/mL soluble anti-CD3 (BD Biosciences) in complete Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum, 10mM HEPES, 4mM L-glutamine, and 50μM 2-mercaptoethanol. Percent suppression was calculated as: [(Tconv division index without Treg cells)-(Tconv division index with Treg cells)]/(Tconv division index without Treg cells). Division index was calculated from CFSE dilution curves using FlowJo software (Tree Star). For *in vitro* cytokine stimulation, $1x10^5$ sort-purified naïve polyclonal CFSE-labeled $CD4^+$ RTEs or MN T cells were cultured with $2x10^5$ irradiated $TCR\beta/\delta^{-/-}$ irradiated splenocytes. Cells were stimulated for 2-6 days at 37°C in 7% CO_2 with 50ng/mL soluble anti-CD3 with or without the addition of 30ng/mL each of exogenous IL-18, IL-1β, and/or IL-33 (all from PeproTech) in RPMI.

Isolation of small intestine intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs)

To examine T cell homing to the gut-associated lymphoid tissue (GALT), $2x10^6$ sort-purified naïve OT-II TCR Tg RTEs and MN T cells were co-transferred into B6 recipients. Two to five days following adoptive transfer, donor T cells were isolated from small intestine IELs and LPLs as previously described (67) with slight modifications. In brief, Peyer's patches (PPs, 2-8 per intestine) were removed, the intestine was opened longitudinally, and the intestinal contents and mucus were removed by gentle scraping. The intestine was cut into 2-cm pieces, washed, and incubated in Hank's balanced salt solution (HBSS) containing 0.5mM dithiothreitol and 12.5% fetal bovine serum at 37°C for 25 min, to remove the IELs. The remaining intestinal tissue pieces were transferred to HBSS containing 1.3mM EDTA and stirred at 37°C for 25 min to remove the epithelium. Intestinal pieces were then incubated in HBSS containing 5% FBS and 150 U/mL collagenase type 2 (Worthington) at 37°C with stirring for 30 min to isolate LPLs. IELs and LPLs were further purified by gradient separation using 44% and 67% Percoll (GE Healthcare).

Depletion of commensal flora

To deplete commensal flora, breeder mice were treated with a cocktail of antibiotics (abx) as previously described, with minor alterations (68). In brief, breeder mice were provided with ampicillin (1g/L; Sigma), vancomycin (500mg/L; Sigma), neomycin (1g/L; AgriLabs), metronidazole (1g/L; Sigma), and amphotericin-b (10mg/L; Sigma) in drinking water prior to mating. Sweet'N Low® was added to antibiotic-treated water to make it more palatable to mice and antibiotic-treated water was changed weekly. Following the birth of a litter, offspring were continued on abx through the duration of the experiment.

Colitis induction

$CD4^{+}CD45RB^{hi}CD25^{-}$ RTEs and MN T cells were sorted by flow cytometry from spleens and peripheral lymph nodes (pLNs) of RAG2p-GFP Tg mice. *Rag1*$^{-/-}$ mice were then injected intravenously with $5x10^{5}$ RTEs or MN T cells and followed for visible signs of disease. Mice were weighed just prior to T cell transfer (time 0) and daily thereafter. Percent weight change was calculated as: (weight at time X – weight at time 0) / (weight at time 0). All mice in the same experimental group were sacrificed when any individual mice showed clinical signs of severe disease or 20 percent weight loss.

Statistics

P values were calculated using a Log-rank (diabetes induction experiments) or paired (adoptive co-transfer experiments) Student's *t* test, and $p<0.05$ was considered significant.

Chapter Three: RTE Tolerance to Commensal-derived Antigens

Introduction

Dysregulated CD4$^+$ T cells play a critical role in the initiation of inflammatory bowel diseases (IBDs) such as Crohn's disease and ulcerative colitis (69). Crohn's disease is characterized by an increase in the production of Th1 cytokines, including IFNγ, IL-12, and TNFα, while ulcerative colitis is characterized by an increase in the production of Th2 cytokines, including IL-4, IL-5, and IL-13 (70). Identification of the immune cell types involved in the initiation of IBDs has benefited from mouse models of intestinal inflammation. In a model similar to Crohn's disease, adoptive transfer of Treg-depleted T cell populations into RAG null recipients results in colitis and wasting disease approximately 5 weeks post transfer (69, 71). Moreover, while adoptive transfer of *in vitro* polarized Th1 cells causes colitis (72-74), adoptive transfer of *in vitro* polarized Th2 cells causes IBD (73). CD8$^+$ T cells are also known to exacerbate IBDs (75).

Although experiments in mice have contributed to our current understanding of the cells involved in IBDs, little is known about where and how these autoreactive T cells originate. Recently it has become clear that not all peripheral antigens are expressed intrathymically, and that autoreactive T cells escape negative selection in the thymus and enter the lymphoid periphery (41). There they are confronted with a collection of previously unencountered innocuous antigens, including those expressed by commensal bacteria of the gut. If the post-thymic maturation period facilitates tolerance induction to antigens not previously encountered, trafficking to the gut would be the logical first step. Indeed, there is evidence that CD8$^+$ RTEs home to the gut within 24 hours of adoptive transfer (76, 77). Based on these observations, and the proliferative and cytokine defects of RTEs (28, 35), we set out to test the hypothesis that RTEs preferentially home to the gut and become tolerized to gut antigens.

Results

$CD8^+$ RTEs express higher levels of gut-homing receptors CCR9 and α4β7

Homing to and retention of lymphocytes in the gut-associated lymphoid tissue (GALT) is associated with lymphocyte expression of the chemokine receptor CCR9, which binds to CCL25, and the integrins α4β7 and αEβ7, which bind to MAdCAM-1 and E-cadherin, respectively (78, 79). To determine whether RTEs express elevated levels of gut-homing receptors, we measured the surface expression of CCR9 and α4β7 on naïve $CD4^+$ and $CD8^+$ RTEs and MN T cells by flow cytometry. While there was no significant difference in the expression of either CCR9 or α4β7 on $CD4^+$ RTE and MN T cells (data not shown), $CD8^+$ RTEs expressed significantly more CCR9 (Figure 3-1A) and α4β7 (Figure 3-1B) than their MN counterparts. In addition, we used the fact that GFP signal strength is inversely correlated with the time since loss of *Rag2* expression (28, 29) to show that gut-homing receptor expression is regulated in a stage-specific and transitory manner. CCR9 expression correlated with GFP brightness, such that the brightest GFP^+ cells expressed the highest levels of CCR9, while the GFP^- cells expressed the lowest levels of CCR9 (Figure 3-1A). Interesting, expression of α4β7 increased from GFP^{hi} to GFP^{lo} cells, both of which were brighter than their MN counterparts (Figure 3-1B). Thus, following egress from the thymus, $CD8^+$ RTEs exhibit increased expression of gut-homing receptors compared to their MN counterparts.

$CD8^+$ RTEs do not preferentially accumulate in the GALT

Due to their increased expression of the gut-homing receptors CCR9 and α4β7, we next asked whether $CD8^+$ RTEs preferentially gather in the GALT. To address this question, we isolated GALT from naïve RAG2p-GFP Tg mice and determined the distribution of $CD8^+$

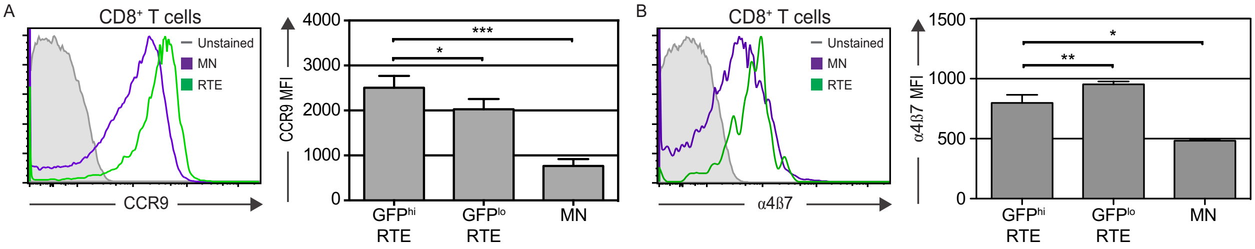

Figure 3-1. Compared to their MN counterparts, CD8$^+$ RTEs express higher levels of the gut-homing receptors CCR9 and α4β7. As GFP brightness is inversely correlated with the time since thymic egress, the total GFP$^+$ gate was divided into the brightest 25% (GFP^{hi} RTE) and the dullest 75% (GFP^{lo} RTE) of cells for the calculation of mean fluorescence intensity (MFI). (A) Representative (left) and mean (right) CCR9 expression levels by splenic CD8$^+$ RTEs and MN T cells directly ex vivo. (B) Representative (left) and mean (right) α4β7 expression levels by splenic CD8$^+$ RTEs and MN T cells directly ex vivo. Error bars denote standard error of the mean (SEM). *$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.001$, using a paired Student's *t*-test. $n = 3$ mice.

RTEs and MN T cells in each tissue. Under steady state, a similar fraction of the CD8$^+$ RTE and MN T cell pools were found in the spleen, pLNs, and mesenteric lymph nodes (mLNs) (Figure 3-2). A slightly larger fraction of the CD8$^+$ RTE pool was found in the small intestine intraepithelial lymphocyte (IEL) compartment compared to the their mature counterparts (1.8% vs. 1.2%), while a slightly larger fraction of the CD8$^+$ MN pool was found within the small intestine lamina propria lymphocyte (LPL) compartment compared to RTEs (2.2% vs. 0.6%) (Figure 3-2). Despite these minor differences, our data do not suggest that RTEs preferentially accumulate in the GALT throughout their period of transition into the MN compartment.

CD8$^+$ RTEs do not preferentially home to the GALT

While the data above suggest that RTEs do not preferentially accumulate in the GALT, these data may be complicated by the high rate of turnover of gut T cells, which may dilute the GFP signal from RTEs prematurely, making it difficult to distinguish RTEs from MN T cells. Although it has been shown that CD8$^+$ RTEs have a tendency to home to the gut within one day of exiting the thymus (76), it is not known whether they settle in the gut or leave following a short stopover. To address this question, we co-transferred an equal number of congenically-marked naïve CD8$^+$ RTEs and MN T cells into WT B6 recipients and used allelic differences as a proliferation-independent marker. Thirty-six hours post-transfer, donor cells from recipient mice were enumerated and the RTE:MN ratio relative to input was calculated. At this time point, RTEs and mature T cells were present at equal proportions in all tissues examined (Figure 3-3). Thus, following egress from the thymus, we found no evidence that CD8$^+$ RTEs preferentially home to the GALT.

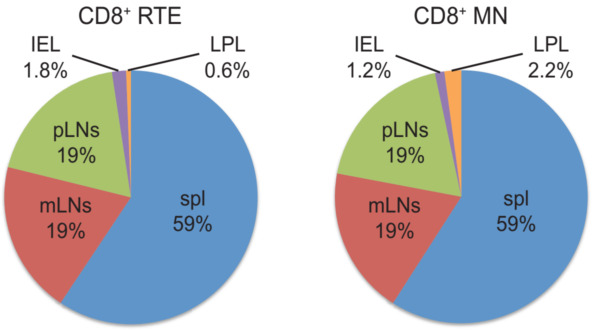

Figure 3-2. CD8$^+$ RTEs do not preferentially accumulate in the GALT. Small intestine IELs and LPLs, and lymphocytes from the spleen (spl), pLNs, and mLNs of unmanipulated RAG2p-GFP Tg mice were isolated. Shown is the percentage of the total CD8$^+$ RTE and MN T cell pool that was allocated into each tissue and quantified. Data are averaged from 3 mice and are representative of 2 independent experiments.

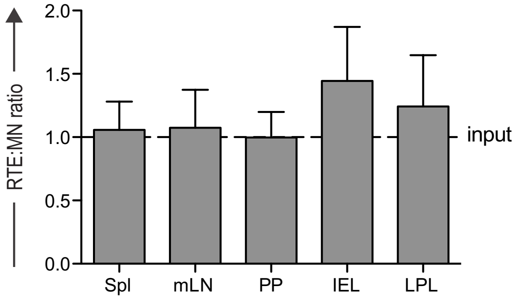

Figure 3-3. Compared to their MN counterparts, CD8$^+$ RTEs do not preferentially home to the GALT. 2x10^6 naive CD8$^+$ RTEs and MN T cells each were co-transferred into B6 recipients. 36 hours post transfer, donor cells from the spl, mLNs, PPs, and small intestine IEL and LPL compartments were enumerated, and the RTE:MN ratio relative to input was calculated. Data are from 4 mice pooled from 2 independent experiments. Error bars denote SEM.

CD8 SP thymocytes preferentially home to the GALT

In order to find donor cells in the GALT of recipient mice we had to transfer large numbers of sort-purified cells. This forced us to draw large GFP gates and collect older RTEs that had potentially already traveled to the GALT. To address this issue, we co-transferred CD8 SP thymocytes ("pre-RTEs") along with sort-purified MN T cells from RAG2p-GFP Tg mice into WT B6 recipients. Thirty-six hours post-transfer, donor thymocytes preferentially located to the small intestine IEL and LPL compartments relative to their mature counterparts (Figure 3-4). Therefore, while $CD8^+$ RTEs do not preferentially home to the GALT (Figure 3-3), CD8 SP thymocytes do.

$CD8^+$ RTE localization to the GALT is altered in the absence of commensal antigens

To determine whether RTE gut-homing might be influenced by the presence of gut antigens, we co-transferred naïve $CD8^+$ RTEs and MN T cells into WT B6 recipients that had been treated *in utero* with antibiotics (abx) to deplete commensal bacteria (68), essentially rendering them "germ-free". Thirty-six hours post-transfer, donor cells were isolated and the RTE:MN ratio relative to input was calculated. Compared to their untreated controls, donor RTEs transferred into antibiotic-treated mice exhibited a slight increase in their preference to home to the small intestine IEL and LPL compartments (Figure 3-5), an increase that did not reach statistical significance.

RTEs and mature T cells do not differ in their capacity to cause colitis

As colitis is often associated with Th1 effector lineages (80), and because $CD4^+$ RTEs are biased against the Th1 lineage (35), we predicted $CD4^+$ RTEs would be less colitogenic than

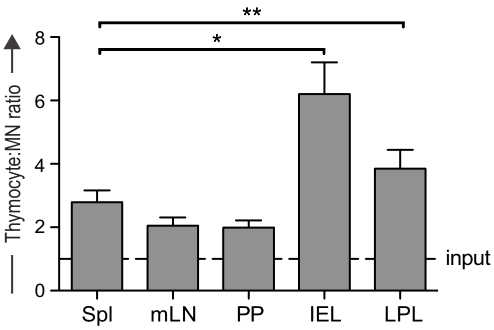

Figure 3-4. Compared to MN T cells, CD8 SP thymocytes preferentially home to the GALT. 1x10^7 enriched CD8 SP thymocytes and sort-purified MN T cells were co-transferred into B6 recipients. 36 hours post transfer, donor cells from the spl, mLNs, PPs, and small intestine IEL and LPL compartments were enumerated, and the thymocyte:MN ratio relative to input was calculated. Data are from 8 mice pooled from 2 independent experiments. Error bars denote SEM. *p≤0.05; **p≤0.01, using a paired Student's *t*-test.

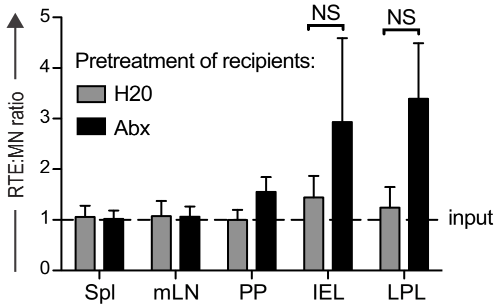

Figure 3-5. In the absence of commensal-derived antigens, CD8$^+$ RTEs exhibit a slight increase in their preference to home to the GALT. 2x10^6 sort-purified naive RTEs and MN T cells were co-transferred into mice treated with antibiotics to deplete commensal bacteria. 36 hours post transfer, donor cells from the spl, mLNs, PPs, and small intestine IEL and LPL compartments were enumerated, and the RTE:MN ratio relative to input was calculated. Data are from 4-5 mice pooled from 3 independent experiments. Error bars denote SEM. NS, not significant.

their MN counterparts. To test this hypothesis, we utilized an established mouse model of colitis (81) in which a minimum of $5\text{x}10^5$ $CD4^+CD45RB^{hi}$ or $CD25^-$ T cells are adoptively transferred into $RAG1^{-/-}$ recipients, resulting in colitis and wasting approximately 5 weeks post transfer. This protocol induces a high incidence of disease in all strains of mice and results in host colon thickening due to hyperplasia of the epithelium and lymphocyte infiltration (69, 82). For these experiments, we transferred $5\text{x}10^5$ $CD4^+CD45RB^{hi}CD25^-$ RTEs or MN T cells into separate congenically marked $RAG1^{-/-}$ recipients that were followed for visible signs of colitis. Once recipients lost 20% of their starting weight, donor cells were quantified and colon weight:length ratios determined. Upon sacrifice, there was no significant difference in the percentage (Figure 3-6A, *left panel*) or number (Figure 3-6A, *right panel*) of RTEs and mature T cells in the spleen or GALT. Recipients of RTEs, however, exhibited slightly lower colon weight:length ratios than recipients of MN T cells (Figure 3-6B), suggesting more severe lymphocyte infiltration. Nevertheless, this did not result in a significant change in host survival (Figure 3-6C).

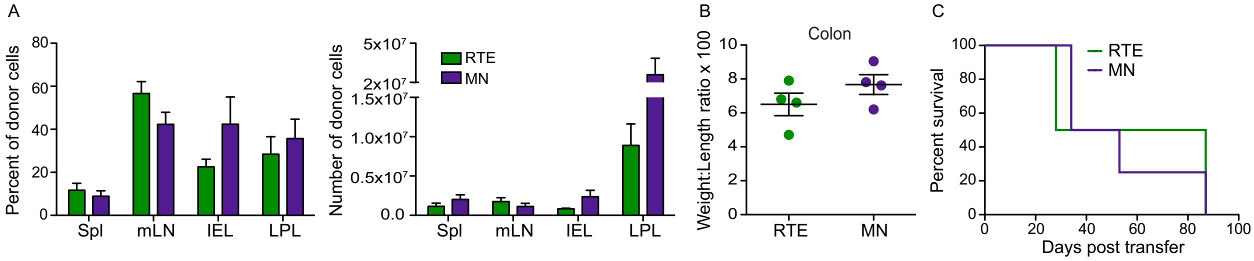

Figure 3-6. RTEs and mature T cells do not differ in their capacity to cause colitis. $5\text{x}10^5$ $CD4^+CD45RB^{hi}CD25^-$ RTEs or MN T cells were transferred into separate $RAG1^{-/-}$ recipients that were followed for visible signs of colitis. Recipients were sacrificed when they lost 20% of their starting weight. (A) Percentage (left panel) and absolute number (right panel) of donor cells isolated from the spl, mLNs, PPs, IEL, and LPL compartments of recipient mice. (B) Colon weight (g)-to-length (cm) ratios and (C) survival curves for recipient mice that received either donor RTEs or MN T cells. Data are from 4 mice per group from 2 independent experiments. Error bars denote SEM.

Conclusions

The experiments described in this chapter suggest that while CD8$^+$ RTEs express higher levels of the gut-homing receptors CCR9 and α4β7, they do not preferentially accumulate in the GALT during their transition from the RTE to the mature T cell pool. However, from our initial experiments, it was unclear whether RTEs show no interest in the GALT or home to the gut immediately after thymic egress and then exit the GALT after a short stay. It was surprising that we found no evidence that CD8$^+$ RTEs preferentially home to the gut, despite previously published reports (76). If RTEs home to the gut and then quickly leave to scan the lymphoid periphery, our purified RTE populations had likely already trafficked to the gut, precluding them from re-entering the gut following adoptive transfer. This idea is supported by our finding that CD8 SP thymocytes ("pre-RTEs") preferentially home to the gut compared to MN T cells (Figure 3-4).

While our data here show a slight increased tendency for CD8$^+$ RTEs to home to the GALT in "germ-free" recipients, we predicted that in the absence of commensal bacteria, RTEs would be unable to become tolerized to gut antigens and therefore they would not traffic to the GALT. However, it is possible that in the absence of commensal bacteria, RTEs might stay longer in the GALT looking for antigen, and this allowed us to catch them in the act. Similarly, we also wanted to use "germ-free" donors to test whether preventing RTEs from recognizing gut antigens would result in greater capacity to induce colitis, but unfortunately, antibiotic-treated mice have very few T cells (83).

Unfortunately, these experiments were carried out through the course of a move to a new facility, including the relocation of mice to a new vivarium. These changes likely altered the commensal flora of the mice, impacting the propensity of T cells to traffic to the GALT and become tolerized to gut-associated antigens. Supporting this idea is the fact that intestinal bacteria are known to contribute to the onset of disease in mouse models of colitis (72, 84).

These issues, along with the fact that it is $CD8^+$ RTEs that express increased levels of gut-homing receptors, but it is primarily $CD4^+$ T cells that contribute to the pathogenesis of IBDs, motivated us to look for alternative models of peripheral tolerance induction. The following chapter will describe the experiments performed to test whether RTEs are more prone to tolerance following encounter with peripherally expressed self-antigen in a diabetes model system.

Chapter Four: RTEs are Tolerized to Peripheral Self-antigen in the Absence of Inflammation

Introduction

To test whether RTEs are tolerized to peripherally expressed self-antigen, we used an established ovalbumin (OVA)-Tg mouse model. RIP-mOVA Tg mice express a membrane bound form of OVA under the control of the rat insulin promoter (64) that drives peripheral expression primarily in the pancreatic β islets and kidney proximal tubules. RIP-mOVA Tg mice have been used extensively to model islet autoantigens and thereby identify the cell types involved in the β islet cell destruction characteristic of autoimmune type I diabetes. Antigen in these mice can be detected by both OVA-specific $CD4^+$ (OT-II) and $CD8^+$ (OT-I) T cells. Tolerance induction of high affinity OT-I T cells in the RIP-mOVA system has been ascribed to cross presentation of OVA by DCs in the pancreatic lymph nodes (pancLNs) (85, 86). Indeed, analysis of autoreactive OT-I T cells in the RIP-mOVA system indicates that $CD4^+$ T cell help impairs their deletion, demonstrating that control of such help is critical for the maintenance of $CD8^+$ T cell tolerance induced by cross-presentation (65).

A functional $CD4^+$ T cell response to antigen is characterized by robust proliferation and activation of appropriate effector functions such as cytokine production, traits that have been manipulated to great effect by the addition of adjuvants to vaccines for over 100 years. Many diverse compounds have been classified as adjuvants, including mineral salts such as alum and emulsions such as Complete Freund's Adjuvant (CFA) (87). Although their precise mechanism(s) of action remains unclear, data suggest adjuvants act by one or more of the following mechanisms: (1) prolonged release of antigen at the site of immunization (depot effect), (2) production of proinflammatory cytokines and chemokines, (3) enhanced recruitment of both innate and adaptive immune cells to the site of injection, (4) improved cellular uptake of antigen, followed by activation and maturation of APCs, and (6) activation of antibody-secreting B cells and cytotoxic T cells (88). Of the many compounds assessed as adjuvants, those that contain aluminum salts are the most widely used in both animal and human vaccines (87).

The current study aimed to determine whether, compared to mature T cells, RTEs are prone to tolerance induction following recognition of their cognate antigen. The use of monoclonal OT-II and OT-I donor RTEs and MN T cells transferred into RIP-mOVA Tg adoptive recipients ensured our analysis was limited to those T cells that had encountered antigen and circumvented any TCR repertoire changes that might occur on a population level. Moreover, co-transferring both RTEs and MN T cells into the same recipient controlled for the antigenic milieu and allowed for more precise donor cell comparisons. In this chapter, we show that following self-antigen encounter *in vivo*, both $CD4^+$ and $CD8^+$ RTEs proliferate less, secrete less IL-2 and IFNγ, and express elevated levels of anergy-associated genes. Relative to mature T cells, stimulated RTEs were also more sensitive to Treg-mediated suppression *in vitro*. Correspondingly, both OT-II and OT-I RTEs were less diabetogenic than their mature T cell counterparts following transfer into RIP-mOVA Tg adoptive hosts. However, in the presence of inflammation, RTEs proliferated to the same extent and secreted as much IL-2 as their mature T cell counterparts. These results place RTEs at a crossroads between tolerance induction and effector cell differentiation, with the presence or absence of inflammation during antigen recognition providing additional guidance to influence their subsequent differentiation.

Results

RTEs exhibit functional defects after in vivo *exposure to peripheral self-antigen*

To quantify the TRA reactivity of peripheral T cells as a function of their maturation stage, an equal number of congenically-marked, naïve carboxyfluorescein succinimidyl ester (CFSE) labeled OT-I (Figure 4-1A-C) or OT-II (Figure 4-1D-F) RTEs and MN T cells were co-transferred from antigen-free donors into RIP-mOVA Tg hosts. Bulk OT-I T cells were co-transferred with OT-II RTEs and MN T cells to enhance antigen release and promote activation of donor cell populations in the draining pancLNs. The absence of donor cell activation in either WT B6 hosts or in the non-draining inguinal (i)LNs of RIP-mOVA Tg hosts underscores the antigen specificity of this activation (Figure 4-2). While there was no significant difference in the expression level of the activation marker CD44 on $CD8^+$ RTEs and mature T cells (Figure 4-1A) harvested from the pancLNs of RIP-MOVA Tg hosts 4 days post transfer, $CD4^+$ RTEs were significantly less activated compared to their mature counterparts (Figure 4-1D). Correspondingly, donor $CD8^+$ RTEs only exhibited proliferative defects at day 7 post transfer (Figure 4-1B), while donor $CD4^+$ RTEs exhibited proliferative defects at both days 4 and 7 (Figure 4-1E).

Next, we examined effector cytokine production by donor $CD8^+$ and $CD4^+$ RTEs and MN T cells following peripheral self-antigen encounter. Seven days post transfer into RIP-mOVA Tg hosts, divided $CD8^+$ and $CD4^+$ RTEs produced less IL-2 and IFNγ than their mature counterparts, both in terms of proportion (Figure 4-1C,F), and on a per cell basis, as measured by MFI (Figure 4-3A). This defect in cytokine production is not simply a downstream consequence of diminished proliferation, as $CD4^+$ RTEs exhibited defects even when IL-2 and IFNγ production were expressed as a function of cell division (Figure 4-3B). Despite the fact that

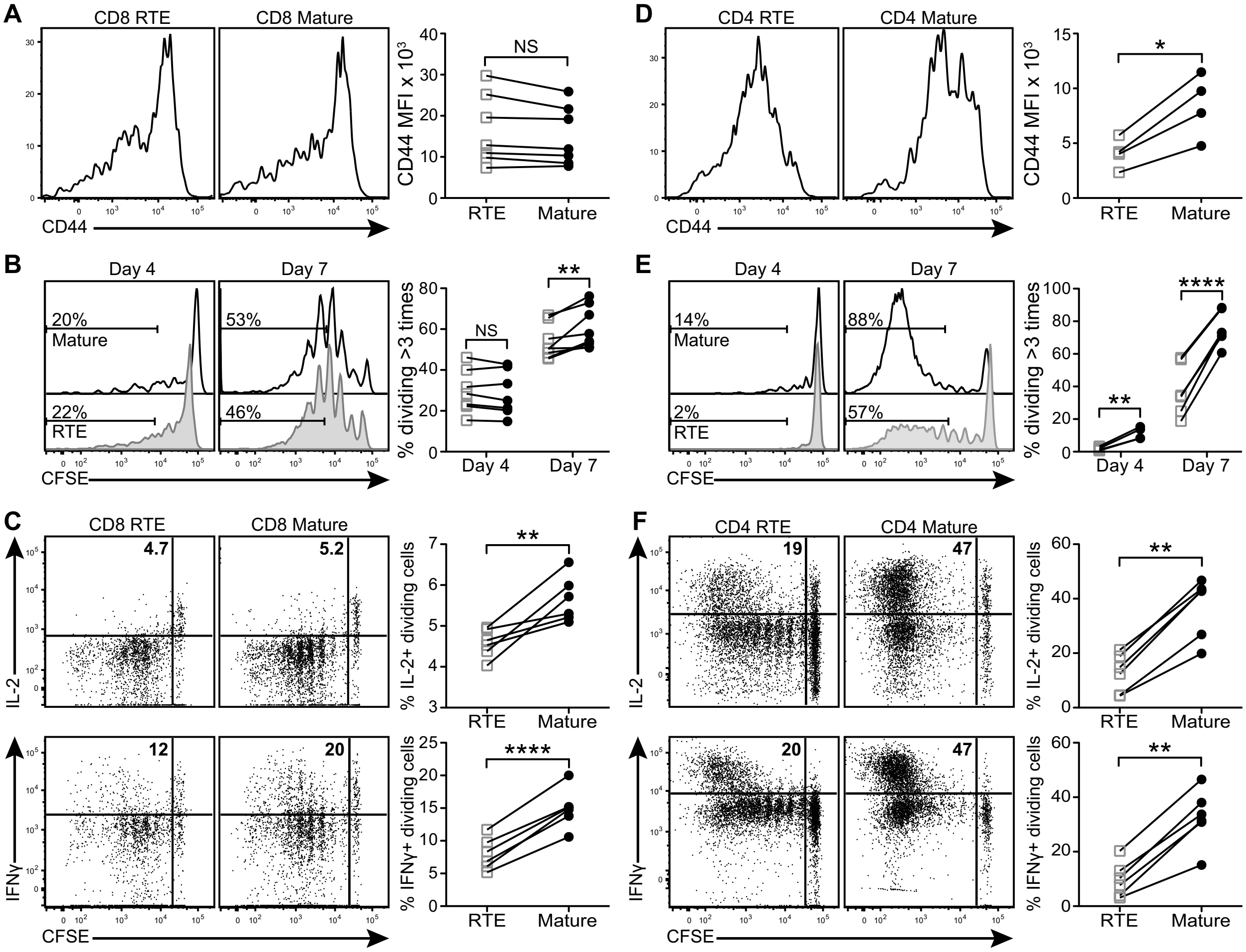

Figure 4-1. CD4$^+$ and CD8$^+$ RTEs proliferate less and secrete less IL-2 and IFNγ than mature T cells in response to peripheral self-antigen *in vivo*. (A-C) 1x10^6 naive OT-I RTEs and MN T cells were co-transferred into RIP-mOVA Tg hosts. (D-F) 0.5x10^6 naive OT-II RTEs and MN T cells were co-transferred along with 2x10^6 bulk naive OT-I T cells into RIP-mOVA Tg hosts. (A and D) Four days following transfer, donor cells from the draining pancLNs were analyzed. Histograms in the left two panels show representative CD44 staining by RTEs and mature T cells. Data in the right panels show compiled (*n* = 4-7) CD44 MFI. (B and E) Four and 7 days following transfer, proliferation of donor cells from the pancLNs was quantified. Numbers in the histograms represent the percentages of donor RTEs (shaded) and mature T cells (open) that have divided >3 times. Data in the right panels show percentages of donor RTEs (open squares) and mature T cells (filled circles) dividing >3 times, compiled from 2 independent experiments (*n* = 4-8). (C and F) Seven days following transfer, cells from recipient pancLNs were restimulated *in vitro* and stained for intracellular cytokines. Numbers in the upper left quadrants of dot plots represent the percentages of IL-2$^+$ or IFNγ$^+$ dividing donor cells. Data in the right panels are compiled from 2 independent experiments (*n* = 6-8). NS, not significant; *p≤0.05; **p≤0.01; ****p≤0.001, using a paired Student's *t*-test.

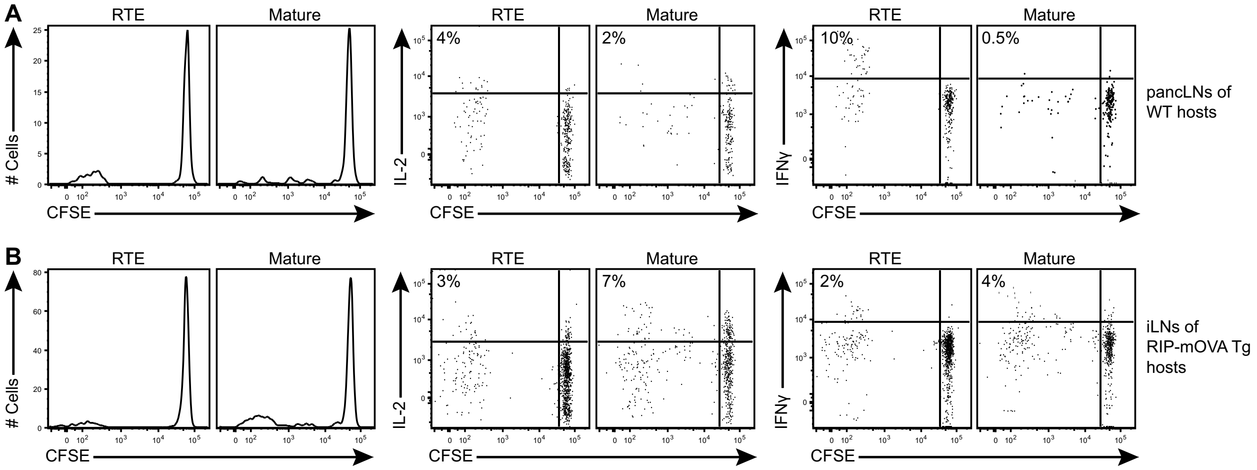

Figure 4-2. RTE lack of proliferation and cytokine production by donor cells from pancLNs of WT or iLNs of RIP-mOVA Tg hosts. (A) $0.5x10^6$ naive OT-II RTEs and MN T cells were co-transferred along with $2x10^6$ bulk naive OT-I T cells into a WT B6 host. Seven days following transfer, proliferation of donor cells from the pancLNs of recipient mice was quantified (left two panels). Following restimulation *in vitro*, donor cells isolated from the pancLNs were stained for intracellular cytokines. Numbers in the upper left quadrants of the dot plots represent the percentages of IL-2^+ (middle two panels) or IFNγ^+ (right two panels) dividing donor cells. (B) $0.5x10^6$ naive OT-II RTEs and MN T cells were co-transferred along with $2x10^6$ bulk naive OT-I T cells into RIP-mOVA Tg hosts. Seven days following transfer, proliferation of donor cells from the non-draining iLNs of recipient mice was quantified (left two panels). Following restimulation *in vitro*, donor cells isolated from the iLNs were stained for intracellular cytokines. Numbers in the upper left quadrants of the dot plots represent the percentages of IL-2^+ (middle two panels) or IFNγ^+ (right two panels) dividing donor cells.

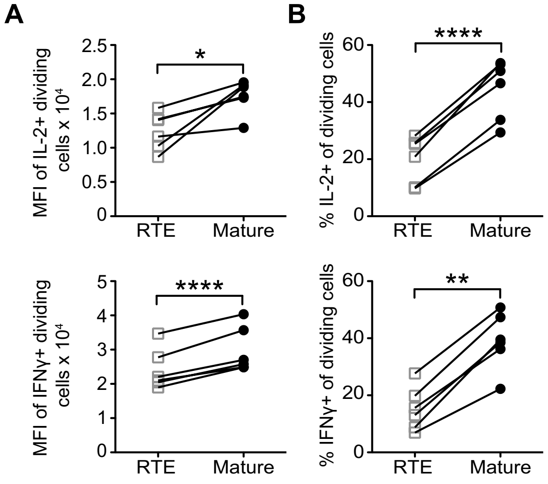

Figure 4-3. Dividing CD4$^+$ RTEs secrete less IL-2 and IFNγ than their mature T cell counterparts in response to peripheral self-antigen *in vivo*. (A-B) 0.5x10^6 sort-purified naive OT-II RTEs and MN T cells were co-transferred along with 2x10^6 bulk naive OT-I T cells into RIP-mOVA Tg recipients. Seven days following adoptive transfer, cells from the recipient pancLNs were restimulated *in vitro* and stained for intracellular cytokines. (A) Data in the top panel show the MFI of dividing cells that are IL-2$^+$. Data in the bottom panel show the MFI of dividing cells that are IFNγ$^+$. (B) Data in the top panel show the percentage of dividing cells that are IL-2$^+$. Data in the bottom panel shows the percentage of dividing cells that are IFNγ$^+$. Data are compiled from 2 independent experiments (*n* = 6-8). *$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.001$, using a paired Student's *t*-test.

$CD4^+$ RTEs are skewed away from the Th1 and toward the Th2 lineage (35), $CD4^+$ RTEs exposed to self-antigen *in vivo* make neither IL-4 nor IL-17 (not shown). Thus, upon self-antigen recognition *in vivo*, both $CD4^+$ and $CD8^+$ RTEs exhibit functional defects similar to those described in murine (35) and human (27) RTEs stimulated *in vitro*.

$CD8^+$ RTEs are less diabetogenic than their mature T cell counterparts

To determine whether the functional defects seen in RTEs exposed to peripheral self-antigen impact their capacity to cause diabetes, we measured diabetes induction following transfer of OT-I RTEs or MN T cells into separate RIP-mOVA Tg hosts. Although high numbers of transferred OT-I T cells alone can induce diabetes in RIP-mOVA Tg mice, help provided by co-transferred OT-II T cells (which alone fail to induce diabetes) facilitates disease initiation by a lower number of OT-I T cells (65). To decrease the number of OT-I T cells needed to initiate autoimmune diabetes, we provided $CD4^+$ T cell help in the form of co-transferred bulk OT-II $RAG1^{-/-}$ T cells. Eight days later, recipients of OT-I MN T cells developed autoimmune diabetes; diabetes was delayed in the recipients of RTEs, and fewer of these hosts became diabetic (Figure 4-4A). Thus, $CD8^+$ RTEs are less diabetogenic than their mature counterparts.

$CD4^+$ RTEs provide less efficient help to $CD8^+$ effectors than their mature counterparts

To determine whether the proliferative and cytokine defects of $CD4^+$ RTEs affect their capacity to help induce $CD8^+$ T cell-mediated autoimmunity, we measured diabetes induction following transfer of OT-II RTEs or MN T cells plus bulk naïve OT-I T cells into separate RIP-mOVA Tg hosts. Fewer of the mice that received RTE helper cells progressed to autoimmune diabetes compared to those receiving mature helpers (Figure 4-4B). Thus, the help provided by

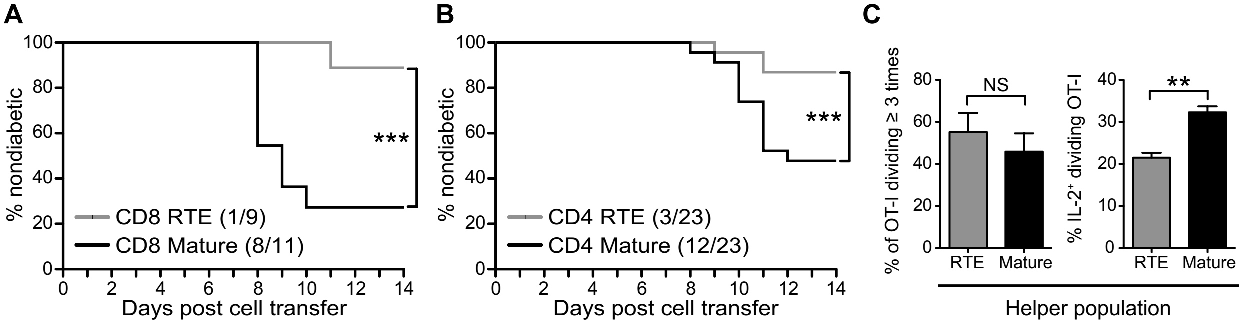

Figure 4-4. CD4$^+$ and CD8$^+$ RTEs are less efficient than their mature T cell counterparts at inducing diabetes. (A) 1x10^6 naive OT-I RTEs or MN T cells were transferred along with 5-8x10^5 bulk naive OT-II RAG1$^{-/-}$ T cells into separate RIP-mOVA Tg hosts. (B) 7x10^5-1x10^6 naive OT-II RTEs or MN T cells were transferred along with 2-3x10^6 bulk naive OT-I T cells into separate RIP-mOVA Tg hosts. Shown is host disease incidence as measured by blood glucose, compiled from 3-5 independent experiments. Numbers in parentheses indicate the number of diabetic mice over the total number of mice per group. ***$p \leq 0.005$, using a Log-rank test. (C) 1x10^6 naive OT-II RTEs or MN T cells were co-transferred along with 2x10^6 bulk naive OT-I T cells into RIP-mOVA Tg hosts. Seven days following transfer, proliferation (left panel) and cytokine production (right panel) by donor OT-I T cells cells from recipient pancLNs was quantified. Data are representative of 2 independent experiments. **$p \leq 0.01$ using an unpaired Student's *t*-test.

$CD4^+$ RTEs is less efficient than that provided by mature T cells at facilitating $CD8^+$ T cell-mediated diabetes. The impact of helper cell source on the $CD8^+$ population is evidenced by the reduction in the proportion of IL-2 producing OT-I effectors that develop in the presence of RTE helpers rather than in their proliferative capacity, which is equivalent regardless of the source of $CD4^+$ T cell help (Figure 4-4C).

Compared to their mature counterparts, $CD4^+$ RTEs are more prone to anergy

Anergy in $CD4^+$ T cells is characterized by an inability to produce IL-2, resulting in a diminished proliferative response (44). The defective proliferation and cytokine (particularly IL-2) production by RTEs in response to self-antigen stimulation, as well as the fact that RTEs receive strong TCR signals but weak costimulation (39) are all properties displayed by anergic cells (44). Therefore, we measured anergy-associated gene expression in naïve OT-II RTEs and MN T cells compared to that in antigen-exposed $CD44^{hi}$ OT-II RTEs and mature T cells sorted from the pancLNs of RIP-mOVA Tg adoptive hosts. Compared to mature T cells, and relative to their naïve counterparts, antigen-exposed $CD4^+$ RTEs expressed increased levels of multiple anergy-associated genes, including Egr2, DGKα, and Cbl-b (Figure 4-5). Similar results were seen in $CD8^+$ RTEs (Figure 4-6).

$CD4^+$ RTEs are more susceptible than mature T cells to Treg cell-mediated suppression

To determine whether the impaired proliferation and cytokine production by $CD4^+$ RTEs could be also due to enhanced susceptibility to Treg cell-mediated suppression, we measured $CD4^+$ RTE and MN T cell proliferation using a standard *in vitro* suppression assay. Polyclonal CFSE-labeled $CD4^+$ RTEs or MN Tconv were co-cultured with Treg cells from Foxp3-IRES-

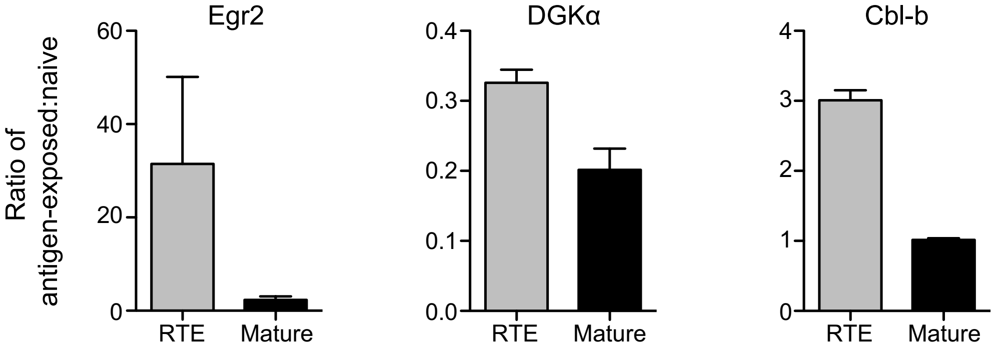

Figure 4-5. CD4$^+$ RTEs express elevated levels of anergy-associated genes following *in vivo* encounter with self-antigen. 1-2x10^6 naive OT-II RTEs and MN T cells were co-transferred along with 2x10^6 bulk naive OT-I T cells into RIP-mOVA Tg hosts. Seven days later, antigen-exposed CD44^{hi} donor OT-II T cells were sorted from host pancLNs and RNA was extracted from these cells and from naïve OT-II RTEs and MN T cells for gene expression analyses. Data are presented as mean ± SD of the ratio of antigen-exposed to naïve samples and are representative of 2 independent experiments.

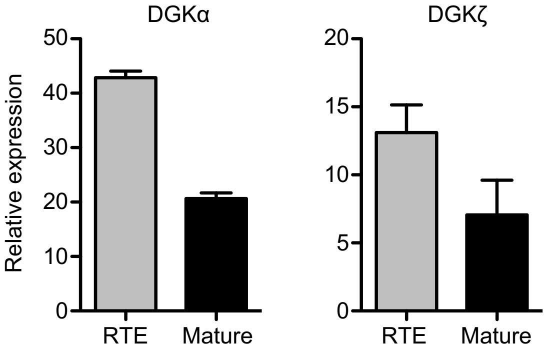

Figure 4-6. CD8$^+$ RTEs express elevated levels of anergy-associated genes following *in vivo* encounter with self-antigen. 1x10^6 sort-purified naïve OT-I RTEs and MN T cells were co-transferred into RIP-mOVA Tg recipients. Seven days following adoptive transfer, donor OT-I T cells were sorted from recipient splenocytes and RNA extracted. Data are presented as mean ± SD of triplicate samples from one of 3 independent experiments and are relative to *Hprt*. These analyses were performed by Dr. Qingyong Ji.

RFP Tg reporter mice (at decreasing Treg/Tconv ratios), irradiated APCs and soluble anti-CD3. After 72 hours, both RTEs and mature T cells proliferated robustly in the absence of Treg cells, although fewer RTEs underwent division (Figure 4-7), as previously reported (28). Furthermore, both RTE and mature T cell proliferation was suppressed in a dose dependent manner by the addition of Foxp3$^+$ Treg cells. Interestingly, RTE proliferation was diminished at lower Treg/Tconv ratios, indicating that CD4$^+$ RTEs are more susceptible to Treg cell-mediated suppression (Figure 4-7). This inhibitory effect was not simply a consequence of reduced RTE proliferation, because suppression was calculated as a function of cell division. Thus, CD4$^+$ RTEs have heightened sensitivity to Treg cell-mediated suppression, a trait that likely impacts their *in vivo* function.

Inflammation converts tolerance-prone CD4$^+$ RTEs into competent effector cells

Knowing that RTEs are more tolerance-prone than their mature T cell counterparts following peripheral self-antigen encounter, we asked whether the RTE response is equally defective following antigen encounter in the presence of inflammation. To test this, an equal number of CFSE-labeled OT-II RTEs and MN T cells were co-transferred into recipients that were immunized subcutaneously the next day with 4-hydroxy-3-nitrophenylacetyl hapten conjugated to OVA (NP-OVA) in alum to induce inflammation. In contrast to the proliferative defects observed in RTEs responding to peripheral self-antigen (Figure 4-1B), robust proliferation was observed in CD4$^+$ RTEs harvested from the draining iLNs of mice immunized with NP-OVA in alum (Figure 4-8A, *top panels*). In fact, fewer RTEs were left undivided, and more RTEs than mature T cells underwent 1-6 rounds of division (Figure 4-8A, *top right panel*), suggesting that in the context of inflammation, CD4$^+$ RTEs outperform their mature T cell

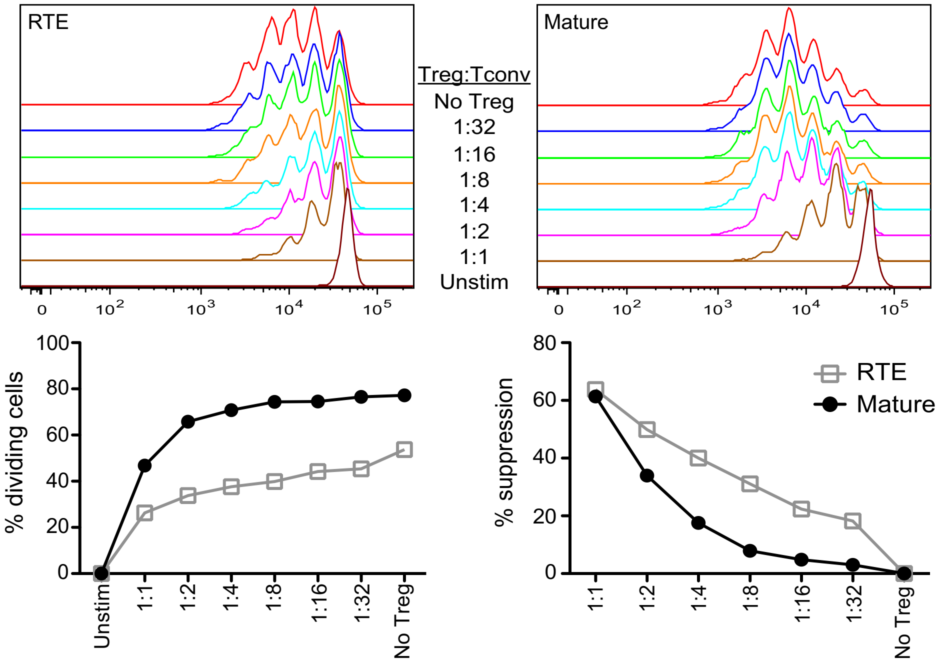

Figure 4-7. CD4$^+$ RTEs are more susceptible to Treg cell-mediated suppression. 1x10^5 naive polyclonal CFSE-labeled CD4$^+$ RTEs or MN T cells were co-cultured *in vitro* with the indicated ratios of Foxp3$^+$ Treg cells from Foxp3-IRES-RFP Tg mice in the presence of 1x10^5 irradiated TCRβ/δ$^{-/-}$ splenocytes. Cells were cultured for 72h without (unstim) or with 50ng/ml soluble anti-CD3 and CFSE dilution measured (top panels). Data in the bottom left panel show the percent of dividing cells, and in the bottom right panel, the percent suppression of T cell proliferation by Foxp3$^+$ Treg cells. Data are representative of 3 independent experiments.

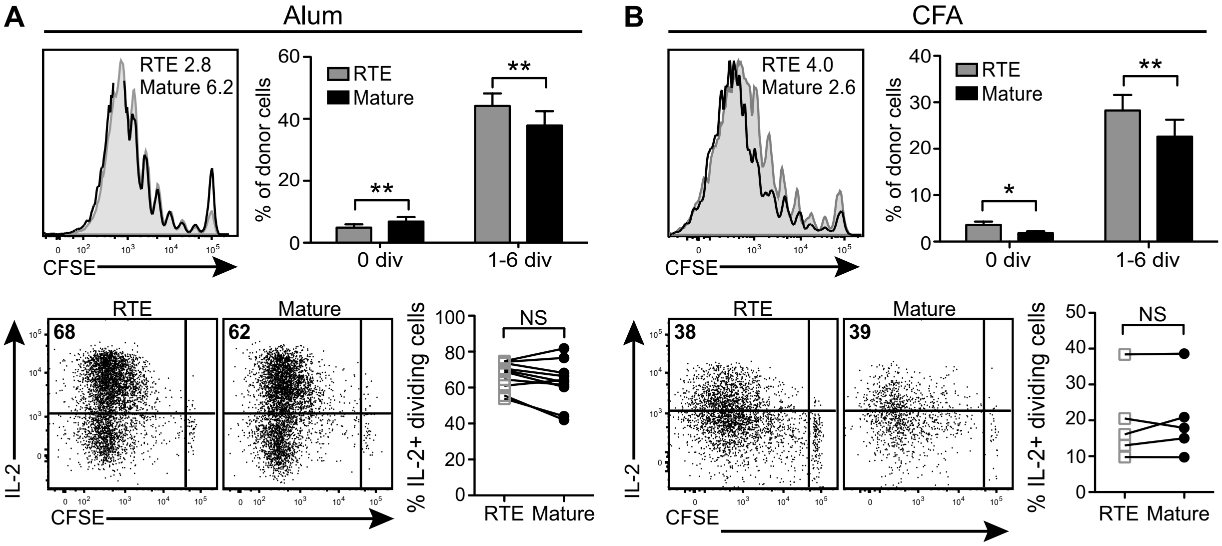

Figure 4-8. Antigen in the presence of inflammation converts tolerance-prone CD4$^+$ RTEs into competent effector cells. 2x10^6 CFSE-labeled OT-II RTEs and MN T cells were co-transferred into WT B6 hosts that were immunized subcutaneously the following day with 100µg of NP-OVA in a 1:1 solution with either alum (A) or CFA (B). Seven days later, donor cell proliferation and cytokine production were quantified in the draining iLNs. Data in the top left panels represent CFSE dilution by RTEs (shaded) and mature T cells (open) and numbers denote the percentages of undivided donor cells. Data in the top right panels show the proportion of donor cells that have undergone 0 or 1-6 divisions (div). Data are presented as mean ± SEM compiled from 3 independent experiments (*n* = 12). Following restimulation *in vitro*, donor cells isolated from the draining iLNs were stained for intracellular cytokines. Numbers in the upper left quadrants of the dot plots represent the percentages of IL-2$^+$ dividing donor cells. Data in the bottom right panels are compiled from 2 independent experiments (*n* = 5-10). NS, not significant.

counterparts. Furthermore, dividing RTEs and mature T cells made similar amounts of IL-2 and IFNγ (Figure 4-8A, *bottom panel and data not shown*), in contrast to the poor response exhibited by RTEs to peripheral self-antigen. Despite alum's reputation for mild Th2 skewing, these cells generated little to no IL-4 (data not shown). A similar recovery of effector cell function was noted in CD4$^+$ RTEs exposed to antigen in CFA (Figure 4-8B). Thus, inflammation converts antigen-exposed, tolerance-prone RTEs into competent effector cells.

Addition of IL-1β, IL-18, and/or IL-33 disproportionately improves RTE function in vitro

To investigate how adjuvant exposure might impact RTE effector function, we focused on alum because of its greater impact, particularly in terms of cytokine production (compare Figure 4-8A and B, *lower panels*). Exposure of cells to alum *in vitro* has been shown to activate caspase-1, resulting in the induction of its known downstream targets, IL-1β, IL-18, and IL-33 (89, 90). To determine the inflammatory cytokines that convert tolerance-prone CD4$^+$ RTEs into competent effector cells, we cultured polyclonal CFSE-labeled CD4$^+$ RTEs or MN T cells with irradiated APCs and soluble anti-CD3, with or without the addition of exogenous IL-1β, IL-18, and/or IL-33. After 48 hours of culture, both RTEs and mature T cells proliferated, although fewer RTEs divided 2 or more times (Figure 4-9A). Although the addition of exogenous IL-18 or IL-1β and IL-33 disproportionately increased the proliferation of RTEs relative to mature T cells, the addition of all three cytokines (IL-1β, IL-18 and IL-33) was required to drive RTE proliferation to mature T cell levels (Figure 4-9A). Following 5-6 days of culture with irradiated APCs and anti-CD3, ~58% of mature CD4$^+$ T cells made IL-2, whereas only ~7% of RTEs did. The addition of exogenous IL-1β and IL-33 resulted in a 1.3-fold increase in mature IL-2-producing T cells, compared anti-CD3 alone (Figure 4-9B). Strikingly, ~32% of RTEs made IL-2 under the

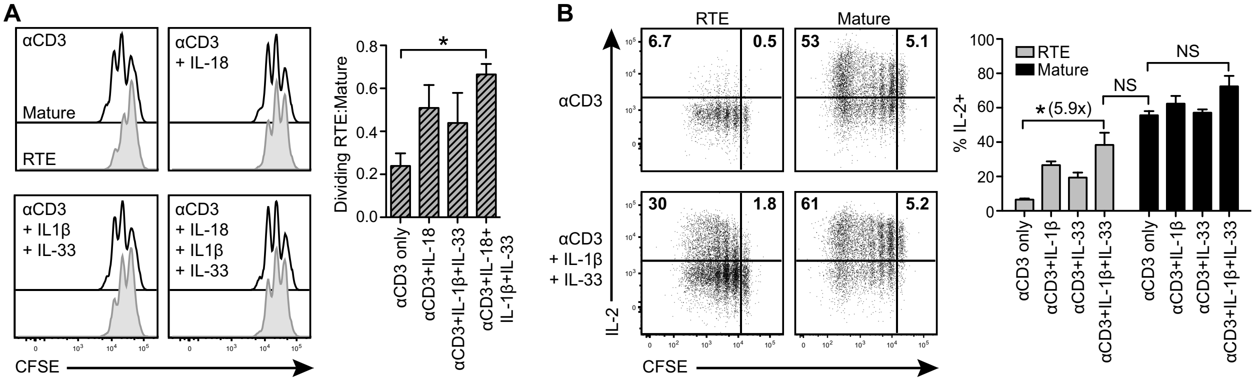

Figure 4-9. Addition of exogenous IL-1β, IL-18, and/or IL-33 disproportionately increases RTE proliferation and cytokine production *in vitro*. (A and B) $1x10^5$ naive polyclonal CFSE-labeled CD4 RTEs or MN T cells were cultured for 2-6 days with $2x10^5$ irradiated $TCR\beta/\delta^{-/-}$ splenocytes and 50ng/mL soluble anti-CD3, with or without 30ng/mL IL-1β, IL-18, and/or IL-33. (A) Histograms show representative CFSE dilution by RTEs (shaded) and mature T cells (open) following 2 days of culture under the indicated conditions. Bar graph data show the ratio of the percentage of RTEs to mature T cells dividing 2 or more times; data are compiled from 2 independent experiments. (B) After 5-6 days of culture, cells were restimulated with PMA and ionomycin, and stained for intracellular IL-2. Numbers in the upper left and upper right quadrants of the dot plots represent the percentage of $IL\text{-}2^+$ divided and non-divided cells, respectively. Data in the right panel are compiled from 2 independent experiments. Numbers in parentheses indicate the fold-change for the indicated condition relative to stimulation with anti-CD3 alone. NS, not significant; $*p<0.05$ by an unpaired Student's *t*-test.

same culture conditions, a dramatic ~6 fold increase over anti-CD3 alone (Figure 4-9B). Thus, the addition of exogenous IL-1β, IL-18, and IL-33 disproportionately increases RTE proliferation and cytokine production relative to their mature T cell counterparts.

Conclusions

Our work demonstrates that following egress from the thymus, RTEs undergo a maturation process in which they are prone to tolerance induction upon exposure to extrathymic self-antigen. This idea was first hinted at by the fact that compared to MN T cells, RTEs express TCRs with longer CDR3 lengths (33), reminiscent of the CDR3s of TCRs expressed by pre-negative selection thymocytes (58). While some RTEs may be deleted prior to entering the mature T cell pool, peripheral T cells undergoing tolerance induction are often rendered anergic, displaying dampened immunocompetence following antigen stimulation. We show here for the first time that upon self-antigen recognition *in vivo*, both $CD4^+$ and $CD8^+$ RTEs exhibit proliferative and cytokine defects similar to those described in murine (35) and human (27) RTEs stimulated *in vitro*. Interestingly, these functional defects exhibited by the youngest peripheral T cells also mirror those of aged T cells near the end of their lifespan (91, 92).

Our data show that the proliferative and cytokine defects exhibited by both $CD4^+$ and $CD8^+$ RTEs following self-antigen encounter, as well as their susceptibility to Treg-mediated suppression, likely contribute to their diminished diabetogenic potential relative to that of mature T cells. However, previous data from our lab showed that, compared to their mature counterparts, OT-I RTEs drive <u>increased</u> diabetes induction in RIP-mOVA Tg adoptive recipients following inflammation induced by pathogen-associated antigen (39). Increased diabetes induced by RTEs in the presence of inflammation can be attributed to their enhanced expression of the integrin VLA-4 (39), which has been shown to drive T cell accumulation in the prediabetic pancreas (93, 94) and inflamed skin (95). Even in the absence of inflammation, naïve $CD8^+$ RTEs preferentially localize to the splenic white pulp (data not shown), entry to which is known to be VLA-4 dependent (96). This may be important for allowing RTEs to access and become tolerized to TRAs not normally encountered by circulating naïve T cells. Increased tissue invasiveness by RTEs in the absence of inflammation may also help explain recent findings that fetal T cells (RTEs) are able to infiltrate the organs of pregnant women, including the spleen and lymph nodes, and may help contribute to the onset of autoimmune disorders following pregnancy (97-99). Therefore, while the increased invasiveness of RTEs may help contribute to host protection, it may also serve to promote autoimmunity when self-tolerance is broken. Thus, it is crucial that the period of post-thymic maturation be coupled with tolerance induction to TRAs not expressed within the confines of the thymus.

The diminished proliferation, IL-2 and IFNγ production, and strong TCR signals but weak costimulation (28, 39) exhibited by RTEs are all properties displayed by anergic T cells (44). In line with these properties, multiple anergy-associated genes are upregulated in CD4$^+$ RTEs following self-antigen stimulation *in vivo*. The upregulation of Egr2 is especially significant, given that Egr2 has been identified as a key transcription factor responsible for regulating anergy induction (100). Although anergy in CD8$^+$ T cells is less well defined, upregulation of anergy-associated genes DGKα and ζ similarly characterized CD8$^+$ RTEs harvested from the pancLNs of adoptive hosts (Figure 4-6). RTEs harvested from the pancLNs of RIP-mOVA Tg hosts show upregulation of folate receptor-4 (FR4) surface expression but downregulation of CD73 expression compared to their mature counterparts (data not shown) and thus do not fully phenocopy anergic mature T cells, which have been reported to be uniformly FR4^{hi}CD73^{hi} (101).

The dampened function of RTEs could be due in part to their heightened sensitivity to Treg cell-mediated suppression. Although the precise mechanism of Treg cell suppression is unclear, Treg cells may act as IL-2 “sinks”, absorbing the IL-2 needed by stimulated T cells to initiate proliferation and activation of effector functions (53, 102). $CD4^+$ RTEs fail to efficiently upregulate the high affinity IL-2Rα following *in vitro* stimulation (28). This suggests they would be less capable of sensing low concentrations of IL-2, levels that would be expected to be encountered when RTEs are the main responders, perhaps explaining their increased susceptibility to Treg cell suppression.

Several recent publications have shown that $CD4^+$ RTEs are uniquely sensitive to Treg cell induction both *in vitro* and *in vivo*, after transfer into either lymphoreplete (103) or lymphopenic (104) recipients. However, using OT-II RAG2p-GFP Tg Foxp3-IRES-RFP reporter mice to deplete RFP^+ donor Treg cells, we were unable to identify any consistent preference for $CD4^+$ RTEs to develop into $Foxp3^+$ Treg cells one week following transfer into RIP-mOVA Tg adoptive recipients (Figure 4-10). It is possible that technical differences, such as the use of high concentrations of exogenous TGFβ to induce Treg differentiation or colony-specific differences in intestinal microbiota might explain these incompatible data, but our current results support our laboratory's previous findings that *in vitro* skewed $CD4^+$ RTEs are defective in their commitment to the Treg cell lineage (35).

Our findings here suggest that inflammation induced by alum disproportionately increases RTE proliferation and cytokine production, converting antigen-exposed, tolerance-prone RTEs into competent effector cells. This response can be at least partially attributed to the action of the IL-1 family of cytokines, which are known to be produced following activation by alum (89, 90, 105), as the exogenous addition of IL-1β, IL-18, and IL-33 to cultures of polyclonal $CD4^+$ RTEs restored RTE proliferation and cytokine production to the level of their mature counterparts. Stimulated RTEs and mature T cells express similar cell surface levels of the IL-1 and IL-33 receptors (data not shown), failing to explain the disproportional response of

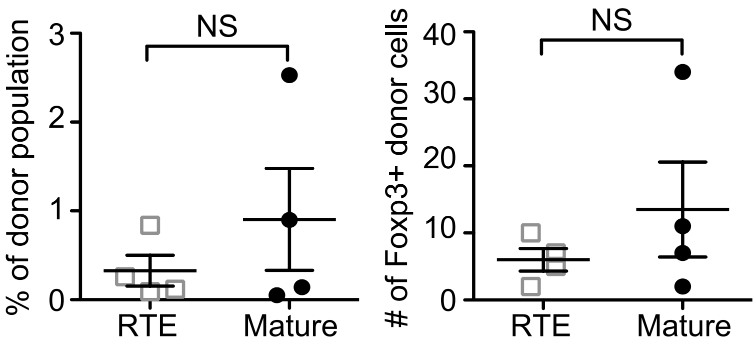

Figure 4-10. CD4$^+$ RTEs fail to upregulate Foxp3 in response to peripheral self-antigen *in vivo*. 1x10^6 sort-purified Treg cell-depleted (from Foxp3-IRES-RFP Tg reporter mice) naïve OT-II RTEs or MN T cells were co-transferred along with 2x10^6 bulk naïve OT-I T cells into RIP-mOVA Tg recipients. Seven days following adoptive transfer, donor OT-II T cells from the recipient pancLNs were harvested and analyzed for Foxp3 expression. Data in the left panel show the percentage of donor cells that are Foxp3$^+$. Data in the right panel show the total number of Foxp3$^+$ donor cells in the pancLN. Data are compiled from 1 of 3 representative experiments. NS, not significant.

RTEs to IL-1β and IL-33. While IL-33 is known to be involved in Th2 cell responses (106), the Th2 skewing of RTEs is largely the result of their inherent bias against the Th1 effector lineage (35), suggesting their helper cell bias may not be dependent on IL-33 receptor expression.

In sum, following encounter with TRAs in the absence of inflammation, RTEs exhibited defects in proliferation, diminished cytokine production, elevated expression of anergy-associated genes, and diminished capacity to initiate diabetes. These properties were mirrored *in vitro* by enhanced RTE susceptibility to Treg cell-mediated suppression. However, in the presence of inflammation, RTEs and mature T cells were equally capable of inducing diabetes, proliferating, and producing cytokines, Thus, recirculating RTEs encounter TRAs during a transitional developmental stage that facilitates tolerance induction, but inflammation converts antigen-exposed, tolerance-prone RTEs into competent effector cells.

Chapter Five: Concluding Remarks

The data presented in this thesis provide important insight into the advantages gained by peripheral T cell maturation. Our data provide evidence that both CD4$^+$ and CD8$^+$ RTEs enter the lymphoid periphery in an attenuated state, but that certain immunological cues may position RTEs to overcome these defects. The dampened immunocompetence exhibited by RTEs following antigen encounter in the absence of inflammation may serve to limit the production of harmful proinflammatory cytokines directed against TRAs not expressed within the confines of the thymus during T cell development. Indeed, phenotypic and functional maturation of RTEs requires contact with secondary lymphoid organs (32), access to which is important for inducing T cell tolerance to self-antigens expressed exclusively in the periphery in a non-inflammatory context (107). Furthermore, a tempered RTE response to antigen may be of particular benefit to neonates, whose entire T cell pools consist of RTEs (18, 56, 57), and in which this interaction takes place within an autoimmune-promoting lymphopenic environment (108). In contrast, following antigen encounter in the presence of inflammation, RTEs are equal to mature T cells in their capacity to proliferate and produce cytokines, suggesting that RTE function is not universally defective, but is instead modulated by an interpretation of the antigenic milieu that is distinct from that of mature T cells. Balancing effector function and tolerance induction within RTEs is key to ensuring that the neonate—all of whose peripheral T cells are RTEs—handles the world of antigens, both harmful and not, to the best of its advantage.

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