Impact of ligand affinity on CD8 recent thymic emigrant responses to bacterial infection

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Abstract

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To explore the relative T cell receptor (TCR) sensitivity of recent thymic emigrants (RTEs) compared to mature naïve T cells, we triggered T cells with altered peptide ligands (APLs) that vary in their affinity for the TCR. Upon peptide stimulation *in vitro*, RTEs exhibited increased TCR signal transduction, and following infection *in vivo* with APL-expressing bacteria, CD8 RTEs expanded to a greater extent in response to low affinity antigens than their mature T cell counterparts. RTEs skewed to short-lived effector cells in response to all APLs but were also characterized by diminished cytokine production. RTEs responding to infection expressed increased levels of VLA-4, with consequent improved entry into inflamed tissue and pathogen clearance. These positive outcomes were offset by the enhanced capacity of RTEs to elicit autoimmunity. While RTEs exhibited increased effector responses, their memory formation differed from that of mature-derived memory T cells, with a lower proportion of central memory

T cells and less efficient homing to the bone marrow. Overall, salient features of CD8 RTE biology should inform strategies to improve neonatal vaccination and therapies for cancer and HIV, as RTEs make up a large proportion of the T cells in the newborn and other lymphodepleted environments.

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List of Abbreviations

APC – antigen presenting cell

APL – altered peptide ligand

BHK – baby hamster kidney fibroblast

CDR – complementarity determining region

CFA - Complete Freund's adjuvant

CFU – colony forming units

CTL – cytotoxic T lymphocyte

D – diversity gene segment

DC – dendritic cell

DMEM – Dulbecco’s modified Eagle's medium

DNA – deoxyribonucleic acid

DP – double positive

Eomes – eomesodermin

ERK – extracellular signal-regulated kinase

GFP – green fluorescent protein

HBSS – Hank’s balanced salt solution

HIV – human immunodeficiency virus

IFNγ – interferon gamma

J – joining gene segment

IL-2 – interleukin-2

IL-7rα – interleukin-7 receptor, alpha chain

KLRG1 – killer cell lectin-like receptor subfamily G, member 1

LFA-1 – leukocyte function-associated antigen-1

Lm.APL – *Listeria monocytogenes* expressing altered peptide ligands

MFI – mean fluorescence intensity

MHC – major histocompatibility complex

MN – mature naïve

MPEC – memory precursor effector cell

N4 – SIINFEKL peptide

OT-I – TCR Tg T cell that recognizes SIINFEKL sequence of OVA or APL

OVA – ovalbumin protein

PBS – phosphate buffered saline

PFU – plaque forming units

pMHC – peptide MHC

Q4 – SIIQFEKL peptide

RAG – recombination activating gene

RIPmOVA – mice that express membrane bound OVA under control of the rat insulin promoter

RPMI – Roswell Park Memorial Institute

RTEs – recent thymic emigrants

SLEC – short lived effector cell

T4 – SIITFEKL peptide

T_{CM} – central memory T cells

TCR – T cell receptor

T_{EM} – effector memory T cells

Tg – transgenic

TNFα – tumor necrosis factor alpha

TREC – T cell receptor excision circle

V – variable gene segment

VLA-4 – very late antigen-4

VSV – vesicular stomatitis virus

VSV.OVA – vesicular stomatitis virus engineered to express OVA

Yop – Yersinia outer protein

Yptb – *Yersinia pseudotuberculosis*

ZAP70 – zeta-chain-associated protein kinase 70

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Chapter One: Introduction

T cells and adaptive immunity

The immune system is comprised of two critical components, the innate and adaptive systems, which mediate coordinated responses to ensure host survival upon infection. The innate system initiates immediate responses to conserved regions of pathogens, but these responses do not improve following multiple pathogen encounters (1). The adaptive immune system provides antigen specificity and long-lasting memory to a diverse array of pathogens, properties that the innate system lacks.

The adaptive immune system largely consists of two distinct cell types; B cells produce antibodies and mediate humoral immunity and T cells provide cell-mediated immunity. T cells recognize cell surface antigen using the T cell receptor (TCR). T cell subsets can be distinguished through expression of either a γδ TCR or an αβ TCR, but γδ T cells have more innate-like properties and respond to conserved antigens. In the periphery, including the secondary lymphoid organs (spleen and lymph nodes) αβ T cells (referred to here as T cells) predominate and are the focus of this work. These T cells can be further distinguished by their expression of the CD4 and CD8 coreceptors and have distinct roles within the immune system. CD4 expression classifies T helper cells which are important for activating B cells, macrophages, and CD8 T cells. CD8 T cells are cytotoxic T lymphocytes (CTL) and are responsible for killing abnormal cells. The generation and activation of these cells are tightly regulated processes.

T cells recognize foreign antigens with their TCRs through recognition of pMHC, peptide bound to molecules encoded by the Major Histocompatibility Complex, presented on the surface of antigen presenting cells (APCs) including the important linker of innate and adaptive immunity, dendritic cells (DCs). It is through this highly specific pMHC-TCR interaction that T

cells can mount a targeted response, as an individual TCR can only recognize a very limited number of pMHC (2). This presents a challenge for the developing T cell repertoire: the generated TCRs must be diverse in order to recognize all possible pathogens; however, the TCR must also be able to interact with a limited number of MHC molecules. This careful balance is achieved through the complex process of TCR gene rearrangement and T cell selection in the thymus.

T cell development

To achieve the diversity of TCRs required to recognize a vast number of pathogens, TCR genes are segmented in the genome and are rearranged in the thymus. In the adult, hematopoietic stem cells enter the thymus from the bone marrow where they proliferate following contact with thymic stromal cells. These double negative thymocytes, which do not express CD4 or CD8, then rearrange the germline TCR genes, beginning with the β-chain gene. Following the expression of the tightly regulated enzymes required for gene rearrangement, recombination activating gene-1 and -2 (RAG-1 and RAG-2), diversity (D) and joining (J) gene segments are combined first before rearrangement occurs to join the variable (V) to the DJ segment (3). After successful β-chain rearrangement, the α-chain gene is rearranged, consisting of Vα and Jα gene segments. Together the large number of β-chain V, D, and J genes (35 for mice and 54 for humans for the V genes alone) and α-chain V and J genes that can be combined exponentially increases the variability of possible TCRs. This diversity is further increased through the addition of non-templated P- and N- nucleotides at the junctions between the V, D, and J or V and J genes (4). The hypervariable regions correspond with loops at the end of the TCR that come in contact with the pMHC, or complementarity determining regions (CDRs), of which there are 3 per TCR

chain. The CDR3 region includes the areas of P- and N- additions, is the most variable of the CDRs, and frequently is the loop in closest contact with the peptide in the binding groove of the MHC (5). Successful rearrangement of a vast number of TCRs is the first hurdle in T cell development.

While the first step of T cell development involves the generation of diverse TCRs, the second part ensures these receptors are useful without being over-reactive to self. To determine if a TCR is useful, it must first be able to recognize the MHC expressed by stromal cells in the thymus, which is determined through a process known as positive selection (6). $CD4^+CD8^+$ double positive (DP) immature thymocytes are positively selected on self-pMHC expressing cortical epithelial cells in the cortex of the thymus. Cells that find their ligand are positively selected and those that do not, die by neglect. Positive selection also determines whether a DP thymocyte will become a CD4 or CD8 T cell, depending on which MHC the thymocyte interacts, MHC-II or MHC-I, respectively (2). Negative selection eliminates T cells that react too strongly with self-pMHC in the thymus and have the potential to cause autoimmunity in the periphery (7). This process occurs mainly at the cortico-medullary junction on dendritic cells and macrophages. Mature thymocytes, having survived both positive and negative selection, leave the thymus and join the pool of recirculating peripheral T cells.

Recent thymic emigrants

The threshold for successful T cell development is high, requiring successful TCR rearrangement, positive selection, and escape from negative selection, but further maturation of naïve T cells is required in the periphery upon thymic egress, termed post-thymic maturation (8). Remaining auto-reactive T cells persist despite regulated selection in the thymus, as not all

antigens are thymically expressed, including commensal antigens (9, 10). Knowledge of this post-thymic maturation process has been best gathered through the study of recent thymic emigrants (RTEs), those cells that have most recently left the thymus. While self-tolerance is critical, a protective immune response is also important, especially in lymphopenic settings such as in the neonate and human immunodeficiency virus (HIV)-infected or chemotherapy-treated patients where RTEs make up a considerable proportion of the peripheral T cells. Therefore, it is crucial that RTEs maintain this delicate balance between host protection and self-tolerance.

Study of RTEs over the years has been aided by technological advances that improved the identification and evaluation of this distinct population (11, 12). One early barrier in the study of RTEs has been the lack of a definitive surface marker to easily identify this population in the mouse. Intrathymic fluorescein isothiocyanate injection, 5-bromo-2-deoxyuridine incorporation, quantification of TCR rearrangement excision circles (TRECs), fetal thymus organ cultures, and thymic lobe grafts were the first techniques used to identify RTEs. These methods allow for variable levels of phenotypic or functional analysis but have many disadvantages including surgical stress, short window for analysis, impure RTE populations, and inability to evaluate function. Unsurprisingly, human RTE studies are more difficult as methods to label thymocytes are unavailable; however, some surface markers for CD4 RTEs in humans have recently been identified (13). The development of a transgenic (Tg) mouse model in which RTEs can be readily identified and isolated from their mature but still naïve (MN) T cell counterparts, allows for phenotypical and functional analysis. This model avoids some of the previously mentioned pitfalls and has dramatically increased our knowledge of RTE biology (14). In RAG2p-GFP Tg mice, green fluorescent protein (GFP) is expressed under control of the RAG2 promoter, and while expression is extinguished in mature thymocytes, a residual GFP

signal can be detected in a population of peripheral T cells defined as RTEs (15). Using this system, unmanipulated RTEs can better be evaluated for their contribution to T cell responses *in vitro* and *in vivo*.

Using RAG2p-GFP Tg mice, we and others have shown that RTEs are phenotypically and functionally distinct from mature T cells (14, 16-23), a distinction that is mirrored by human RTEs (13, 24). Upon egress from the thymus, RTEs occupy this transitional state for ~3 weeks, during which time they undergo progressive maturation to MN T cells (14, 16). Age-dependent involution of the thymus has been well established, and can be observed using RAG2p-GFP Tg mice; while neonates have the highest proportion of GFP^+ RTE, the number of GFP^+ RTEs peaks at 6-weeks of age, and reduced numbers of GFP^+ cells are seen in the periphery of aged mice (16). Phenotypic differences that have been associated with RTEs include increased CD24 and TCR/CD3 expression and lower expression of Qa2, IL-7Rα, CD28, and CD45RB compared to MN T cells (14). CD8 RTEs in mice display increased levels of gut homing molecules such as CCR9 and αE integrin (25), while human RTEs identified as $TREC^+$ CD8 naïve T cells are enriched for αE $integrin^+$ cells (24). Following activation, CD8 RTEs generate fewer IL-7Rα^{hi} killer cell lectin-like receptor subfamily G, member 1 $(KLRG1)^{lo}$ memory precursor effector cells (MPECs) compared to their MN-derived counterparts (17). Functionally, both CD4 and CD8 RTEs produce less cytokine and exhibit decreased *in vitro* proliferative capacity (14, 17-19). While the mechanisms for these functional differences remain to be elucidated, increased deoxyribonucleic acid (DNA) methylation of promoter regions within some cytokine loci and increased expression of DNA modifying enzymes in CD4 RTEs suggest a unique poised state of RTEs (19). Much more work is required to fully understand the regulation of post thymic T cell maturation and RTE biology.

CD8 T cells: cytotoxic T lymphocytes

CD8 T cells are the CTL of the immune system and are known for their role in protection from intracellular bacteria and viruses. These cells recognize peptide presented on MHC-I, expressed on nearly every cell in the body. To maintain homeostasis, self-pMHC is presented and CD8 T cells are not cytolytic. During infection three signals initiate CTL activation: 1) recognition of pMHC via the TCR, 2) co-stimulatory signals through CD28, and 3) signal 3, mediated by inflammatory cytokines (26). All three of these signals are required for an effective CD8 T cell response. DCs are particularly good at picking up exogenous antigen, cross presenting it to CD8 T cells, and providing the appropriate co-stimulation and cytokine signals. An effective CD8 T cell response requires robust expansion of effector T cells, access to peripheral tissues, activation of appropriate effector functions such as the production of interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), and generation of durable memory to protect the host from reinfection (26, 27). When this CD8 T cell response goes awry, autoimmunity rather than host protection can result. Due to tight selection in the thymus, any remaining T cells reacting to self-antigens are typically of low affinity (28). The ability of the CD8 RTE population to maintain the proper balance between protection and self-destruction is largely unstudied and is the focus of this thesis work.

OT-I Tg T cells and altered peptide ligands

To investigate RTE responses to low affinity ligands, we utilized altered peptide ligands (APLs), peptide variants that differ in their TCR binding affinity, which can probe monoclonal T cell responses to high and low affinity antigens (29). OT-I TCR Tg T cells have been an

invaluable model utilized to study antigen specific T cell responses (30). In this model, OT-I T cells recognize the antigen ovalbumin (OVA) in the context of H-2K^b (31). Using this OT-I TCR Tg system, APLs of OVA have been well studied in thymic selection, in which distinct affinity thresholds have been defined for positive and negative selection (32), and in the mature T cell response to Lm.APL, *Listeria monocytogenes* that express OVA containing APLs (33, 34). The APLs studied here include the N4 variant, which is the canonical SIINFEKL peptide recognized by OT-I Tg T cells, Q4, with an 18-fold lower functional avidity for the OT-I TCR, and T4 that has a 71-fold lower functional avidity for the OT-I TCR (33). Previous studies probing the response of mature T cells to low affinity ligands found that low affinity ligands drive reduced expansion and an increased rate of contraction by OT-I T cells; however, these low affinity antigens were able to drive functional memory responses (33). This study aims to determine how the RTE response differs from that of the mature T cell response to low affinity TCR stimulation through the use of OVA APLs.

While some characteristics of the CD8 RTE response are known, we specifically wanted to elucidate the response of RTEs to low affinity antigens. We aimed to determine whether 1) TCR signal transduction by RTEs in response to reduced ligand affinity was different from that of mature T cells, 2) the acute response of RTEs differs from that of mature T cells following infection with bacteria expressing low affinity ligand, and 3) the memory response to bacterial infection by RTEs was differentially impacted by stimulation with low affinity ligand.

Chapter Two: Materials and Methods

Mice

RAG2p-GFP Tg (15) OT-I TCR Tg mice were backcrossed in our laboratory at least 12 generations onto the C57BL/6 (B6) background with both the CD45.1 and CD45.2 congenic markers. B6xB6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (CD45.2xCD45.1) and C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA) mice that express membrane-bound OVA in pancreatic β cells were bred in house. All mice were housed and bred under specific pathogen free conditions and used in accordance with the University of Washington Institutional Animal Care and Use Committee.

Reagents

The OVA APL peptides SIINFEKL (N4, a potency of 1), SIIQFEKL (Q4, a potency of 1/18), and SIITFEKL (T4, a potency of 1/71) were obtained from Genemed Synthesis Inc. at >98% purity and used for *in vitro* stimulations. To assess T cell homing to sites of inflammation, Complete Freund's adjuvant (CFA), obtained from Sigma-Aldrich and emulsified 1:1 with phosphate buffered saline (PBS), and anti-VLA-4 (PS/2, UCSF Monoclonal Antibody Core) were used as indicated.

Flow cytometry antibodies

Cells were stained with fluorochrome-conjugated antibodies against the following surface and intracellular molecules: CD16/32 (2.4G2), CD45.1 (A20), CD45.2 (104), NK1.1 (PK136), CD4 (RM4-5), CD8α (53-6.7), Ter119 (Ter119), CD11b (M1/70), B220 (RA3-6B2), CD44 (IM7), CD62L (MEL-14), CD127/IL-7Rα (A7R34), KLRG1 (2F1), CD49d/α4 (R1-2), CD29/β1

(HMβ1-1), Ly6C (HK1.4), IL-2 (JES6-5H4), TNFα (MP6-XT22), IFNγ (XMG1.2), Tbet (4B10), Eomes (Dan11mag), pZAP70 (17A/P-ZAP70), and pERK (E10), all from eBioscience, BD Biosciences, BioLegend, or Cell Signaling.

Cell sorting and adoptive transfers

Single cell suspensions were generated from spleens and peripheral lymph nodes of RAG2p-GFP Tg OT-I TCR Tg mice and red blood cells water lysed. Cells were first enriched with a no-touch method using CD8+ T cell isolation kits (StemCell Technologies), and Fc receptors were blocked (anti-CD16/32) before cells were sorted to >98% purity as GFP^+ or GFP^-, $NK1.1^-CD4^-$ $Ter119^-CD11b^-B220^-$ (dump gate) $CD44^{lo}CD62L^{hi}$ cells. Sorted RTE and MN T cells were counted, mixed 1:1 or kept purified for single transfers, and 10,000 total cells were injected intravenously in 200mL PBS into congenically marked hosts.

Bacterial growth and infections

Lm.APL, provided by Dietmar Zehn (Swiss Vaccine Research Institute), were grown until mid log phase (0.3-0.7 OD600). Then 2000 colony forming units (CFU) were injected intravenously one day post T cell transfer. *Yersinia pseudotuberculosis* expressing OVA (Yptb.N4) was streaked from frozen stock on a plate with 24μg/mL tetracycline and grown at 26°C for 2 days (from Tessa Bergsbaken and Michael Bevan). An isolated colony was selected and cultured in 2 mL brain heart infusion media and 24μg/mL tetracycline at 26°C with shaking for ~18 hours. Yptb.N4 was centrifuged in table-top centrifuge at 16,000xg for 1 minute and washed once with PBS. Yptb.N4 was resuspended in 3 mL, diluted 1:20 in PBS, and OD600 measured to determine CFU/mL. 10,000-20,000 CFU were injected intraperitoneally one day post cell

transfer. For both Lm.APL and Yptb.N4, injected bacteria were diluted and plated to determine actual CFU inoculated.

Tissue inflammation

For inducing ear inflammation or challenging with vesicular stomatitis virus (VSV), mice were sedated with a mixture of ketamine/xylazine (130mg/kg/8.8mg/kg) 5.5 days post infection and 10μL 1:1 CFA/PBS mixture or 10μL with 10^4 PFU VSV Indiana strain or VSV engineered to express OVA (VSV.OVA) from Michael Bevan (University of Washington, originally from reference 35) were injected intradermally into one ear. Where indicated, 100-150μg anti-VLA-4 blocking antibody was administered intraperitoneally 12 hours before and 12 hours after CFA treatment.

Cell preparation and flow cytometry

Bone marrow was harvested by flushing one femur and one tibia with Hank's balanced salt solution (HBSS) and the bone marrow plug was resuspended before being centrifuged and red blood cells lysed as with splenocytes. To isolate skin infiltrating T cells, ears were harvested and cut into tiny pieces, digested for three rounds using Liberase (0.14U/mL, Roche) media, and stirred at medium speed for 40-45 minutes at 37°C. The supernatant containing released cells was filtered into Roswell Park Memorial Institute (RPMI) 1640 media containing 10% fetal calf serum in between digestions. Splenocytes were isolated from mice infected 7 days earlier with Lm.APL. Cells were surface stained for 20 minutes at 4°C. For intracellular cytokine staining, 2-4x10^6 cells were incubated with 10nm SIINFEKL peptide and brefeldin A (GolgiPlug; BD Biosciences) for 5 hours at 37°C, surface stained, then fixed and permeabilized by resuspending

with BD Cytofix/Cytoperm according to the manufacturer's instructions. For intranuclear staining, cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Single color compensation controls and experimental samples were identically fixed. All samples were run on an LSRII or FACSCanto (BD Biosciences) and analyzed by FlowJo software (TreeStar).

In vitro peptide stimulation and intracellular staining for phosphorylated signaling molecule

Single cell suspensions from RAG2p-GFP Tg OT-I TCR Tg mice were counted and $3x10^6$/50μL were aliquoted per well in a 96 well plate in HBSS containing no serum. Prewarmed peptide was added at the indicated times for a final peptide concentration of 0.1μM (total volume: 100μL) and cells were placed at 37°C for the duration of the stimulation. Cells were fixed all at once by adding 100μL Fix Buffer I (BD Bioscience) for 10 minutes at 37°C and permeablized with 200μL Perm Buffer III (BD Bioscience) on ice for 30 minutes. Cells were stained for both surface and phosphorylated signaling molecules for 30 minutes at room temperature then run on an LSRII or FACSCanto (BD Biosciences) and analyzed by FlowJo software (TreeStar).

Plaque assay for viral titers

Ear tissue or lymph nodes were harvested into 1mL 2% Dulbecco's modified Eagle's medium (DMEM) in 2 mL screw top tubes containing ~250μL sterile glass beads. Ears were minced with scissors in the harvest tube and samples were homogenized by bead-beater for 90 seconds; ear samples required 2 rounds of homogenization, and were cooled on ice in between rounds. Samples were kept at -80°C until plaque assays were performed. For plaque assays, baby hamster kidney fibroblast (BHK) cells were grown in 10% DMEM until 60-80% confluent in a

6-well plate. Medium was removed by aspiration and wells were washed with 2 mL DMEM. Frozen tissue homogenates were thawed, centrifuged, and filtered before serial dilutions were made. To each well of BHK cells, 0.8 mL of sample were added and plates were incubated at 37°C for 1 hour. One part 3% agarose in PBS was combined with two parts 2% DMEM for agarose plug overlay and 3 mL was added to each well after incubation. 24 hours later, 3 mL 10% formalin was added to wells for one hour, the agarose plug was removed, and wells were stained with enough 0.2% crystal violet to cover the well to enumerate plaques. Plaque forming units (PFU) were calculated using the following equation: Number of plaques/(dilution factor X 0.8) = PFU/mL.

Diabetes induction

For diabetes induction, 10^4 sorted RTEs or MN T cells were transferred intravenously into RIPmOVA mice which were infected the following day with Lm.APL. Mice were monitored for blood glucose daily beginning 4-5 days post infection and were considered diabetic with a reading >350mg/dL.

Statistics

Unpaired or paired 2-tailed Student's *t* tests were used for comparisons or log rank test for survival as indicated.

Chapter Three: CD8 Recent Thymic Emigrants Exhibit Increased Responses to Low Affinity Ligands

Introduction

This study aimed to determine whether the maturation state of peripheral T cells impacts the response to bacterial infection due to intrinsic differences in TCR signaling. Proper T cell activation requires TCR stimulation, co-stimulation, and signals delivered by inflammatory cytokines (26). Initiation of T cell responses requires TCR recognition of peptide in the context of MHC. Membrane proximal events, such as phosphorylation of zeta-chain-associated protein kinase 70 (ZAP70), propagate this signal through an intracellular cascade that results in distal activation of kinases, such as extracellular signal-regulated kinase (ERK), which play significant roles in regulating gene expression to drive the appropriate effector T cell response (5, 36). Previous work demonstrated through the use of OT-I APLs that low affinity ligands induce lower levels of pZAP70 and pERK in thymocytes (32).

Diverse CD8 T cell subsets are commonly defined through their expression of KLRG1 and IL7Rα into short lived effector cells (SLECs) or MPECs (37, 38). SLECs are terminally differentiated CD8 T cells that, following the peak of the immune response, rapidly contract and (unlike MPECs) contribute minimally to the memory T cell pool (38). The factors contributing to skewing toward one cell fate or the other have been extensively investigated. The cytokine signals of signal 3 in T cell activation prove very important in determining whether a naive T cell generates more SLECs or MPECs. Cytokines interleukin-2 (IL-2), IL-12, and type I IFN promote generation of SLECs (38, 39) while TGFβ inhibits SLEC formation (40). IL-12 signals upregulate the T-box transcription factor, T-bet, which is a key regulator of SLEC formation (38). More recent evidence indicates that signals through the TCR also impact CD8 phenotype as

stimulation of the TCR with high affinity ligand skews the T cell response to a SLEC phenotype (34, 41). Despite these known regulators of the CD8 T cell response, how RTEs are modulated by these factors has not been fully investigated. RTEs are skewed to a SLEC phenotype in both non-inflammatory and inflammatory conditions (17) but the impact of the TCR signal on cell fate skewing of this distinct T cell population has not been investigated.

In addition to the phenotypic markers that predict CD8 T cell fate, other important CD8 T cell functions include expansion of effector T cells, access to peripheral tissues, and activation of appropriate effector functions such as the production of IFNγ and TNFα. Access to peripheral tissues can be gained through the expression of numerous adhesion markers including lymphocyte function-associated antigen -1 (LFA-1), very late antigen-4 (VLA-4), CD44 and Ly6C. LFA-1 and VLA-4 are homodimeric integrins composed of an α and β chain that are transmembrane glycoproteins with a short cytoplasmic tail (42). Integrins can signal in both an inside-out and outside-in capacity. Notably for our interests, inside-out signaling includes the ability of the TCR to activate integrins and induce the integrin to undergo a conformational change to a form that binds ligand with high affinity. These activated integrins can mediate arrest and firm adhesion of T cells, key steps in the ability of T cells to access non-lymphoid tissues. Outside-in signaling has been hard to study due to the difficulty in uncoupling the signaling events, but is thought to induce T cell proliferation and IL-2 production as well as stabilizing the T cells/APC interaction (42). VLA-4 mediates firm cellular adhesion via binding to VCAM-1 and contributes to T cell recruitment to the skin, brain, and bone marrow (43-46). CD44 is a molecule classically known for its expression on activated T cells and while its function is less well known, it has been implicated in T cell adhesion processes as well (47). Ly6C is another marker on activated CD8 T cells, classified as an adhesion molecule and implicated in CD8 T

cell homing though the mechanisms of this process are not well understood (48). Disparate regulation of these molecules allows for the differential localization of T cells and protection within distinct anatomical sites.

While CD8 T cells are classically known for their ability to protect the host from intracellular bacteria and viruses, recent evidence suggests they can have a role in protection from extracellular bacteria as well, specifically *Yersinia pseudotuberculosis* (Yptb) and the related pathogen and mediator of the plague, *Y. pestis* (49). *Yersinia* species subvert the immune system and avoid phagocytosis by injecting *Yersinia* outer proteins (Yops) into the cytosol of host cells via a type III secretion system (50). *Yersinia* express 6 different effector Yop with varying functions. The protein YopE has been shown to elicit a robust CD8 T cell response (51), suggesting these proteins can be processed and presented on MHC of the bacterially infected cell despite the lack of phagocytosis. Early work suggested a role for CD8 T cell mediated protection from Yptb because depleting CD8 T cells resulted in reduced host survival, but the mechanism remained unknown (52). Recent work has determined that following protein immunization, mice are protected from *Yersinia* and while CD8 T cells can kill *Yersinia* targets *in vitro* in a perforin dependent manner, perforin is not required for this protection, but TNFα and IFNγ are required (53).

We hypothesized that RTEs would have differential TCR signal transduction which would impact their response to *in vivo* infection with bacteria expressing ligands of high and low affinity. Thus, we utilized the OT-I Tg T cell model with APLs to test the response of RTEs to peptide stimulation *in vitro* and evaluated the response to infection with Lm.APL to determine the impact of ligand affinity on T cell proliferation, cell fate skewing, cytokine production, expression of transcriptional regulators, and surface adhesion molecule expression.

Results

CD8 RTEs exhibit increased TCR signal transduction

Splenocytes from RAG2p-GFP Tg OT-I TCR Tg mice were stimulated *in vitro* with 0.1μM N4, Q4, or T4 peptide and TCR signaling quantified by phosphorylation of downstream signaling mediators using flow cytometry (Figure 3-1A and B). ZAP70 phosphorylation was detected following stimulation with the N4 high affinity ligand in as little as 5 minutes (Figure 3-1A, bottom); but interestingly, only within RTEs. ZAP70 phosphorylation peaked in the RTE population at about 75% in response to N4 peptide, while the mature T cells only reached 26%. The response to low and high affinity peptides occurred with similar kinetics, with the peak of ZAP70 phosphorylation for both RTEs and mature T cells at 60-90 minutes. Q4 stimulation induced about 40% of RTEs and 10% of mature T cells to phosphorylate ZAP70 (Figure 3-1A). T4, the lowest affinity ligand tested, generated very little pZAP70, but RTEs still displayed increased levels over the barely detectible response of mature T cells (Figure 3-1A).

We also measured ERK phosphorylation to determine whether the increased proximal signals in RTEs are propagated downstream. ERK activation following stimulation with the highest affinity peptide could be detected by 5-10 minutes (Figure 3-1B, bottom). This response peaked for RTEs at 90 minutes with 93% of cells phosphorylating ERK, while mature T cells peaked at 120 minutes, with 80% pERK+. Stimulation with the lower affinity peptides, Q4 and T4, resulted in a detectable pERK signal at 30 minutes that peaked at 60 minutes (Figure 3-1B). RTEs also displayed increased ERK activation compared to mature T cells in the low affinity peptide stimulation across all time points examined. Thus, RTEs are better able than mature T cells to transduce both proximal and distal TCR signaling events *in vitro* in response to low and high affinity peptide stimulation.

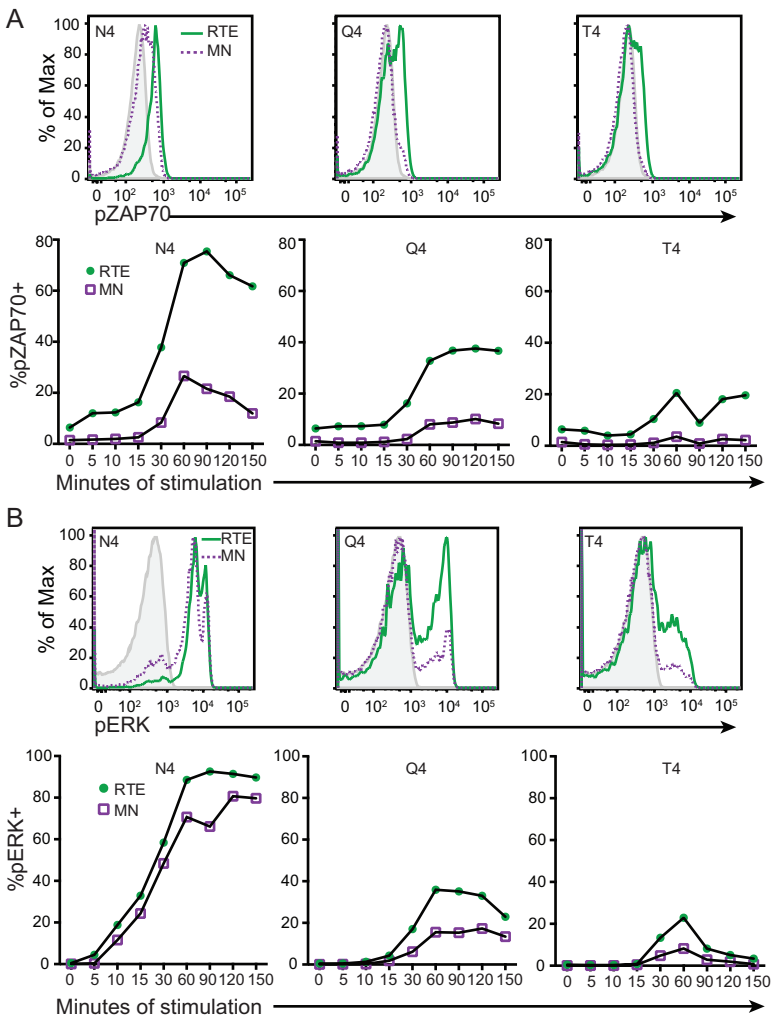

Figure 3-1. RTEs have heightened TCR signal transduction following stimulation with high and low affinity peptide antigens *in vitro*. RAG2p-GFP Tg OT-I TCR Tg splenocytes were stimulated in vitro with 0.1μM of the indicated peptide. A and B) Representative histograms (top) of pZAP70 (A) and pERK (B) staining following activation of OT-I TCR Tg RTEs (solid green line) and MN T cells (dashed purple line) for one hour with N4 (left), Q4 (middle), or T4 (right) OVA.APLs. The "no peptide" control is shown in gray. Bottom curves in A and B show a full time course for RTE (green circle) and MN T cell (purple box) activation with the indicated peptides as measured by pZAP70 (A) and pERK (B) expression. Data are representative of 2 independent experiments.

CD8 RTEs expand to a greater extent and are skewed toward a SLEC phenotype

To determine the effect of increased TCR signal transduction in RTEs, we used an *in vivo* model of bacterial infection, Lm.APL. Using this model, RTEs expand to a greater extent than mature T cells in the same inflammatory environment as measured both by frequency (Figure 3-2A, left) and absolute number (Figure 3-2A, right) of transferred cells in the spleen. Expansion to low affinity ligands is diminished (as has been shown previously in reference (33)), yet RTEs expanded to a greater extent in response to low affinity ligands compared to mature T cells (Figure 3-2A). This increased relative expansion is in part due to the skewing of RTEs toward a SLEC phenotype as determined by KLRG1 and IL-7Rα expression (Figure 3-2B and C). Lower affinity ligands drive the generation of a diminished proportion of SLECs and an elevated proportion of MPECs; RTEs follow this trend with a lower proportion of SLECs in response to Lm.T4 compared to Lm.N4 infection. However, RTEs still skew to a SLEC phenotype when compared to the mature T cell response to low affinity stimulation (Figure 3-2C). Taken together, these data suggest that the enhanced TCR signal transduction in RTEs drives increased cell expansion and skews cell fate toward the SLEC compartment in response to bacterial infection.

A lower proportion of CD8 RTEs makes effector cytokines

When evaluated for their ability to generate effector cytokines (Figure 3-3A), a significantly lower proportion of RTEs compared to mature T cells produced IL-2, TNFα, and IFNγ (Figure 3-3B) in response to infection with bacteria expressing a high affinity ligand, Lm.N4. No difference was seen in IFNγ production between RTE- and MN-derived T cells following stimulation with low affinity ligands (Figure 3-3B, right), although the majority of

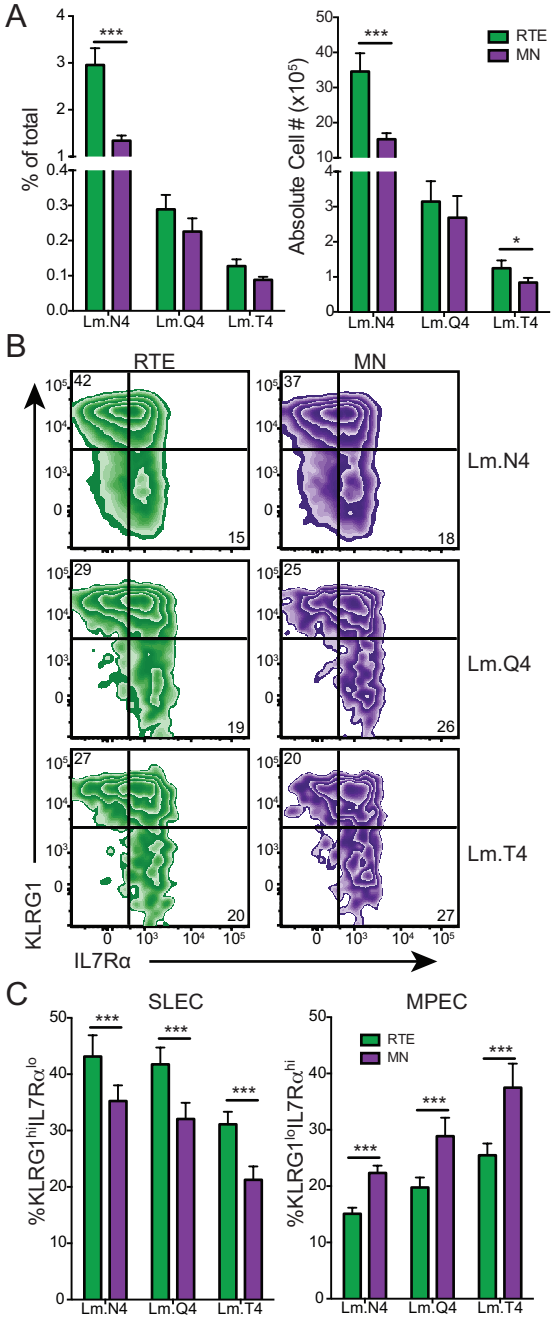

Figure 3-2. RTEs display greater expansion and are skewed to a SLEC phenotype in response to infection with *Listeria* expressing high and low affinity ligands. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Splenocytes were analyzed on d7 post infection. A) Proportion (left) and absolute number (right) of RTEs and mature T cells. B) Representative flow plots to determine relative SLEC ($KLRG1^{hi}IL7R\alpha^{lo}$) and MPEC ($KLRG1^{lo}IL7R\alpha^{hi}$) phenotype in RTEs vs mature T cells. Numbers represent the percentage of transferred cells within that quadrant. C) Proportional SLEC (left) or MPEC (right) phenotype. Data are presented as mean ± SEM compiled from 8 independent experiments, n=19 (N4), 20 (Q4), and 22 (T4). *p<0.05, ***p<0.001 by paired Student's *t* test.

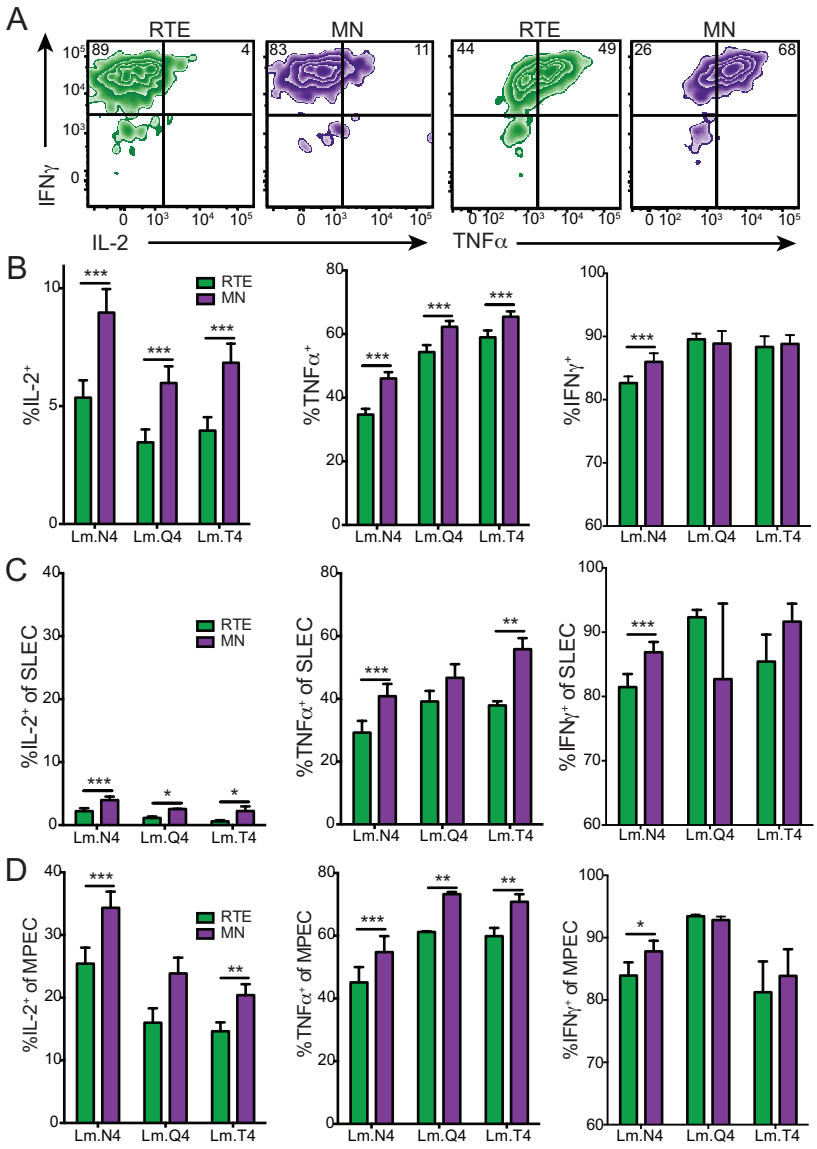

Figure 3-3. A lower proportion of RTEs produces cytokines in response to bacterial infection, regardless of cell fate. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Splenocytes were analyzed on d7 post infection after restimulation *in vitro* with SIINFEKL for 5 hours in the presence of brefeldin A. A) Representative flow plots of cytokine staining; gates were set using cells from an infected recipient that were not stimulated *in vitro*. Numbers represent the percentage of transferred cells within that quadrant. B) RTE- or MN-derived cells from mice infected with the indicated Lm.APL producing IL-2 (left), TNFα (middle), and IFNγ (right). C) and D) IL-2 (left), TNFα (middle), and IFNγ (right) production by RTE- or MN- derived SLECs (C) or MPECs (D). Data are presented as mean ± SEM compiled from B) 8 independent experiments, n=19 (N4), 20 (Q4), and 22 (T4) and C) and D) 2 independent experiments, n=6/group. *p<0.05, **p<0.01, ***p<0.001 by paired Student's *t* test.

transferred cells made IFNγ. RTEs exhibited a reduced ability to make IL-2 and TNFα in response to low affinity Lm.Q4 and Lm.T4 infection (Figure 3-3B). RTE occupants of both the SLEC and MPEC compartments were comprised of a lower proportion of cytokine producing cells (Figure 3-3C and D), indicating that the differences are not due to cell fate skewing alone. A higher proportion of MPECs were cytokine producers (Figure 3-3D), most noticeably so for IL-2. These data suggest that some differences in cytokine production by RTEs can be attributed to their increased SLEC skewing, but their cell fate phenotype cannot fully account for their cytokine defects and is, therefore, intrinsic to RTEs.

RTEs produce less cytokines on a per-cell basis

We evaluated the cytokine production by RTEs to determine if, in addition to being less likely to become cytokine-producing cells, they produced lower amounts of cytokines per cell. Cytokine production per cell was determined by mean fluorescence intensity (MFI) of cytokine positive cells. While around half the proportion of RTEs made IL-2 compared to mature cells (Figure 3-3), the RTEs that did make IL-2 made an amount similar to that generated by mature T cells (Figure 3-4). However, RTEs produced less TNFα and IFNγ on a per cell basis than mature cells in response to infection with bacteria expressing ligands of all affinities tested (Figure 3-4).

CD8 RTEs express higher levels of the transcription factor T-bet but not Eomes

Important transcriptional regulators of the CD8+ T cell response include T-bet and Eomesodermin (Eomes). These master regulators contribute to both effector responses and memory T cell formation. On d7 post Lm.APL infection, intranuclear staining for T-bet and Eomes revealed robust upregulation of T-bet compared to the overall endogenous CD8+

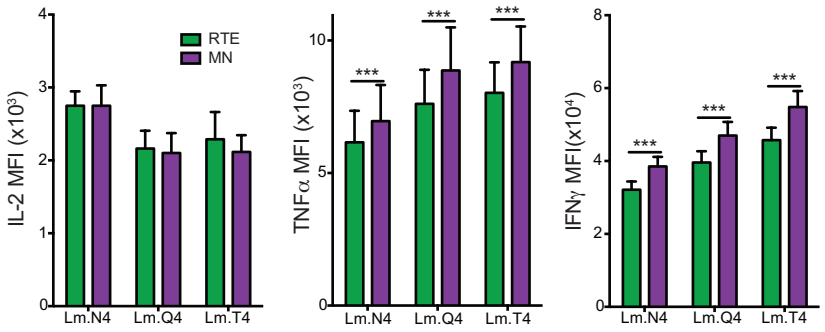

Figure 3-4. RTEs make less TNFα and IFNγ on a per cell basis in response to infection with bacteria expressing high and low affinity ligands. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Splenocytes were analyzed on d7 post infection after restimulation *in vitro* with SIINFEKL peptide for 5 hours in the presence of brefeldin A. Cytokine production by RTE- or MN-derived cells as measured by mean fluorescence intensity (MFI) of IL-2+ (left), TNFα+ (middle), and IFNγ+ (right) cells. Data are compiled from 8 independent experiments, n=19 (N4), 20 (Q4), and 22 (T4). ***$p<0.001$ by paired Student's *t* test.

response (Figure 3-5A). On the other hand, expression of Eomes was more diverse. RTEs display increased T-bet expression compared to mature cells following both high and low affinity stimulation (Figure 3-5B, top). Eomes expression by RTEs is either the same or lower than mature cells (Figure 3-5B, bottom), suggesting these cells differ at the level of transcriptional regulation.

Increased VLA-4 and other adhesion molecule expression by RTEs compared to mature T cells in response to low affinity antigens

RTE- and MN-derived T cells encountering Lm.N4 highly upregulate VLA-4 expression, with RTEs exhibiting slightly increased expression when compared to mature T cells (Figure 3-6A and C). This difference is magnified in response to the low affinity ligand, as only 75% of mature T cells upregulate VLA-4 expression while 90% of RTEs do (Figure 3-6C). Similarly, RTEs maintain higher levels of the activation and adhesion marker CD44 in response to low affinity antigen (Figure 3-6B and C). Finally, RTEs have higher expression of Ly6C in response to low affinity ligand stimulation, while MN-derived cells maintain a steady Ly6C expression pattern in response to infection with bacteria expressing ligands of varying affinities (Figure 3-6B and C). Overall, the adhesion molecule expression by RTEs responding to bacterial infection is increased over that of mature cells.

RTEs enter inflamed tissue and control infection in a VLA-4 dependent manner

Knowing that VLA-4 plays an important role in homing, we sought to evaluate the impact of this disparate VLA-4 expression by inducing non-specific inflammation in the ear. Effector cells were generated as before using T cell adoptive transfer and Lm.T4 infection; inflammation was

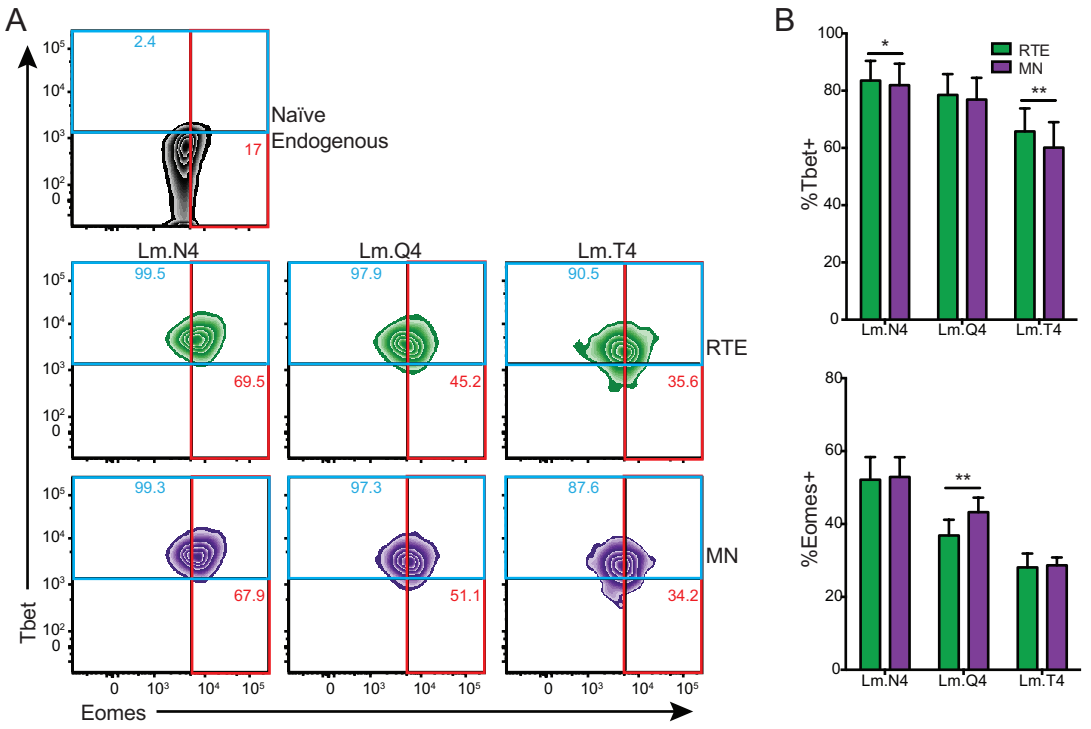

Figure 3-5. Compared to mature T cells, RTEs better upregulate expression of the transcription factor Tbet but not Eomes. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Splenocytes were analyzed on d7 post infection through intranuclear staining. A) Representative flow plots of transcription factor staining; gates were set using endogenous naïve CD8+ T cells from infected mice (top, black). Numbers represent the percentage of transferred cells within the gate. B) Proportion of Tbet+ (top) or Eomes+ (bottom) RTEs or mature T cells. Data are presented as mean ± SEM compiled from 8 independent experiments, n=19 (N4), 20 (Q4), and 22 (T4). *p<0.05 and **p<0.01 by paired Student's *t* test.

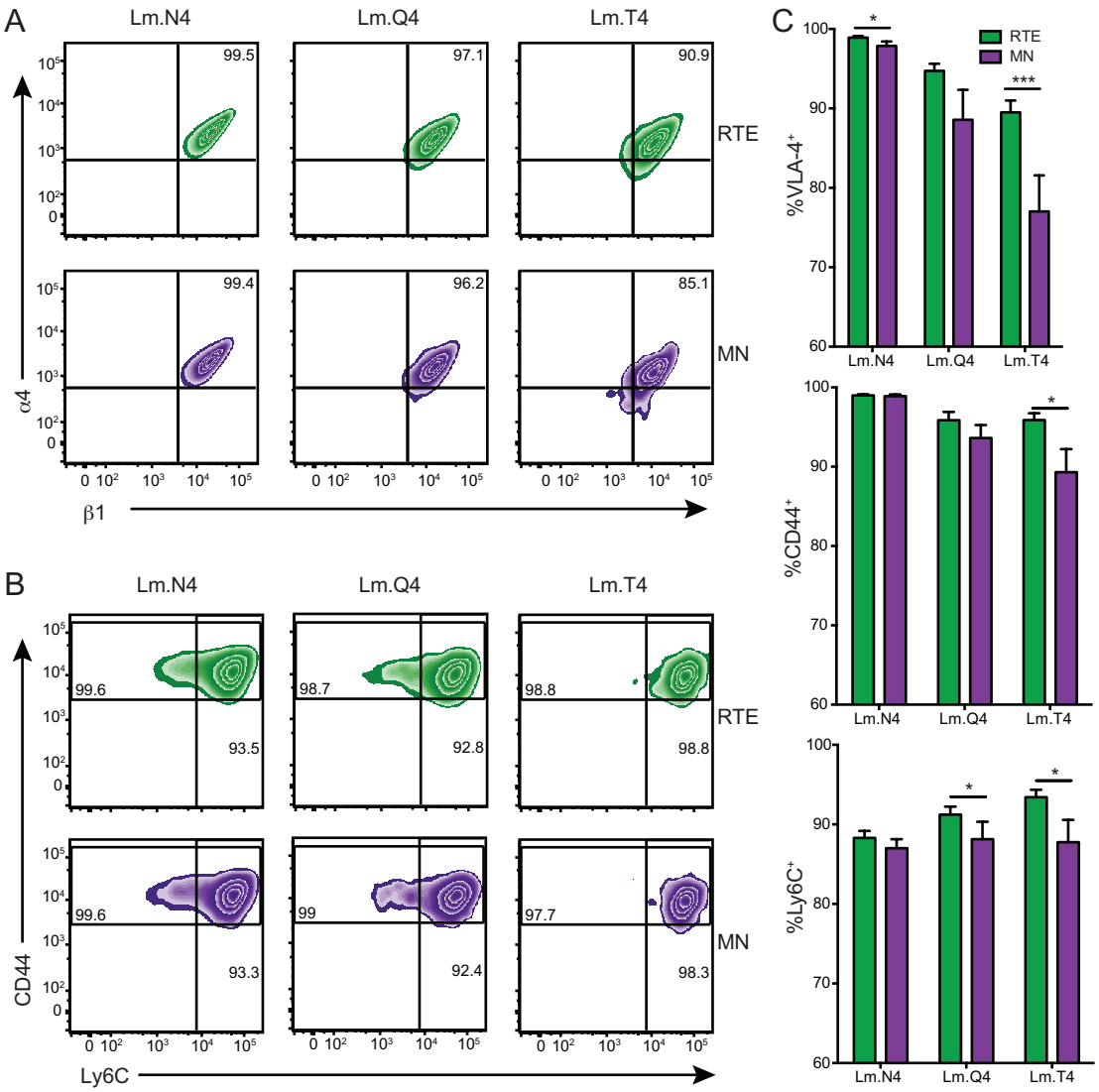

Figure 3-6. RTEs maintain adhesion molecule expression following infection with bacteria expressing a low affinity ligand. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Splenocytes were analyzed on d7 post infection by surface staining. A) and B) Representative flow plots of A) VLA-4 (α4β1) and B) CD44 and Ly6C staining; gates were set using naïve endogenous CD8+ T cells from infected mice. Numbers represent the percentage of transferred cells within the gate. C) Proportion of VLA-4+ (top), CD44+ (middle) or Ly6C+ (bottom) RTEs or mature T cells. Data are presented as mean ± SEM compiled from 8 independent experiments, n=19 (N4), 20 (Q4), and 22 (T4). *$p<0.05$ and ***$p<0.001$ by paired Student's *t* test.

then induced by administering a small amount of CFA intradermally into one ear 5 days post infection. The inflamed and control ears were separately digested 36 hours later and the transferred effector cells quantified. The number of transferred cells in the CFA-treated ear was increased >30 fold over that in the control ear (Figure 3-7A). The majority of this homing to the inflamed tissue was VLA-4 dependent, as it was significantly reduced by treatment with blocking anti-VLA-4 (Figure 3-7A). Most interestingly, RTEs were better able to enter the inflamed ear compared to mature T cells when activated by a low affinity ligand (Figure 3-7B). To determine whether this increased invasiveness is protective, RTEs and MN T cells were separately transferred to recipient mice, which were then infected with Lm.APL. These mice were challenged in the ear with VSV or VSV.OVA on day 5 post-Lm.APL infection and viral PFU in the ear quantified 24 hours later. Mice challenged with VSV had similar virus levels between mice receiving RTEs or MN T cells (data not shown). Mice receiving RTEs had significantly reduced virus in the ear following VSV.OVA challenge compared to recipients of MN T cells (Figure 3-7C). Therefore, despite the reduced capacity of RTEs to produce cytokines, their increased invasiveness allows them to better access sites of inflammation and control viral challenge in an antigen dependent manner.

Increased invasiveness by RTEs drives disease when cells recognize self-antigens

To determine whether this increased invasiveness of RTEs could also contribute to increased autoimmunity, we measured diabetes induction in mice expressing a model pancreatic antigen. Sort-purified OT-I TCR Tg RTEs or MN T cells were separately transferred into RIPmOVA hosts that were then infected with Lm.N4 or Lm.T4 the following day. After Lm.N4 infection, all recipients of RTEs rapidly developed diabetes, while fewer of the mice receiving

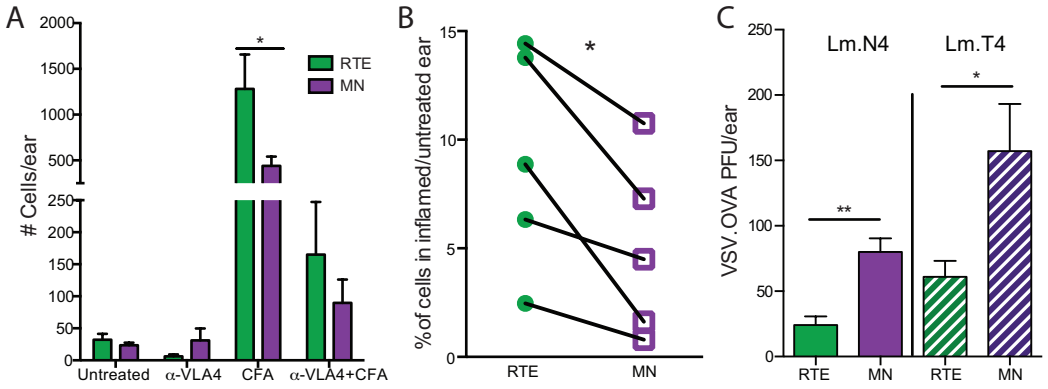

Figure 3-7. RTEs demonstrate both increased VLA-4 dependent homing to inflamed tissues and viral clearance in the ear following challenge. Sort-purified OT-I TCR Tg RTEs and MN T cells were transferred together (A and B) or separately (C) into congenically-marked hosts which were infected the following day with the indicated Lm.APL. A) Number of cells in the ear on d7 post Lm.T4 infection following CFA treatment on d5.5 and/or anti-VLA-4 administration. Data are presented as mean ± SEM and are representative of 2 independent experiments. B) Proportion of cells in the CFA-treated (inflamed) ear corrected for their proportion in the untreated ear of Lm.T4 infected mice. Data are compiled from 5 independent experiments, 3 pooled ears/data point. C) VSV PFU/ear 24 hours following injection of 10^4 PFU VSV.OVA intradermally in the ear on day 5.5 post Lm.APL infection in recipients of individually transferred RTEs or MN T cells. Data are presented as mean ± SEM compiled from 2-3 independent experiments *$p<0.05$, **$p<0.01$,***$p<0.001$ by paired or unpaired (C) Student's *t* test.

MN T cells became diabetic, and did so with slower kinetics (Figure 3-8A). Infection with the low affinity Lm.T4 strain drove less overall diabetes but RTEs still appeared more diabetogenic (Figure 3-8B), consistent with their increased VLA-4 expression following stimulation with low affinity ligand. Thus, the increased invasiveness of RTEs, while contributing to host protection, can also be detrimental when self-tolerance is lost.

Yersinia pseudotuberculosis infection drives similar responses by RTE- and MN-derived cells but a lower proportion of cytokine producing RTEs

To complement our studies on the response to the intracellular pathogen *Listeria*, the ability of RTEs to respond to extracellular bacteria was evaluated using Yptb.N4 engineered to express OVA as one of the translocated Yop effector proteins. These responses to Yptb.N4 could be compared to the Lm.N4 infection with matching SIINFEKL sequence expression. Sort-purified RTEs and MN T cells were adoptively transferred into congenically marked hosts that were intraperitoneally infected the following day with Yptb.N4. The CD8 T cell response was analyzed at the peak of the response on d11/12. RTEs did not expand to a greater extent compared to mature T cells, inconstant to the response to Lm.N4 infection (Figure 3-9A), nor were they skewed toward a SLEC phenotype (Figure 3-9B, left). The majority of both transferred RTE and MN OT-I Tg T cells were of the MPEC lineage (Figure 3-9B, middle). Similar VLA-4 expression levels were seen on RTE- and MN-derived cells (Figure 3-9B, right). However, similar to the Lm.N4 response, fewer RTEs than mature T cells produced cytokines (Figure 3-9C). Overall, these data suggest that the responses seen by RTEs following Lm.N4 infection are not translatable to all infection types. However, the decreased ability to produce cytokines by RTEs seems to be a universal trait of RTE biology.

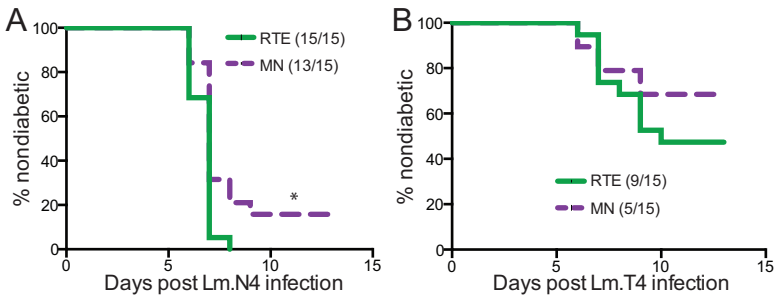

Figure 3-8. RTEs drive increased diabetes in RIPmOVA recipients following Lm.APL infection. 10,000 sort-purified OT-I TCR Tg RTEs or MN T cells were separately transferred into RIPmOVA mice which were infected the following day with Lm.N4 (A) or Lm.T4 (B). Shown is disease incidence in infected RIPmOVA recipients as measured by a blood glucose reading >350mg/dL. Numbers in parentheses indicate diabetic/total mice. Data are combined from 3 independent experiments. *$p<0.05$ by log rank test.

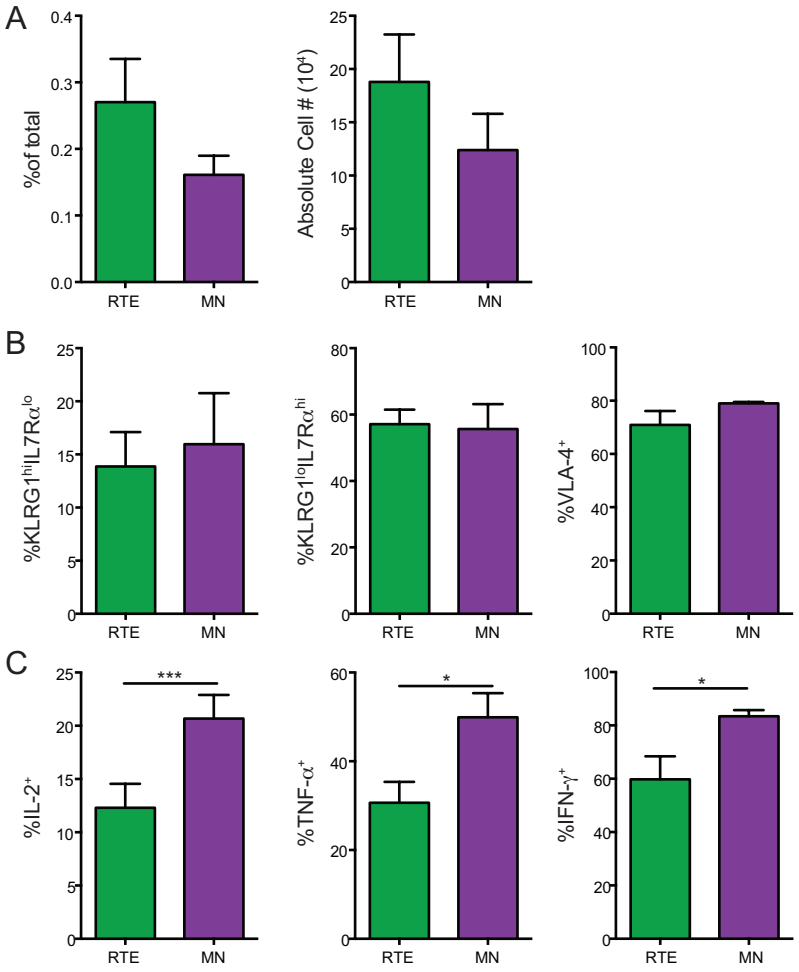

Figure 3-9. Following infection with *Yersinia pseudotuberculosis*, RTEs are deficient in cytokine production but do not differ in cell accumulation or cell fate phenotype from their mature counterparts. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected i.p. the following day with *Y. pseudotuberculosis* expressing OVA. Splenocytes were analyzed on d11/12 post infection. A) Proportion (left) and absolute number (right) of RTEs and mature T cells. B) Proportional SLEC (left) or MPEC (middle) phenotype and VLA-4 expression (right) by responding RTE and mature T cells. C) The proportion of RTE- or MN-derived T cells producing IL-2 (left), TNFα (middle), and IFNγ (right). Data are presented as mean ± SEM compiled from 2 independent experiments, n=6. *$p<0.05$, ***$p<0.001$ by paired Student's *t* test.

Conclusions

The experiments described in this chapter provide evidence for increased TCR signaling and invasiveness of CD8 RTEs, which results in either enhanced protection in an infectious setting or an increased risk of autoimmunity. Our results support the importance of peripheral tolerance mechanisms as part of the process of post-thymic maturation. The increased TCR signal transduction by RTEs has many potential implications. The first is evidenced through the differential responses observed using the model systems presented here, such as increased number, altered cell fate skewing, and enhanced tissue invasiveness. Higher affinity peptides have previously been shown to induce increased pERK compared to low affinity peptides in thymocytes (32), but this surprising increase in TCR signal transduction by RTEs suggests that they may be able to respond to a broader range of peptides as well as to receive increased homeostatic survival signals (54). The increased average CDR3 length of TCRs expressed by RTEs (55) and upregulated TCR/CD3 expression by RTEs (14, 23) may help explain the enhanced signal transduction of polyclonal RTEs (5). However, results from studies using TdT-/- mice, in which the CDR3 loops of the TCRs are shorter due to the lack of N-nucleotides, are conflicting as to whether short CDR3 regions lead to increased (56) or reduced (57) breadth of the T cell response. Further characterization of molecules involved in modulating TCR signaling, such as phosphatases and miR181a, will be necessary to better understand the driving force behind the enhanced TCR signal transduction in RTEs. The microRNA miR181a is involved in TCR tuning in immature and mature T cells and its expression correlates with T cell sensitivity to antigen (58). Regulation of T cell sensitivity by miR181a acts in part through down regulation of phosphatases, thereby reducing the TCR sensitivity threshold. The expression levels of miR181a in human cord blood (enriched for RTEs) was increased over that in adult peripheral

blood (59). Future studies addressing the expression level of miR181a in murine RTEs would be of interest, as this could play a role in the differential TCR signaling of RTEs and mature T cells we have demonstrated here.

Our data are in agreement with recent work demonstrating that increased TCR signaling skews cell fate toward a SLEC phenotype (34), and we extend these studies by evaluating the differential response of RTEs. The increased TCR signaling by RTEs observed *in vitro* correlated with their expansion and skewing towards a SLEC phenotype following Lm.APL infection *in vivo*. Though low affinity ligands had reduced skewing toward SLECs, RTEs always had an increased proportion of SLECs compared to mature T cells. The propensity of RTEs to skew to a SLEC phenotype may not be a completely universal response, however, as RTEs responding to Yptb did not skew to a SLEC phenotype. It is hard to state this definitively, as there is little known about the role of CD8 T cells in Yptb infection and we observed very little skewing to SLECs in the overall T cell response. Results from the Palmer group would suggest that RTEs might maintain longer contacts with APC and perhaps have more asymmetric division, generating polarized proximal daughter cells (34). Evaluation of the synapse in RTEs would be an interesting question to address, as many players in the immune synapse, including the TCR, CD28, and integrins (14, 23), have been shown by us to be differentially expressed in RTEs and mature T cells. Overall, these data suggest that RTEs are better able to respond to ligands with a range of affinities and should be able to respond to a peptide that falls below the classically defined response threshold of mature cells, an interesting suggestion in lieu of the autoimmunity data presented here as well.

The decreased cytokine production by RTEs shown here is a characteristic of this distinct population found in nearly all experimental models examined thus far, including our model

looking at Yptb responses presented here. To our knowledge, this is the first time IL-2 and TNFα cytokine production has been associated with an MPEC phenotype, though decreased cytokine production by RTEs is not dependent on cell phenotype. Previous data in CD4 RTEs indicate that decreased cytokine production may be due to increased methylation patterns at cytokine promoter regions (19) but this has not been assessed directly in CD8 RTEs. The increased Tbet expression seen by RTEs is somewhat contradictory, given that IFNγ is a direct target of Tbet. This expression pattern may suggest that IFNγ expression in RTEs is regulated by additional mechanisms. Given that RTEs in a neonatal environment encounter many antigens for the first time (10), this decreased cytokine production may protect the lymphopenic host from any undue cytokine destruction.

The deficiency seen in cytokine production by RTEs seems to be, at least in part, remedied by their ability to better access inflamed tissues. When mounting an immune response in a lymphopenic environment, it would be desirable for the effector cells guarding barrier surfaces to prevent pathogens from establishing an infection. In addition to looking in the skin, we evaluated whether RTEs were present at greater numbers in the brain or bone marrow, as VLA-4 expression has also been shown to regulate homing to these tissues (44-46, 60). However, we did not see differences in cell numbers in these tissues, at least at the time point evaluated. It could be that the similar localization of RTEs and mature T cells in these tissues was the result of evaluating a single time point, as the kinetics of this homing are not well established. Our work establishes that the increased invasiveness by RTEs can also be translated to increased autoimmunity. These responses by RTEs highlight the necessity of completing this peripheral T cell tolerance process to remove or inhibit T cells prone to autoimmunity. Further

work is required to determine the interactions required for RTEs to undergo tolerance induction to peripherally expressed antigens.

Chapter Four: Reduced Central Memory T Cell Formation from RTE-derived Memory Cells

Introduction

Development of a long lasting memory response is a hallmark of adaptive immunity and the CD8 T cell memory response has been well characterized as a means to define correlates of immune protection as targets for vaccine design. Cells during the acute response to infection are classified as SLECs or MPECs on the basis of their KLRG1 and IL-7Rα expression pattern and these subsets of T cells have been shown to differentially contribute to the memory T cell pool (38).

Memory cells can also be classified into different subsets based largely on expression of the chemokine receptor CCR7 and the homing molecule CD62L. CD62L mediates entry and retention of naïve and memory T cells in lymph nodes. Cells expressing high levels of CD62L are classified (61) as central memory T cells (T_{CM}), whereas cells with low levels of CD62L are effector memory T cells (T_{EM}). T_{CM} localize to lymph nodes, spleen, blood, and bone marrow and are thought have greater proliferative capacity as well as enhanced ability to make IL-2. In particular, the majority of CD8 T cells in the bone marrow are T_{CM} (60) and this site has been suggested to be an important niche for homeostatic proliferation by CD8 memory T cells (62, 63). T_{EM} are present to a lesser degree in lymph nodes, but are also present in the spleen and peripheral tissues (64). T_{EM} functions are important to limit initial pathogen expansion despite their limited proliferative capacity, and are thought to have more immediate effector functions as well as and to rely on their localization to peripheral sites.

The conditions surrounding memory formation have been extensively studied, and yet the complexity behind generation of a diverse memory T cell response is still not fully understood. It

is clear that the starting frequency of antigen specific naïve T cells can influence the expansion of the effector response as well as the generation of memory cells (65). While we know from previous work using OVA APL that the TCR signal strength regulates the magnitude of the effector response, ligands of all affinities were able to generate a memory population (33). Inflammation also plays a role in the generation of a memory T cell response, and reduced inflammation leads to a greater formation of T_{CM} (66). On the other hand, repeated infection, vaccination, or priming generates successively more T_{EM} (67), though it is unclear how this affects the protective ability of the memory T cell response.

The acute response of RTEs is skewed towards a SLEC phenotype, yet previous data suggest they form a functional memory population (17). Here we aim to test the hypothesis that RTEs have differential memory generation compared to mature T cells due to their increased TCR signal transduction and enhanced acute T cell response. We utilized the OT-I TCR Tg system with OVA APLs to evaluate the *in vivo* response of RTEs to bacterial antigens of varying affinity. In this chapter we show that while RTEs generate a memory response, this response is skewed to the T_{EM} compartment, which may impacts the RTE-derived long-term memory responses.

Results

RTEs are not deficient in memory formation despite reduced proportions of MPECs at acute time points

To evaluate the ability of RTEs to form long lived memory cells, experiments were designed as previously described, transferring sort-purified OT-I TCR Tg RTEs and MN T cells into congenically marked hosts before infecting them with Lm.APL the following day. Cells were analyzed on day 60, following resolution of infection and T cell contraction, to evaluate the responses of memory cells that originally encountered high or low affinity antigen as RTEs or MN T cells. Although the proportion of RTEs with a MPEC phenotype at an acute time point was significantly lower than that of mature cells (Figure 3-2), RTEs were not deficient in their ability to form memory cells, measured by percent of total cells (Figure 4-1, left) or absolute cell number (Figure 4-1, right). In fact, RTE-derived cells were still increased over MN-derived cells at this memory time point. This was true for infection with bacteria expressing ligands of all affinities tested.

At memory time points, a lower proportion of RTE-derived cells are producing IL-2

RTE- and MN-derived T cells that encountered antigen >60 days ago still produced cytokines upon restimulation (Figure 4-2A). Cells that originally encountered bacteria expressing high affinity ligands were more likely to be IL-2 producers at a memory time point than cells that encountered low affinity ligands (Figure 4-2B, left). Although this is true for RTE- as well as MN-derived cells, a lower proportion of RTEs produced IL-2 compared to mature T cells in response to ligands of all affinities tested. RTE- and MN-derived cells that made IL-2 produced

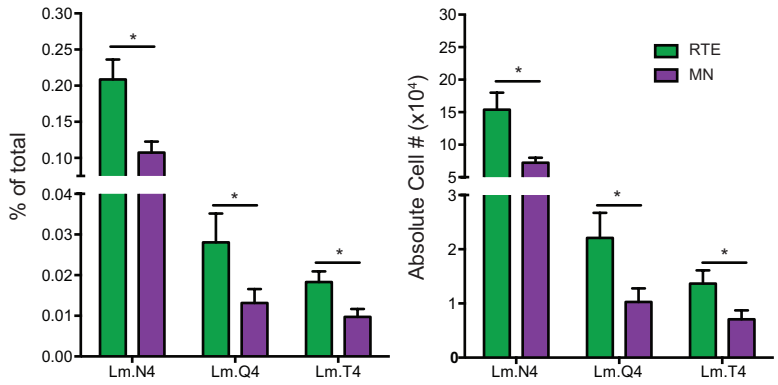

Figure 4-1. RTEs are not deficient in their ability to form memory cells despite a reduced proportion of MPECs during acute infection. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Splenocytes were analyzed on d60 post infection. Proportion (left) and absolute number (right) of RTE- and MN-derived T cells. Data are presented as mean ± SEM compiled from 3 or 4 independent experiments; n=12 (N4), 9 (Q4), and 12 (T4). *$p<0.05$, by paired Student's *t* test.

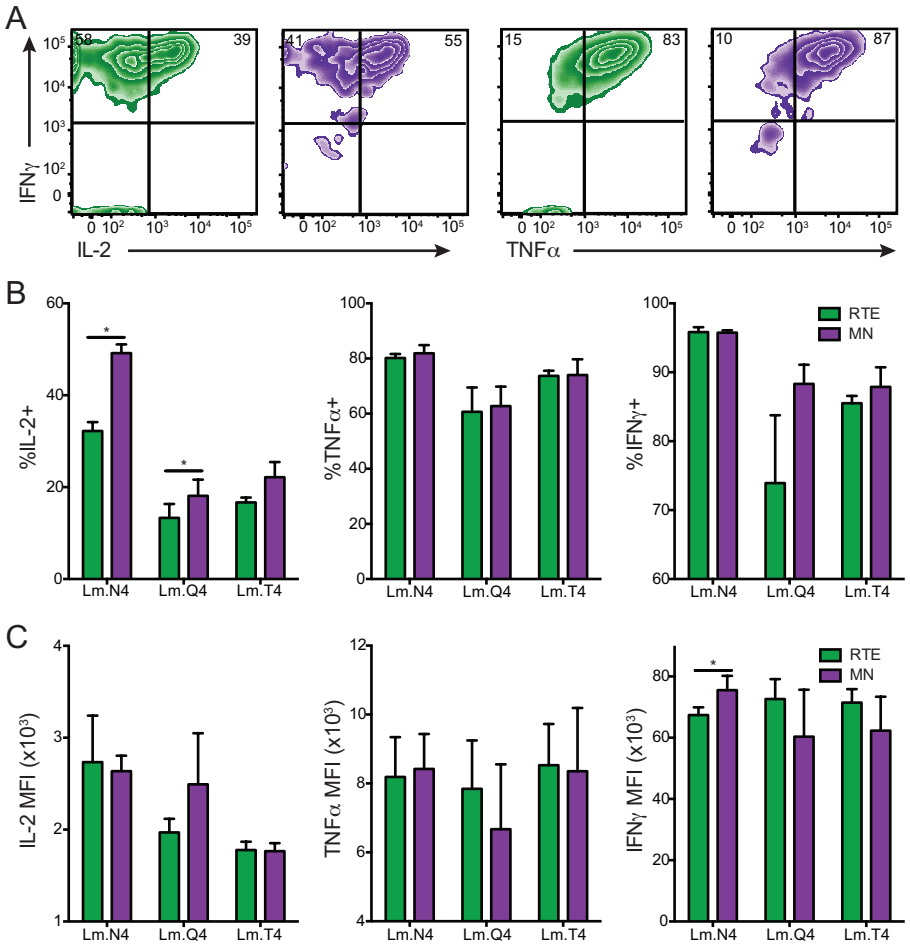

Figure 4-2. Reduced IL-2, but not TNFα, production by RTE-derived memory cells. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Splenocytes were analyzed on d60 post infection after restimulation *in vitro* with SIINFEKL peptide for 5 hours in the presence of brefeldin A. A) Representative flow plots of cytokine staining from Lm.N4 infected mice; gates were set using cells from an infected recipient that were not stimulated *in vitro*. Numbers represent the percentage of transferred cells within that quadrant. B) RTE- or MN-derived cells from mice infected with the indicated Lm.APL producing IL-2 (left), TNFα (middle), and IFNγ (right). C) Cytokine production by RTE- or MN-derived cells as measured by mean fluorescence intensity (MFI) of IL-2+ (left), TNFα+ (middle), and IFNγ+ (right) cells. Data are presented as mean ± SEM compiled from 2 independent experiments, n=6 for N4, Q4 and T4, *p<0.05, by paired Student's *t* test.

similar amounts on a per cell basis at this memory time point (Figure 4-2C, left). TNFα production was not different between RTE- and MN-derived cells (Figure 4-2B and C, middle). While the proportion of RTE-derived cells making IFNγ was similar to that of mature cells, IFNγ+ RTE-derived cells primed by high affinity ligand made less IFNγ on a per cell basis than their mature counterparts (Figure 4-2C, right). Together, these data demonstrate that the maturation state of the T cell at the time of original antigen encounter can have long lasting impacts on cell function.

RTE- and MN-derived memory cells express similar levels of adhesion molecules but differ in memory phenotype

Increased VLA-4 expression is a feature of RTEs during the acute response to bacterial infection and the greatest differences between RTE and mature cells are observed following low affinity ligand stimulation (Figure 3-6C, top). VLA-4 expression at a memory time point was lower in cells that were primed with a low affinity ligand; however, differences between RTE- and MN-derived cells were no longer statistically significant at this memory time point (Figure 4-3A). Most of the cells at this memory time point were $CD44^{hi}$, although this defined a slightly lower proportion of the cells originally primed with low affinity ligands, and expression levels were not different between RTE- and MN-derived cells (Figure 4-3B). CD62L expression, however, was much higher in MN- compared to RTE-derived cells (Figure 4-3C). In addition to expressing reduced CD62L levels compared to MN-derived memory cells, RTE-derived cells also expressed lower levels of the transcription factor Eomes, important for the formation of T_{CM} (Figure 4-3D). The reduced Eomes expression was limited to high affinity infections however, as

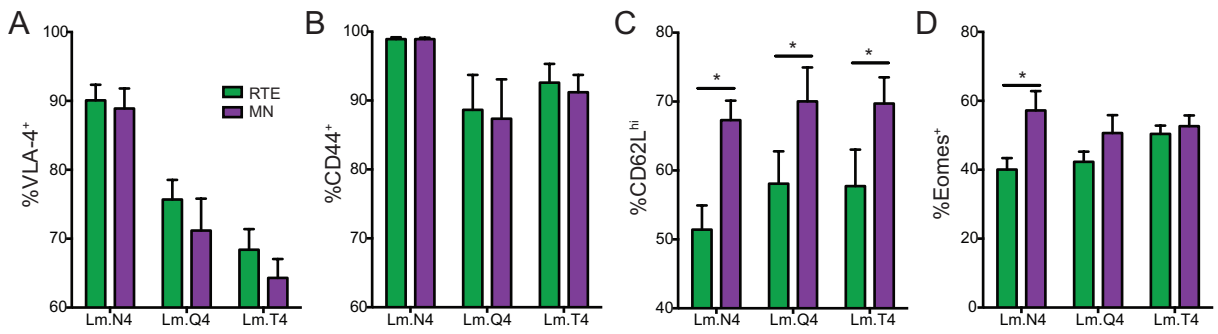

Figure 4-3. Differences in adhesion molecule expression were less dramatic between RTE- and MN-derived memory cells, but RTEs show a skewed memory phenotype. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Splenocytes were analyzed on d60 post infection. Proportion of VLA-4+ (A), CD44+ (B), CD62L^{hi} (C), or Eomes-expressing (D) RTEs and mature T cells. Data are presented as mean ± SEM compiled from 3 or 4 independent experiments, (A-C) n=12 (N4), 9 (Q4), and 12 (T4) or (D) n=6/group. *$p<0.05$, by paired Student's *t* test.

these differences were lost in response to low affinity infections. These data suggest that fewer RTEs than mature T cells have converted to or become T_{CM} $CD44^{hi}CD62L^{hi}$ cells. While differences in adhesion marker expression do not hold at memory time points, RTE- and MN-derived memory T cells differ in their phenotype.

RTE-derived memory cells following Yptb.N4 infection mirror the Lm.APL response

While RTE responses seen in the acute response to Yptb.N4 infection did not completely recapitulate the response of RTEs to Lm.N4, the memory responses are more similar. Similar proportions and numbers of RTE- and MN-derived cells were present 60 days post Yptb.N4 infection (Figure 4-4A). Although there was a similar number of memory cells in response to Yptb.N4, there was a reduced proportion of IL-2 and IFNγ producing cells (Figure 4-4B). VLA-4 expression was not increased on RTE- compared to MN-derived cells at a memory time point in response to Yptb.N4 (Figure 4-4C). This result was expected given that RTEs and MN T cells at an acute time point did not show differential expression of this integrin (Figure 3-9B). Interestingly, CD62L expression was decreased on RTE- compared to MN-derived memory cells, but moreover, the overall the CD62L expression on cells responding to Yptb.N4 infection was lower than on cells responding to Lm.N4 (Figure 4-4C and 4-3). Therefore, cells responding to Yptb.N4 infection are slower at their conversion to T_{CM} compared to those generated in response to Lm.N4 infection.

Delayed conversion to T_{CM} phenotype by RTEs

To evaluate the conversion of effectors to T_{CM}, we tracked transferred RTE and MN T cells over time for their CD62L expression in the blood. Following infection with Lm.APL, cells

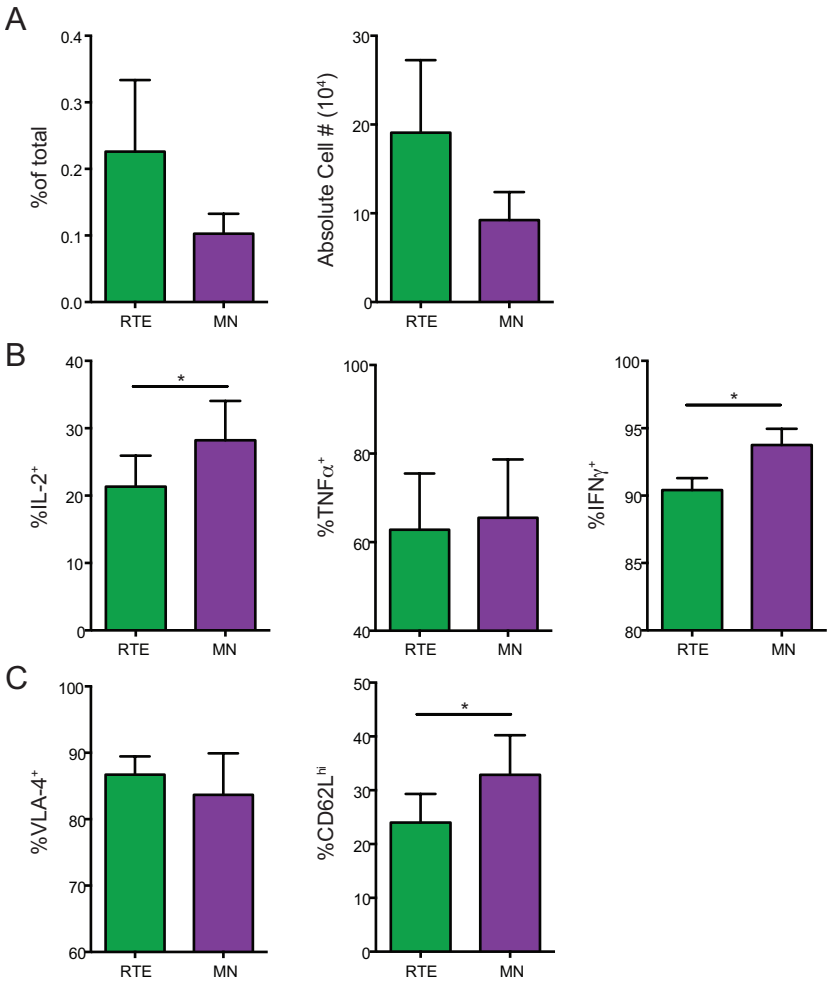

Figure 4-4. Following infection with *Yersinia pseudotuberculosis*, RTE-derived memory cells are deficient in cytokine production and CD62L expression but do not differ in cell accumulation from their mature counterparts. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected i.p. the following day with *Y. pseudotuberculosis* expressing OVA. Splenocytes were analyzed on >d60 post infection. A) Proportion (left) and absolute number (right) of RTE- and mature-derived memory T cells. B) RTE- or MN-derived cells producing IL-2 (left), TNFα (middle), and IFNγ (right). C) Proportional VLA-4 (left) or CD62L^{hi} (right) expression. Data are presented as mean ± SEM compiled from 2 independent experiments, n=6. *p<0.05, ***p<0.001 by paired Student's *t* test.

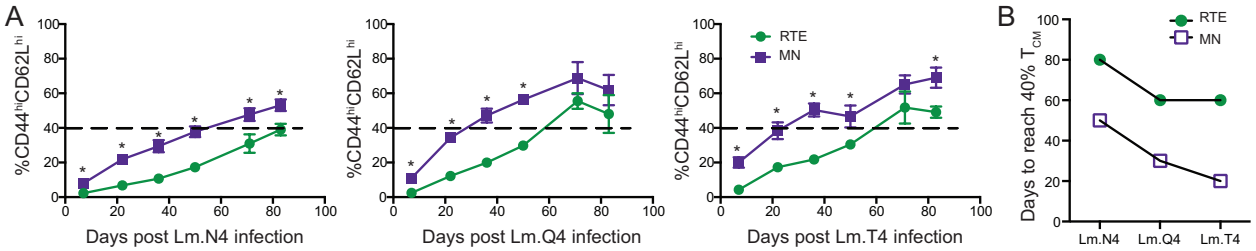

Figure 4-5. RTE-derived memory cells are delayed in their conversion to T_{CM} compared to MN-derived cells in response to bacterial infection. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. A) Blood was analyzed at the indicated time points for the percent of RTE- or MN-derived memory T cells that are $CD44^{hi}CD62L^{hi}$ T_{CM} cells. The dashed line indicates 40% $CD44^{hi}CD62L^{hi}$. B) Graphical summary of the number of days post infection for the transferred population to reach 40% T_{CM}. Data are presented as mean ± SEM and are representative of 3 independent experiments, n=4-10 (N4), 3-9 (Q4), and 4-10 (T4). *$p<0.05$, by paired Student's *t* test. Experiments are ongoing and some data were generated by K. Deets.

gradually converted to T_{CM} cells over time, but the rate was dependent on the affinity of the priming ligand, with responders to low affinity ligands converting to T_{CM} faster (Figure 4-5A). Independent of ligand affinity, RTE-derived cells had a lower proportion of $CD44^{hi}CD62L^{hi}$ T_{CM} compared to cells that originally encountered antigen as mature T cells (Figure 4-5A). By looking at the approximate number of days for the cells to reach 40% $CD44^{hi}CD62L^{hi}$ (Figure 4-5B), MN T cells responding to the lowest affinity antigen were the quickest to convert to T_{CM}, reaching this threshold at about 20 days. It took responding RTEs 60 days to reach a similar proportion of T_{CM} cells. Following Lm.N4 infection, RTE-derived cells took much longer (80 days) to reach the 40% T_{CM} threshold. RTEs responding to low affinity more resembled the high affinity response of MN T cells (Figure 4-5B). Therefore, RTE-derived cells are again more sensitive to low affinity ligands and at a memory time point, this is reflected in their delayed conversion to T_{CM} cells.

Reduced relative numbers of RTE-derived memory cells in the bone marrow

Because RTEs have a reduced capacity to become T_{CM}, the bone marrow was evaluated for the proportion of memory T cells. The bone marrow is an important niche for T_{CM} and a preferential site for homeostatic proliferation of memory cells (62, 63). At day 60, while RTEs are present at greater proportions in the spleen, the proportion in the bone marrow is comparable to that of the MN-derived cells (Figure 4-6). The ratio of RTE:MN-derived cells in the bone marrow was lower than the ratio in the spleen (Figure 4-6), indicative of a reduced accumulation specifically in the bone marrow. This differential localization likely reflects the lower proportion of T_{CM} in RTE-derived memory T cells compared to their mature counterparts. These data

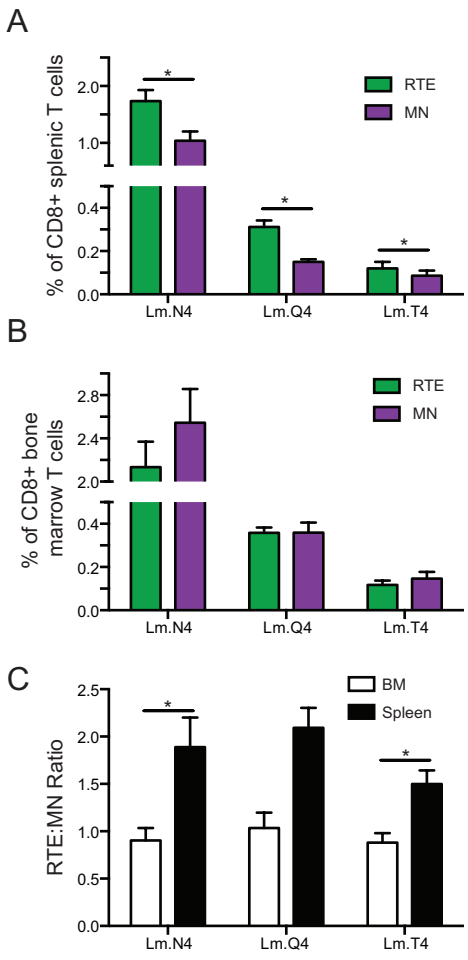

Figure 4-6. RTE-derived memory cells localize less efficiently to the bone marrow than do MN-derived cells following bacterial infection. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Spleen (A) and bone marrow (B) were analyzed at >d60. Ratios compare percent RTE of CD8 T cells:percent MN T cells of CD8 T cells (C). Data are presented as mean ± SEM and are compiled from 1 or 2 independent experiments, n=6 (N4), 3 (Q4), and 6 (T4). *$p<0.05$, by paired Student's *t* test.

demonstrate the surprising long-term impact on memory T cells of the maturation stage at which T cell first meets antigen.

RTE-derived memory cells in the blood contract more than their mature counterparts

As RTE-derived cells had a reduced number of T_{CM} cells and, more specifically, a reduced number in the important memory T cell niche of the bone marrow, we wanted to evaluate the impact on the overall number of memory CD8 T cells in the blood over time. RTE-derived cells were present at greater proportions in the blood compared to MN-derived T cells on days 21-50 after infection with all Lm.APL (Figure 4-7). At later time points (days 71 and beyond), MN- and RTE-derived cells were present in the blood in similar proportions (Figure 4-7). Slopes indicated on Figure 4-7 represent the rate of contraction between days 21 and 35, with the rate of RTEs being larger following all infections expressing ligands of varying affinity. Together, these data suggest that RTE-derived memory cells are not as long lived as MN-derived memory cells, perhaps due to their decreased T_{CM} conversion and failure to localize to the bone marrow.

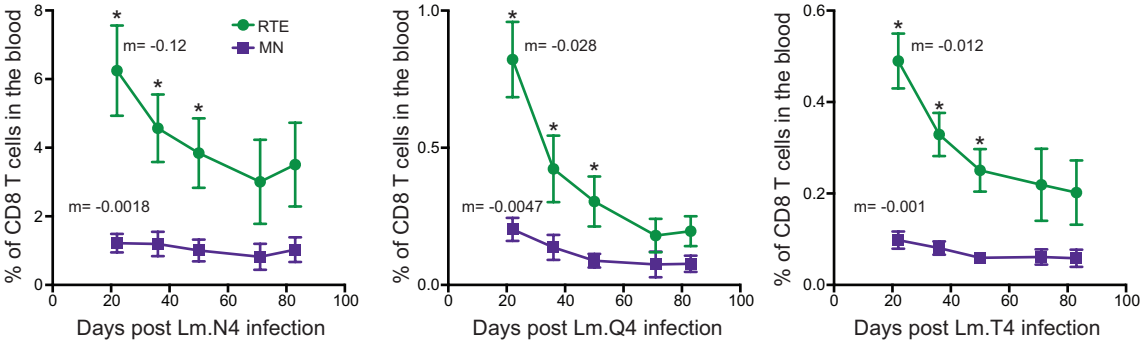

Figure 4-7. RTE-derived memory cells in the blood contract to a greater extent than their mature counterparts in response to bacterial infection. Sort-purified OT-I TCR Tg RTEs and MN T cells were co-transferred into congenically-marked hosts which were infected the following day with the indicated Lm.APL. Blood was analyzed at the indicated time points for the percent transferred cells of total CD8 T cells. Slopes (m) are shown for linear regression analysis. Data are presented as mean ± SEM and are representative of 3 independent experiments, n=4-10 (N4), 3-9 (Q4), and 4-10 (T4). *$p<0.05$, by paired Student's *t* test. Experiments are ongoing and some data were generated by K. Deets.

Conclusions

The experiments from this chapter provide evidence supporting the decreased ability of RTEs to contribute to the long-term memory pool. The formation of functional memory responses are the ultimate goal of a T cell response to better contain future pathogen challenges. While early phenotypic predictors of memory cells, MPECs, can imply memory potential, data from our group and others suggest that only a few MPECs are required to generate a functional memory population (17, 33). Memory responses however, are more complex than just elicitation of a recall response; location, function, and durability are other critical components of immunological memory.

RTE-derived memory cells are present at greater numbers compared to mature cells at 60 days post Lm.APL infection, but this response begins to wane at later memory time points. The data presented here suggest the time dependent loss of RTEs could be due to their decreased IL-2 production, lower proportion of T_{CM}, decreased Eomes expression, and impaired localization to the bone marrow. Any one or a combination of these factors could contribute to poor memory maintenance by cells that first meet antigens as RTEs, even though these cells enter the mature T cell compartment 3 weeks or so after exit from the thymus.

Decreased cytokine production by RTEs is maintained well after these cells have left the RTE compartment, suggesting that seeing antigen in this transitional state reduces their ability to produce cytokines for their lifetime and impacts their progeny as well. Here we show that RTEs responding to both Lm.APL and Yptb.N4 infection have a decreased proportion of cells producing IL-2 and to some extent IFNγ. Autocrine IL-2 is required for secondary CD8 memory responses (68) so the decreased ability of RTEs to produce this survival cytokine could impact their recall memory response. Previous evaluation of the ability of RTEs to respond to re-

challenge showed no defect in the secondary effector response; however, these experiments were done in the presence of MN-derived cells that could provide IL-2 to co-localized neighbors. That mature T cells can provide cytokine help to RTEs is not an unfounded idea; co-culture experiments indicate that CD4 RTE responses are impacted by surrounding MN T cells (18). Further investigation of the ability of RTE-derived memory cells to mount a secondary response is certainly warranted.

The inability of cells primed as RTEs to localize to the bone marrow at a memory time point is compelling and yet somewhat puzzling in light of their increased VLA-4 expression at acute time points. The bone marrow is an important niche for homeostatic proliferation of memory CD8 T cells, providing survival signals such as IL-7 and IL-15 (62, 63). From the data presented here, it is unclear whether RTEs do home to the bone marrow and die there because they fail to receive these survival signals or whether they fail to efficiently home to the bone marrow in the first place. VLA-4 has been shown to be required for firm arrest of T_{CM} in bone marrow microvessels (60), and while RTEs in the acute response expressed increased levels of VLA-4, they do not differ from mature T cell counterparts at a memory time point. However, we do see decreased VLA-4 expression in both memory RTE- and MN-derived T cells responding to low affinity ligands, and yet do not see impaired localization within the bone marrow. This suggests that some other homing molecule contributes to this localization. CXCR4 expression on memory cells could be one mechanism of bone marrow entry (60, 69), however CXCR4 expression on memory RTE-derived cells is currently unknown. Blocking with anti-CXCL12, the ligand for CXCR4, failed to prevent all memory cell homing to the bone marrow (60), so other currently unknown mechanisms should be investigated as well. Further support for the possibility of reduced CXCR4 expression by RTEs is suggested by the fact that Eomes null mice

showed reduced T_{CM} formation, diminished access to the bone marrow, and down regulated CXCR4 expression (70).

It is possible that memory RTEs are able to access the bone marrow but are unable to receive the IL-7- and IL-15-mediated survival signals provided there. We know that RTEs express reduced levels of IL-7Rα at acute time points (17), but the expression of this cytokine receptor has not been extensively characterized at memory time points. Expression of IL-15R (CD122) is not different on naïve RTE and MN T cells (14) but expression on memory cells is unknown.

In sum, memory cells derived from RTEs display reduced cytokine production, decreased T_{CM} formation, impaired localization to the bone marrow, and increased contraction. Together, these data indicate that RTEs are skewed in their memory response and that this skewing might impact their long-term memory potential.

Chapter Five: Concluding Remarks

The data presented in this thesis define important differences in CD8 RTEs and mature T cells, highlighting the downstream consequences when T cells are activated prior to completing post thymic maturation processes. Some features of the RTE response can be beneficial, such as efficient effector cell generation and subsequent protective effect at peripheral sites. But these increased responses can come at a price if RTEs recognize self-antigen and thus exhibit an increased propensity to autoimmunity. Similarly, the efficient effector generation seems to come at the expense of a full central memory T cell response, as RTEs fail to localize to the bone marrow or undergo efficient T_{CM} conversion compared to MN-derived memory cells. Overall, it is clear that RTEs must carefully maintain balance between mediating protection from pathogen and driving autoimmunity, and appear perfectly poised to do so.

Further research is required to determine whether these distinctions in the response of RTEs and mature T cells hold true across multiple models. One model of particular interest would be the ability of RTEs to invade tumors. Accurate evaluation the RTE response in a model simulating a lymphopenic chemotherapy patient or HIV-infected individual would be interesting as well, to see how RTEs respond to pathogen challenges in a lymphoid compartment that is not full.

Applying what we have learned to vaccine design could prove helpful to the field of vaccinology. It may be possible to manipulate the intrinsic differences between the mature T cell and RTE responses to antigen so as to achieve a desired outcome. What is required however, is a better understanding of what the desired vaccine response is as different infections require different correlates of immunity. Further knowledge of RTE biology will add to this understanding of protective immune responses and benefit future vaccine development.

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