CD8+ recent thymic emigrants exhibit increased responses to low affinity ligands and improved access to peripheral sites of inflammation

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Abstract
To explore the TCR sensitivity of recent thymic emigrants (RTEs), we triggered T cells with altered peptide ligands (APLs). 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 capacity of RTEs to elicit autoimmunity. 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 lymphodepleted environments.

INTRODUCTION
Development of functional and self-tolerant T cells requires selection in the thymus and further maturation in the periphery. Recent thymic emigrants (RTEs) comprise the subset of peripheral T cells that have most recently undergone thymic maturation and egress. RTEs, although an understudied population, contribute significantly to the T cell pool in neonates, during immune reconstitution following HIV infection or chemotherapy, and to T cell diversity throughout life.

A mouse model in which RTEs can be readily identified and isolated from their mature but still naïve (MN) T cell counterparts has expanded our understanding of RTE biology (reviewed in 1). In RAG2p-GFP transgenic (Tg) mice, GFP is expressed under the 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 (2, 3). We and others have used RAG2p-GFP Tg mice to show that RTEs are phenotypically and functionally distinct from mature T cells (2–10), dissimilarities that are mirrored by human RTEs (11, 12). The full extent of these differences remains an open question.

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An effective CD8 T cell response to infection requires robust expansion of effector T cells, access to peripheral tissues, activation of appropriate effector functions such as the production of IFN\(\gamma\) and TNF\(\alpha\), and generation of durable memory to protect the host from reinfection (13, 14). The pattern of killer cell lectin-like receptor subfamily G member 1 (KLRG1) and IL7R\(\alpha\) expression is commonly used (14) to define CD8 T cells as short-lived effector cells (SLECs) or memory precursor effector cells (MPECs). SLECs are terminally differentiated T cells that rapidly contract after the peak of the immune response and contribute minimally to the memory T cell pool. When CD8 T cell responses go awry, autoimmunity rather than host protection can result. The ability of RTEs to maintain the proper balance between protection and self-destruction is largely unstudied.

Initiating T cell activation requires TCR recognition of peptide presented by MHC. Membrane proximal events, such as phosphorylation of ZAP70, propagate this signal via an intracellular cascade that results in distal activation of kinases, such as ERK, which help regulate gene expression to drive the appropriate effector T cell response (15, 16). Recent evidence suggests that the strength of the TCR signal can impact the relative SLEC/MPEC skewing of the CD8 T cell response (17). Altered peptide ligands (APLs), peptide variants that differ in their TCR binding affinity, can probe monoclonal T cell responses to high and low affinity antigens (15). In the OVA-specific OT-I TCR Tg system, APLs of OVA have been well studied in thymic selection (18) and in the mature T cell response to Lm.APL, Listeria monocytogenes that express OVA containing APLs (17, 19).

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. Here we show that RTEs have increased TCR signal transduction and enhanced skewing toward the SLEC compartment during the acute effector response to a pathogen. Independent of T cell fate, RTEs are less able to produce cytokines compared to mature cells, but this dampened function is balanced by the enhanced sensitivity of RTEs to low affinity ligands, reflected in upregulation CD44 and VLA-4 expression, facilitating RTE migration into inflamed tissues and pathogen clearance. These distinct traits suggest RTEs are able to clear a broad spectrum of pathogens without causing undue cytokine-mediated destruction, although these advantages can be undermined by an enhanced tendency to autoimmune destruction through efficient tissue localization.

**MATERIALS AND METHODS**

**Mice**

RAG2p-GFP Tg OT-I TCR Tg mice were backcrossed in our laboratory 12 generations onto CD45.1+ or CD45.2+ C57BL/6J (B6) backgrounds. These mice, as well as B6×B6.SJLPterc\(^{aPepc}\)/BoyJ, and B6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA) mice that express membrane-bound OVA in pancreatic \(\beta\) cells were housed under specific pathogen free conditions and used in accordance with the University of Washington Institutional Animal Care and Use Committee guidelines.
Reagents

Cells were stained with fluorochrome-conjugated antibodies against: CD45.1 (A20), CD45.2 (104), NK1.1 (PK136), CD4 (RM4-5), Ter119 (Ter119), CD11b (M1/70), B220 (RA3-6B2), CD44 (IM7), CD62L (MEL-14), CD8α (53-6.7), CD127/IL-7Rα (A7R34), KLRG1 (2F1), CD49d/α4 integrin (R1-2), CD29/β1 integrin (HMβ1-1), IL-2 (JES6-5H4), TNFα (MP6-XT22), IFNγ (XMG1.2), pZAP70 (17A/P-ZAP70), and pERK (E10), all from eBioscience, BD Biosciences, BioLegend, or Cell Signaling. The OVA APLs 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 for in vitro stimulations. *Listeria monocytogenes* expressing OVA containing APLs (Lm.APL) were obtained from Dietmar Zehn (19). Vesicular stomatitis virus Indiana strain (VSV) and VSV engineered to express OVA (VSV.OVA, reference 20) were both obtained from M. Bevan (University of Washington).

Cell preparation, flow cytometry, and sorting

Water lysed single cell suspensions from spleens and lymph nodes were negatively enriched using CD8 T cell isolation kits (StemCell Technologies), and Fc receptors blocked (anti-CD16/32; 2.4G2) before sorting to >98% purity as NK1.1−CD4−Ter119−CD11b−B220− (dump gate) CD44loCD62Lhi cells that were either GFP+ (RTEs) or GFP− (MN T cells). On d7 post infection, splenocytes were stained for surface markers or intracellular cytokines as previously described (4). All samples were acquired on an LSRII or FACSCanto (BD Biosciences) and analyzed using FlowJo software (TreeStar).

In vitro peptide stimulation and staining for phosphorylated signaling molecules

Splenocytes from RAG2p-GFP Tg OT-I TCR Tg mice were resuspended (3×10⁶/50µL) in 37°C serum-free HBSS and prewarmed peptide was added at the indicated times to a final concentration of 0.1µM. Cells were fixed in Fix Buffer I (BD Bioscience), permeablized with Perm Buffer III (BD Bioscience), and stained for both surface and phosphorylated signaling molecules for 30 minutes at room temperature.

Adoptive transfers, infections, tissue inflammation, and diabetes induction

A total of 10⁴ sorted RTEs and MN T cells were transferred i.v. either separately or as a 1:1 mix into congenic hosts. Lm.APL strains were grown until mid log phase and 2000 CFU injected i.v. into mice one day after cell transfer. For inducing ear inflammation or VSV.OVA challenge, mice were sedated with ketamine/xylazine 5.5 days post infection and one ear injected intradermally with 10µL 1:1 CFA/PBS (Sigma-Aldrich) or 10⁴ PFU VSV.OVA. Equivalent application of PBS or VSV served as respective controls. Where indicated, 100–150µg anti-VLA-4 blocking antibody (PS/2, UCSF Monoclonal Antibody Core) was administered i.p. 12 hours before and 12 hours after CFA treatment. To isolate skin infiltrating T cells, ears were harvested, minced, and digested 3 times using 0.14U/mL Liberase (Roche) in medium for 40–45 minutes at 37°C. For diabetes induction, 10⁴ sorted RTEs or MN T cells were transferred i.v. 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.
RESULTS AND DISCUSSION

RTEs exhibit increased TCR signal transduction

RTEs and MN T cells from RAG2p-GFP Tg OT-I TCR Tg mice were stimulated *in vitro* with 0.1µM N4, Q4, or T4 peptide and phosphorylation of downstream mediators of TCR signaling measured using flow cytometry (Figure 1A and B). ZAP70 phosphorylation was detected in RTEs stimulated for as little as 5 minutes with N4, the high affinity ligand (Figure 1B, left). ZAP70 phosphorylation peaked in N4-stimulated RTEs at about 75%, while mature T cells lagged behind at 26%. Q4 stimulation induced about 40% of RTEs and 10% of mature T cells to phosphorylate ZAP70. T4, the lowest affinity ligand tested, generated very little pZAP70, but RTEs displayed increased levels over the barely detectable mature T cell response (Figure 1B). The response kinetics to low and high affinity peptides were similar in both populations.

ERK phosphorylation was examined to determine whether the enhanced proximal signals in RTEs are propagated downstream. ERK activation after N4 stimulation was detected by 5–10 minutes (Figure 1B, right). This response peaked in RTEs at 90 minutes with 93% of cells phosphorylating ERK compared to 80% of mature T cells, peaking at 120 minutes. Stimulation with Q4 and T4 resulted in a detectable pERK signal at 30 minutes that was enhanced in RTEs relative to mature T cells at all timepoints thereafter. Thus, RTEs are better able than mature T cells to transduce both proximal and distal TCR signaling events upon stimulation with low and high affinity ligands. The surprising increase in TCR signal transduction by RTEs suggests they may be able to respond to a broader range of peptides as well as to receive increased homeostatic survival signals (21). While the elongated CDR3 regions of TCRs expressed by RTEs (22) and their elevated TCR/CD3 expression (2) may help explain this enhanced signal transduction (15), further characterization of molecules that modulate TCR signaling is called for.

RTEs expand more and are skewed towards a SLEC phenotype

We turned to an *in vivo* model of Lm.APL infection to explore the impact of this increased TCR signal transduction in RTEs. In response to Lm.APL infection, RTEs expanded to a greater extent than mature T cells in the same inflammatory environment, as measured both by frequency (Figure 2A, left) and absolute number (Figure 2A, right) of transferred cells in the spleen. As expected, mature T cell expansion in response to low affinity ligands was diminished (19), although even in this case, RTEs predominated (Figure 2A). This increased expansion was correlated with the skewing of RTEs toward a SLEC phenotype, as determined by KLRG1 and IL-7Rα expression (Figures 2B and C). Lower affinity ligands drive the generation of a diminished proportion of SLECs and an elevated proportion of MPECs among mature CD8 T cell responders (17); RTEs follow this trend, generating a lower proportion of SLECs in response to infection with Lm.T4 compared to Lm.N4. However, RTEs still skew to a SLEC phenotype relative to mature T cells responding to low
affinity stimulation (Figure 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. This SLEC-centric response would be advantageous in a neonatal setting, in which mounting a strong effector response to ligands of a broad range of affinities could help ensure survival of the infected lymphopenic individual.

**A lower proportion of CD8 RTEs makes effector cytokines**

A significantly lower proportion of RTEs than mature T cells produced the effector cytokines IL-2, TNFα, and IFNγ upon Lm.N4 infection (Figures 3A and B). RTEs also exhibited reduced production of IL-2 and TNFα following Lm.T4 infection, although no notable differences in IFNγ production between RTE- and MN-derived T cells were apparent. A higher proportion of MPECs were cytokine producers, most noticeably so for IL-2 (Figures 3C and D). A lower proportion of RTEs produced cytokines within both the SLEC and MPEC compartments, indicating that while some differences in cytokine production by RTEs can be attributed to their increased SLEC skewing, cell fate phenotype cannot fully account for these cytokine defects, which are therefore intrinsic to RTEs. The lower cytokine production by RTEs may prove beneficial in a neonatal host in which RTEs predominate and unrestrained release of inflammatory cytokines in response to commensal antigens would be problematic (23), although these cells did not produce more of the anti-inflammatory cytokine IL-10 than their mature counterparts (data not shown). It remains to be determined whether the dampened cytokine production by CD8 RTEs, also seen directly ex vivo (9), is a result of increased methylation at cytokine promoter regions, as has been shown for CD4 RTEs (7).

**CD8 RTEs upregulate VLA-4 expression and are better able to enter inflamed tissue**

RTE- and MN-derived T cells encountering Lm.N4 highly upregulate VLA-4 expression (measured by the expression of CD49d/α4 and CD29/β1 integrin subunits), with RTEs exhibiting slightly increased expression relative to their mature counterparts (Figure 4A). This difference is magnified in response to the low affinity ligand, during which only 75% of mature cells upregulate VLA-4 expression, compared to 90% of RTEs (Figure 4A). The heightened sensitivity of RTEs to low affinity ligands is also reflected in their enhanced upregulation of CD44 (Figure 4A).

Knowing that the integrin VLA-4 is important in promoting access to peripheral tissues (24), we induced ear inflammation by CFA administration to evaluate the impact of this disparate VLA-4 expression on effector T cells generated upon Lm.T4 infection. Transferred effector cells were quantified in separately digested inflamed and control ears. The number of transferred cells in the CFA treated ear was increased by >30-fold over that in the control ear (Figure 4B). T cell homing to the inflamed tissue was largely VLA-4 dependent, being significantly reduced by anti-VLA-4 treatment (Figure 4B). Most interestingly, RTEs were better able to enter the inflamed ear compared to mature T cells (Figure 4C). To determine whether this increased invasiveness is protective, RTEs and MN T cells were separately transferred into recipient mice, which were then infected with Lm.APL, challenged 5.5 days later in the ear with VSV or VSV.OVA, and assessed for viral PFU in the ear the next day.
VSV challenged recipients of RTEs and mature T cells had comparable virus levels (not shown). In contrast, mice receiving RTEs had significantly reduced viral titers in the ear following VSV.OVA challenge (Figure 4D). Therefore, despite the impaired capacity of RTEs to produce effector cytokines, their increased invasiveness allows them to better access sites of inflammation and control viral challenge in an antigen dependent manner. Thus, efficient tissue localization compensates for the dampened cytokine production by RTEs. Whether the enhanced tissue invasiveness that RTEs demonstrate impacts their relative contribution to the pool of skin resident memory T cells remains to be determined.

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

To determine whether the enhanced invasiveness of RTEs could contribute to increased autoimmunity, we measured diabetes induction in mice expressing a model pancreatic antigen, in which the diabetogenic capacity of mature CD8 T cells correlates with VLA-4 expression (17). 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. All Lm.N4 infected recipients also receiving RTEs rapidly developed diabetes, while fewer of the mice receiving MN T cells became diabetic, and did so with slower kinetics (Figure 5A). Infection with the low affinity Lm.T4 strain drove less diabetes overall, but RTEs still appeared more diabetogenic (Figure 5B), 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. It is clearly crucial that post-thymic maturation be coupled with the induction of tolerance to (often low affinity) self-antigens whose expression is restricted to the lymphoid periphery. This facet of RTE biology is a focus of current research.

We show here for the first time that RTEs demonstrate increased TCR signal transduction, notably even in response to low affinity ligands, and that this response can result in host protection through better access to peripheral tissues via enhanced VLA-4 expression. This positive outcome is balanced by the risk of increased autoimmunity when the response is directed to self-antigen. These newly described properties that distinguish RTE responses from those of mature cells have important implications for neonatal vaccine design and anti-cancer therapy, as RTEs comprise the majority of the responding T cells in these lymphopenic environments.

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Abbreviations

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<tr>
<td>APLs</td>
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**KLRG1** killer cell lectin-like receptor subfamily G member 1  
**Lm.APL** *Listeria monocytogenes* expressing APLs of OVA  
**Lm.N4** Lm.T4, *Listeria monocytogenes* expressing OVA containing N4 or T4  
**MN** mature naïve  
**MPECs** memory precursor effector cells  
**N4** SIINFEKL peptide  
**Q4** SIIQFEKL peptide  
**RIPmOVA** transgenic mice expressing membrane bound OVA under the control of the rat insulin promoter  
**RTEs** recent thymic emigrants  
**SLEC** short-lived effector cells  
**Tg** transgenic  
**T4** SIITFEKL peptide  
**VSV** vesicular stomatitis virus

**References**


Figure 1. RTEs have heightened TCR signal transduction following in vitro stimulation with high and low affinity peptide antigens

RAG2p-GFP Tg OT-I TCR Tg splenocytes were stimulated with 0.1µM peptide. A) Representative histograms of pZAP70 (top) and pERK (bottom) staining following activation of RTEs (solid black line) and MN T cells (dashed line) for 1 hour with N4 (left), Q4 (middle), or T4 (right). The “no peptide” control for MN T cells is shown in gray. B) A full time course for RTE (black circle) and MN T cell (gray box) activation with the indicated peptide as measured by pZAP70 (left) and pERK (right) expression. Data are representative of 2 independent experiments.
Figure 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) Percentage (left) and absolute number (right) of RTEs and mature T cells. B) Representative flow cytometric plots of the relative SLEC (KLRG1\textsuperscript{hi}IL7Rα\textsuperscript{lo}) and MPEC (KLRG1\textsuperscript{lo}IL7Rα\textsuperscript{hi}) phenotype. Numbers represent the % 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) or 22 (T4). *p<0.05, ***p<0.001 by paired Student’s t test.
Figure 3. Regardless of cell fate, a lower proportion of RTEs produces cytokines 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. Splenocytes were analyzed on d7 post infection after a 5 hour restimulation in vitro with SIINFEKLE peptide. A) Representative flow cytometric plots of cytokine staining; gates were set using cells from an infected recipient that were not stimulated in vitro. Numbers represent the % of transferred cells within that quadrant. B) RTE- or MN-derived cells

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producing IL-2 (left), TNFα (middle), and IFNγ (right). C) and D) IL-2, TNFα, and IFNγ 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) or 22 (T4) and C) and D) 2 independent experiments, n=6 (N4 and T4). *p<0.05, **p<0.01, ***p<0.001 by paired Student’s t test.
Figure 4. 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–C) or separately (D) into congenically-marked hosts which were infected the following day with the indicated Lm.APL. A) VLA-4 (α4β1 integrin, left) and CD44 (right) expression on splenocytes 7 days post infection. Data are presented as mean ± SEM compiled from 8 independent experiments, n=19–22. B) Number of cells in the ear on d7 post Lm.T4 infection following CFA treatment on d5.5 and/or anti-VLA-4 (PS/2) administration. Data are presented as mean ± SEM and are representative of 2 independent experiments. C) 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. D) 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 or 3 independent experiments *p<0.05, **p<0.01, ***p<0.001 by paired or unpaired (D) Student’s t test.
Figure 5. RTEs drive increased diabetes in RIPmOVA recipients following Lm.APL infection

$10^4$ 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 $>350$mg/dL. Numbers in parentheses indicate diabetic/total mice. Data are combined from 3 independent experiments. *$p<0.05$ by log rank test.