Cutting Edge: Contact with Secondary Lymphoid Organs Drives Postthymic T Cell Maturation

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T cell development, originally thought to be completed in the thymus, has recently been shown to continue for several weeks in the lymphoid periphery. The forces that drive this peripheral maturation are unclear. The use of mice transgenic for GFP driven by the RAG2 promoter has enabled the ready identification and analysis of recent thymic emigrants. Here, we show that recent thymic emigrant maturation is a progressive process and is promoted by T cell exit from the thymus. Further, we show that this maturation occurs within secondary lymphoid organs and does not require extensive lymphocyte recirculation. The Journal of Immunology, 2008, 181: 5213–5217.

Cell function has been thought to occur in its entirety in the thymus, giving rise to mature naive T cells that circulate in the lymphoid periphery, patrolling for foreign antigenic insults. However, recent studies have suggested that to attain full immunocompetence, further development must occur in T cells that have recently entered the peripheral environment. Such recent thymic emigrants (RTEs) are a subset of naive T cells that help maintain the diversity of the peripheral T cell repertoire and are of particular importance for recovery from lymphopenia, when thymic output is needed to replenish the peripheral repertoire and, in infants, when RTEs first seed the periphery.

A challenge to studying RTEs is their lack of a unique cell surface marker. Recent work from our laboratory (1, 2) characterized a new model system that allows unambiguous identification of RTEs from unmanipulated mice and enables the ready isolation and analysis of their function and phenotype. This system uses mice transgenic (Tg) for GFP under control of the RAG-2 promoter (3). Thymocytes from such RAG2p-GFP Tg mice express high levels of GFP, mirroring endogenous RAG expression. Although RAG expression in RAG2p-GFP Tg mice is extinguished by the single positive (SP) stage, a residual, decaying GFP signal remains in cells following thymic egress. Thymectomy studies have indicated that the GFP signal declines with time in the periphery, such that GFP<sup>high</sup> and GFP<sup>low</sup> RTEs have been in the periphery for up to 1 and 2–3 wk, respectively (1). GFP<sup>−</sup> naive T cells (non-RTEs) have exited the thymus at least 3 wk previously.

Using this system, we demonstrated that RTEs exhibit a CD2<sup>high</sup>Q<sub>8</sub><sup>low</sup>CD45<sup>R</sup><sub>B</sub><sup>low</sup>IL-7R<sub>A</sub><sup>low</sup>TCR<sup>high</sup>CD3<sup>high</sup>CD28<sup>low</sup> phenotype relative to non-RTEs (1). We also showed that RTEs differ functionally from non-RTEs, exhibiting a dampened response to stimulation, with decreased IL-2 and IFN-γ production, proliferation, and high-affinity IL-2R up-regulation (1). These striking differences between RTEs and non-RTEs hint that definable forces may drive cells from RTE status into the mature naive T cell compartment. We now show that RTE maturation occurs progressively, requires egress from the thymus, and is driven to completion not in the blood, but within secondary lymphoid organs (SLOs).

**Materials and Methods**

**Mice**

C57BL/6 mice were from The Jackson Laboratory and RAG2p-GFP Tg mice (3), originally a gift from M. Nussenzweig (The Rockefeller University, New York, NY), were backcrossed in our laboratory at least 10 generations onto the C57BL/6 background. Mice were used between 6 and 12 wk of age. All experiments were performed in compliance with the University of Washington’s Institutional Animal Care and Use Committee.

**Mouse procedures**

Mice were splenectomized (4) and thymectomized (1) as described previously. For blockade of thymic and lymph node (LN) egress, mice were i.p. injected daily for 3 or 6 days with 1 μg/g body weight 2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL-R), a sphingosine 1-phosphate (SIP) mimetic, or AAL-S (gifts from Novartis), a biologically inactive enantiomer of AAL-R, made up at 1 mg/ml in water plus 0.25% DMSO. For blockade of LN entry, mice were given 200 μg of laboratory-purified anti-CD62L (clone MEL-14) plus 100 μg of anti-very late Ag 4 (VLA-4) plus 200 μg of IgG2a isotype control Ab (eBioscience) i.p. on days 0, 2, and 4.
Cell preparation, staining, enrichment, and sorting

Single cell suspensions of thymus, LNs (brachial, axillary, inguinal, cervical, and mesenteric), and water-lysed blood and splenocytes were prepared and counted. Where noted, cells were labeled for 10 min at 37°C with 4 μM of CFSE. For flow cytometric analysis, Fc receptors were blocked with anti-CD16/32 (clone 3G8; BD Biosciences) and cells were stained as described (1) with Abs against the following molecules: CD3 (clone 145.2C11), CD4 (clone RM4-5), CD11c (clone N418), CD24 (clone M1/69), CD25 (clone PC61), CD44 (clone 17G1), CD45.1 (clone A20), CD45.2 (clone 104), CD45RB (clone 16A3), CD62L (clone ME-L14), CD69 (clone H1.2F3), Qa2 (clone 1-1-2), and CD90.2 (clone 53-2-1), all from eBioscience or BD Pharmingen. Biotinylated Abs were detected with allophycocyanin-conjugated streptavidin (eBioscience). Events were collected on a FACSAnA flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (Tree Star) after excluding doublets from live-gated samples. Fluorescence-minus-one (5) samples were run where appropriate. For sorting, untouched T cells were enriched with an EasySep kit (StemCell Technologies) and stained to eliminate non-T cell lineages with PE-conjugated anti-CD11b (clone M1/70), anti-NK1.1 (clone PK136), anti-B220 (clone RA3-6B2), and anti-Ter119 (clone Ly-76) (all from eBioscience or BD Biosciences). Staining with anti-CD62L was used as a positive marker for naïve cells. Cells were sorted on a FACSAnA cell sorter (BD Biosciences) as PE− CD62L− and either GFP+ or GFP− to >97% purity (for blood sorts, purity was >95% for RTE and >80% for non-RTE).

Quantification of IL-2 secretion and cell proliferation

Per well, 25,000 sorted CD4 T cells were stimulated with 30 ng/ml anti-CD3 and 1 μg/ml anti-CD28 (BD Biosciences) in the presence of 175,000 irradiated splenocytes depleted of T cells by treatment with anti-CD4 (RC/24G2), anti-CD8 (3.168.8), and anti-CD90.2 (3.4.6) plus rabbit complement (Cedarlane Laboratories). Cells were cultured in 96-well plates (BD Biosciences) in complete RPMI 1640 at 37°C in 7% CO2. IL-2 secretion was measured in 24-h supernatants with the OptEIA IL-2 ELISA kit (BD Biosciences). Where noted, cells were labeled for 10 min at 37°C with 4 μM of CFSE. For flow cytometric analysis, Fc receptors were blocked with anti-CD16/32 (clone 3G8; BD Biosciences) and cells were stained as described (1) with Abs against the following molecules: CD3 (clone 145.2C11), CD4 (clone RM4-5), CD11c (clone N418), CD24 (clone M1/69), CD25 (clone PC61), CD44 (clone 17G1), CD45.1 (clone A20), CD45.2 (clone 104), CD45RB (clone 16A3), CD62L (clone ME-L14), CD69 (clone H1.2F3), Qa2 (clone 1-1-2), and CD90.2 (clone 53-2-1), all from eBioscience or BD Pharmingen. Biotinylated Abs were detected with allophycocyanin-conjugated streptavidin (eBioscience). Events were collected on a FACSAnA flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (Tree Star) after excluding doublets from live-gated samples. Fluorescence-minus-one (5) samples were run where appropriate. For sorting, untouched T cells were enriched with an EasySep kit (StemCell Technologies) and stained to eliminate non-T cell lineages with PE-conjugated anti-CD11b (clone M1/70), anti-NK1.1 (clone PK136), anti-B220 (clone RA3-6B2), and anti-Ter119 (clone Ly-76) (all from eBioscience or BD Biosciences). Staining with anti-CD62L was used as a positive marker for naïve cells. Cells were sorted on a FACSAnA cell sorter (BD Biosciences) as PE− CD62L− and either GFP+ or GFP− to >97% purity (for blood sorts, purity was >95% for RTE and >80% for non-RTE).

Results and Discussion

Altersations in RTE phenotype result from progressive maturation

To determine whether the phenotypic changes that occur in RTEs are due to selective survival and outgrowth of a subpopulation of RTEs bearing a mature CD24lowQa2highCD45RBhigh phenotype or are due to selective survival and outgrowth of a subpopulation of RTEs bearing a mature CD24lowQa2highCD45RBhigh phenotype or are due to progressive maturation of the bulk of the RTE population, we adoptively transferred equal numbers of sorted CD4 RTEs and non-RTEs into lymphoreplete recipients on day 0 (2 × 106 per mouse, >97% purity). At the indicated times thereafter, recipient splenocytes were stained for donor cell analysis. A. Mean donor cell numbers are expressed as a percentage of the number of cells transferred on day (d) 0. Error bars represent SD. Differences were not statistically significant; p > 0.05, two-tailed Student’s t test with equal variance. B and C, Representative CD24, Qa2, and CD45RB expression in B and GFp and CD44 expression in C by donor cells from T-enriched splenocytes at the indicated times posttransfer. Transferred cells were CFSE labeled in B, and data were collected from CFSEhigh undivided cells. Data are from three recipients of each cell type per time point.

RTE maturation requires thymic egress

To explore whether the RTE maturation that occurs in the periphery is a cell-intrinsic program or one that is triggered by signals from the lymphoid periphery, we sequestered RTEs in the thymus by treating RAG2p-GFP Tg mice with AAL-R (8, 9). AAL-R is a biologically inactive enantiomer of AAL-R (8, 9). To compensate for the unequal cellular age of RTEs from the lymphoid periphery, Sorted populations of CD4 RTEs (GFP+ CD62Lhigh) and naive non-RTEs (GFP− CD62Lhigh) from RAG2p-GFP Tg mice were transferred to separate congenic lymphoreplete recipients on day 0 (2 × 106 per mouse, >97% purity). At the indicated times thereafter, recipient splenocytes were stained for donor cell analysis. A. Mean donor cell numbers are expressed as a percentage of the number of cells transferred on day (d) 0. Error bars represent SD. Differences were not statistically significant; p > 0.05, two-tailed Student’s t test with equal variance. B and C, Representative CD24, Qa2, and CD45RB expression in B and GFp and CD44 expression in C by donor cells from T-enriched splenocytes at the indicated times posttransfer. Transferred cells were CFSE labeled in B, and data were collected from CFSEhigh undivided cells. Data are from three recipients of each cell type per time point.

mucocyte compartments (Fig. 2A). RTE “wannabes” accumulated in the thymus of AAL-R-treated mice, as these most developmentally mature CD4 and CD8 SP compartments increased ~5-fold by percentage (Fig. 2B) and ~3-fold by number (data not shown) relative to the thymus of untreated mice or mice treated with AAL-S, the biologically inactive enantiomer of AAL-R (8, 9).

Non-GFP level in RTEs is a faithful indicator of their age, as the decrease in GFP brightness correlates well with residence time in the periphery (7). To compensate for the unequal cellular age of RTE “wannabes” and control RTEs, we normalized GFP mean fluorescence intensities (MFIs), excluding GFPlow older RTEs from the control group and GFPbright RTE precursors from the “wannabe” group (7). After GFP normalization, RTE “wannabes” were phenotypically immature relative to control RTEs by the indicated times posttransfer.

However, the phenotype of RTE “wannabes” is intermediate, as the decrease in GFP brightness correlates well with residence time in the periphery (7). To compensate for the unequal cellular age of RTE “wannabes” and control RTEs, we normalized GFP mean fluorescence intensities (MFIs), excluding GFPlow older RTEs from the control group and GFPbright RTE precursors from the “wannabe” group (7). After GFP normalization, RTE “wannabes” were phenotypically immature relative to control RTEs by the marker CD24 (Fig. 2, C and D). These results suggest that RTEs must exit the thymus to fully complete their phenotypic maturation within this time frame.

To explore whether the RTE maturation that occurs in the periphery is a cell-intrinsic program or one that is triggered by signals from the lymphoid periphery, we sequestered RTEs in the thymus by treating RAG2p-GFP Tg mice with AAL-R (8, 9). AAL-R is a synthetic mimetic of S1P, blocking S1P receptor 1 (S1P1), influencing S1P3, S1P4, and S1P5 receptors as well as to S1P1, influencing
T cells from AAL-S treated mice (control RTEs). GFP normalization was performed to treat mice (RTE “wannabes”) and GFP test with equal variance.

experiments, with error bars representing SD. In "homeless" RTEs and control RTEs for both CD4 and CD8 T cells, there was a statistically significant difference between

sion level on "homeless" RTEs was normalized to that of control (Fig. 3) and CD45RB (data not shown). When the Qa2 expression by GFP+ CD62LhighTCRhigh SP thymocytes (Mature SP thymocytes) and GFP− CD44low/mid splenic CD4 and CD8 T cells from AAL-S treated mice (control RTEs), GFP normalization was performed on RTE "wannabes" and control RTEs are gated on GFP+ cells to match the age of these two comparison groups (GFP MFI = −1300 for CD4 and GFP MFI = −625 for CD8 RTE "wannabes" and control RTEs). Thymocytes and splenocytes from an untreated age-matched mouse were analyzed on the same day for CD24 expression by GFP+ CD62LhighTCRhigh SP thymocytes (mature SP thymocytes) and GFP− CD44low/mid splenic CD4 and CD8 T cells (naive GFP− peripheral T). Representative data are shown in C, and data in D are averaged MFIs from four to five mice per group from two independent experiments, with error bars representing SD. *, p < 0.01, two-tailed Student’s t test with equal variance.

RTE maturation requires access to SLOs

SLOs are the sites where naive T cells encounter many other cell types, such as dendritic cells, and cytokines, such as IL-7 (14). To test whether RTE maturation takes place in SLOs, we blocked RTE access to SLOs through a combination of splenectomy and administration of anti-CD62L plus anti-VLA-4 to block LN entry, creating "homeless" RTEs. There was no statistically significant difference in the CD24 expression levels between "stuck" and control RTEs for either CD4 or CD8 (Fig. 3C). When RTEs were prevented from entering SLOs for 6 days, numbers of “homeless” blood naive T cells, with a specific increase in the RTE compartment (data not shown). Thus, 6-day deprivation of SLO access leaves the naive T cell compartment largely intact but does promote the survival of the younger RTE compartment relative to that of the older non-RTEs. We matched GFP levels to ensure that our phenotypic comparison of “homeless” and control RTEs was limited to cells that spent the same period of time in the periphery (Fig. 3B).

RTE maturation does not require continuous recirculation in the lymphoid periphery

Naive T cells scan for Ag presented by dendritic cells in SLOs, recirculating to another SLO if Ag is not found within 12–18 h (15). To test whether RTE maturation requires continuous recirculation in the lymphoid periphery, we compared the phenotype of RTEs that were “stuck” in SLOs for 6 days with that of control RTEs continually recirculating for 6 days (Fig. 4A). Because AAL-R treatment blocks thymic egress, we thymectomized control mice at the onset of the experiment to age-match the RTEs in each group and thereby matched GFP levels of “stuck” and control RTEs. There was no statistically significant difference in the CD24 or Qa2 MFIs between “stuck” and control RTEs for either CD4 or factors such as heart rate (12). Our results using the S1P1-specific agonist SEW2871 (13) were comparable to those obtained with AAL-R (data not shown), suggesting that the maturation defects are specific to blockade of thymic egress.

FIGURE 2. RTEs require contact with the lymphoid periphery to mature. A, Diagram of experimental conditions. RAG2p-GFP Tg mice were treated daily for 6 days with AAL-R or control AAL-S and analyzed on day 7. B, Flow cytometric analysis of thymocytes from untreated, AAL-S-treated, and AAL-R-treated mice gated on the indicated cell populations. C and D, CD24 expression by GFP+ CD62LhighTCRhigh CD4 and CD8 SP thymocytes from AAL-R treated mice (RTE “wannabes”) and GFP+ CD44low/mid splenic CD4 and CD8 T cells from AAL-S treated mice (control RTEs). GFP normalization was performed on RTE “wannabes” and control RTEs are gated on GFP+ cells to match the age of these two comparison groups (GFP MFI = −1300 for CD4 and GFP MFI = −625 for CD8 RTE "wannabes" and control RTEs). Thymocytes and splenocytes from an untreated age-matched mouse were analyzed on the same day for CD24 expression by GFP+ CD62LhighTCRhigh SP thymocytes (mature SP thymocytes) and GFP− CD44low/mid splenic CD4 and CD8 T cells (naive GFP− peripheral T). Representative data are shown in C, and data in D are averaged MFIs from four to five mice per group from two independent experiments, with error bars representing SD. *, p < 0.01, two-tailed Student’s t test with equal variance.

FIGURE 3. RTEs require SLO access to mature. A, Diagram of experimental conditions. RAG2p-GFP Tg mice were untreated or splenectomized and treated every other day with anti-CD62L plus anti-VLA-4 for 4 days and analyzed on day 6. Other controls included eusplenic mice treated with both Abs and splenectomized mice treated with isotype control Ab. B and C, Representative Qa2 expression by GFP+ CD62LhighTCRhigh CD4 and CD8 T cells from the blood of splenectomized, anti-CD62L plus anti-VLA-4-treated mice (“Homeless” RTEs), anti-CD62L plus anti-VLA-4-treated euthymic mice (“No LN” RTEs), splenectomized, isotype control Ab-treated mice (“No Spl!” RTEs), or untreated mice (Control RTEs). GFP levels of “homeless” and control RTEs were normalized in B. Thymocytes and splenocytes from an untreated age-matched mouse were analyzed on the same day for Qa2 expression by GFP+ CD62LhighTCRhigh SP thymocytes (Mature SP thymocytes) and GFP− CD44low/mid splenic CD4 and CD8 T cells (Naive GFP− peripheral T). CD24 expression levels could not be assessed on cells in the blood because of high background staining. Data are representative of at least five mice per condition in two independent experiments.
Our data do suggest that phenotypic and functional maturation of CD45RB, an isoform of the CD45 glycoprotein phosphatase, in T cells of Qa2, a nonclassical class Ib MHC molecule, or CD24 has roles in both naive T cell homeostasis and tolerance. Although CD24 has roles in naive T cell maturation, CD24 expression levels upon RTE maturation could modulate immune responses in the gut. RTEs express less IL-2 (Fig. 5A) and proliferate less relative to control RTEs (Fig. 5B) and data not shown), demonstrating that CD24highQa2low peripheral T cells, although ~85% of RTEs are excluded by this gating system (data not shown).

In conclusion, we show in this study that not only does T cell development continue after thymic egress, but that this process is dynamically regulated. While RTEs are adjusting to the lymphoid periphery, their immune competence is dampened for a period of 2–3 wk and they rely on signals received in SLOs to drive them to the full competence of the non-RTE subset, ready to defend against invading pathogens.

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Disclosures
The authors have no financial conflict of interest.

References


