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Homeostatic signals do not drive post-thymic T cell maturation

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ABSTRACT

Recent thymic emigrants, the youngest T cells in the lymphoid periphery, undergo a 3 week-long period of functional and phenotypic maturation before being incorporated into the pool of mature, naïve T cells. Previous studies indicate that this maturation requires T cell exit from the thymus and access to secondary lymphoid organs, but is MHC-independent. We now show that post-thymic T cell maturation is independent of homeostatic and costimulatory pathways, requiring neither signals delivered by IL-7 nor CD80/86. Furthermore, while CCR7/CCL19,21-regulated homing of recent thymic emigrants to the T cell zones within the secondary lymphoid organs is not required for post-thymic T cell maturation, an intact dendritic cell compartment modulates this process. It is thus clear that, unlike T cell development and homeostasis, post-thymic maturation is focused not on interrogating the T cell receptor or the cell's responsiveness to homeostatic or costimulatory signals, but on some as yet unrecognized property.
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1. Introduction

Recent thymic emigrants (RTEs), those peripheral T cells that have most recently completed thymic development and egress, are the subject of much current interest. These young T cells help replenish the diversity of the naïve peripheral T cell repertoire, and are of particular consequence both in adults recovering from lymphopenia, and in infants, whose lymphoid periphery is first being seeded with T cells (reviewed in [1]).

The study of RTE biology has been facilitated by the development of a tractable model system that allows unambiguous identification of RTEs from unmanipulated mice, enabling their ready isolation for functional and phenotypic analysis [2,3]. Thus, in mice transgenic (Tg) for green fluorescent protein (GFP) under control of the RAG-2 promoter [4], GFP^+ peripheral T cells are RTEs [2]. Furthermore, the intensity of the GFP signal can be used as a clock, being inversely proportional to the time the cells have spent in the lymphoid periphery, such that GFP^{hi} and GFP^{lo} RTEs have resided in the lymphoid periphery for ~1 and ~2 weeks, respectively [2,5].

Using RAG2p-GFP Tg mice, we and others have demonstrated that RTEs are both phenotypically and functionally distinct from their mature, yet still naïve (MN), peripheral T cell counterparts. Lower IL-7Rα, CD28, Qa2, and CD45RB, and higher TCR, CD3, and CD24 cell surface expression characterize RTEs from mice of all ages [2,3]. The functional distinctions between RTEs and MN T cells are equally stark. Stimulated RTEs exhibit dampened proliferation and cytokine production compared to MN T cells [2,3,6,7], defective generation of memory precursor cells [8], and impaired skewing to effector lineages upon *in vitro* polarization [9]. In addition to RAG2p-GFP Tg mice, multiple other methods to tag and identify RTEs in both mice [10–13] and humans [14,15] have been used to reach a similar conclusion: RTEs represent a T cell subset that is both functionally and phenotypically distinct from the bulk population of mature, naïve peripheral T cells (reviewed in [1]).

Using phenotypic markers as faithful indicators of RTE function, it has become clear that the transition from RTE to MN T cell is a result of cellular maturation, rather than selection and subsequent outgrowth of a small population of RTEs that already bear an MN-like surface phenotype. Thus, maturation occurs in the absence of selective survival or proliferation [16]. What triggers the maturational process that characterizes the first few weeks of post-thymic life for a T cell? Our previous work [16] has demonstrated that RTE maturation is an active process that requires both thymic egress and access to secondary lymphoid organs (SLOs). Given the need for tonic signaling through the T cell receptor (TCR) to regulate the survival and homeostasis of naïve peripheral T cells [17,18] and our findings that RTEs and MN T cells interpret these homeostatic signals somewhat differently [19], we suspected that these signaling pathways might control post-thymic maturation. The

Abbreviations: B6, C57BL/6; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; iDTR, inducible DTR; GFP, green fluorescent protein; LN, lymph node; MFI, median fluorescence intensity; MN, mature naïve peripheral T cell; RTE, recent thymic emigrant; SLO, secondary lymphoid organ; TCR, T cell receptor; Tg, transgenic.

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central role played in thymocyte development by signals mediated through the TCR strengthened these suspicions. However, our previous work revealed that while RTE maturation is associated with subtle modulation of the TCR repertoire, the maturation process itself is unexpectedly MHC-independent [20]. Thus, signaling through the TCR initiated by recognition of self MHC/self peptide does not drive RTE maturation.

We now extend these studies to ask whether the obligatory entry of RTEs into SLOs facilitates delivery of either IL-7- or costimulation-dependent maturation signals, whether maturation requires RTE homing to specific T cell microenvironments, and whether the presence of an intact dendritic cell (DC) compartment is required to trigger RTE maturation. Our results offer the surprising conclusion that while IL-7, costimulation, and CCR7/CCL19,21-driven microenvironmental homing by T cells are all dispensable for post-thymic T cell maturation, DCs do modulate the transition of RTEs to the MN T cell compartment.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were bred on site. RAG2p-GFP Tg mice [4] were originally a gift from M. Nussenzweig (The Rockefeller University) and were backcrossed in our lab at least 12 generations onto the B6 background. B6 mice Tg for human CD2 promoter-driven IL-7R [21], a gift from K. Elkon (University of Washington), were maintained as heterozygotes and crossed onto the RAG2p-GFP Tg background. CCL19/21$^{-/-}$ mice [22] on the B6 background [23] were a gift from J. Cyster, (University of California, San Francisco), and were crossed onto the RAG2p-GFP Tg background. CD11c-diphtheria toxin receptor (DTR) Tg B6 mice [24] were a gift from M. Bevan (University of Washington), and CD11c-Cre [25] $\times$ inducible (i)DTR [26] Tg B6 mice were a gift from A. Rudensky (then at the University of Washington). Mice were used at 6–12 weeks of age, except for radiation chimeras, which were reconstituted at 6–8 weeks of age and analyzed $\geqslant$8 weeks later. RTE maturation follows a similar trend in mice throughout these age ranges [3]. All experiments were performed in compliance with University of Washington Institutional Animal Care and Use Committee guidelines.

2.2. Mouse procedures

For blockade of IL-7R signaling, mice were given 200 μg each of anti-IL-7 (M25; BioXCell) and anti-IL-7Rα (A7R34; lab-purified) i.p. on days 0, 2, and 4. For blockade of CD28 signaling, mice were given 100 μg each of anti-CD80 (16-10A1) and anti-CD86 (GL-1) i.p., both purchased from the University of California, San Francisco Monoclonal Antibody Core, on days 0, 2, and 4. For DC depletion, mice were given 60 μg/kg body weight of diphtheria toxin (DT, from Sigma) in PBS i.p. on days 0, 1, 3, and 5. After titering the DT dose from 12.5 to 150 μg/kg, we judged this dose to mediate effective DC ablation with acceptable weight loss.

To generate radiation chimeras, ~5×10^6 T cell-depleted bone marrow cells from femurs and tibia were injected i.v. into lethally irradiated (1000 rads) RAG2p-GFP Tg recipient mice. Recipients were maintained on water containing neomycin sulfate (Mediatech, Inc.) and Polymyxin B (Invitrogen) from 1 day before to 14 days after irradiation. T cell depletion was achieved by incubating a single-cell suspension of bone marrow with lab-generated anti-CD4 (RC172.4R6), anti-CD8 (3.168.8), and anti-CD90.2 (13.4.6), followed by incubation with rabbit complement (Cedarlane).

2.3. Cell preparation, staining, enrichment and sorting

Single cell suspensions of brachial, axillary, inguinal, cervical, and mesenteric lymph nodes (LNs) and water-lysed splenocytes were prepared and counted. Where indicated, T cells were enriched using an EasySep mouse T cell enrichment kit (StemCell Technologies) according to the manufacturer's protocol. For flow cytometric analysis, FcR were blocked with anti-CD16/32 (2.4G2, BD Biosciences), and cells were stained as previously described [16] with antibodies conjugated to FITC, Phycoerythrin, Peridin chlorophyll protein-Cyanine 5.5, Phycoerythrin-Cy7, Allophycocyanin, Allophycocyanin-eFluor 780, or biotin and against the following molecules: CD4 (RM4-5), CD8 (53-6.7), CD11c (N418), CD24 (M1/69), CD44 (Pgp-1), CD45RB (16A), CD62L (MEL-14), CD80 (16-10A1), CD86 (GL-1), Qa2 (1-1-2), and I-A^b (M5/114.15.2), all from eBioscience or BD Pharmingen. Biotinylated antibodies were detected with Allophycocyanin- or Phycoerythrin-conjugated streptavidin (eBioscience). Events were collected on a FACSCanto (BD Biosciences) and data were analyzed on FlowJo software (TreeStar) after excluding doublets from live-gated samples. Fluorescence-Minus-One [27] samples were run when appropriate. While IL-7R, CD28, Qa2, TCR, CD3, CD24, and CD45RB levels all differ on RTEs and MN T cells, maturation can be most reliably assessed by tracking CD24, Qa2, and CD45RB expression. Data are shown for splenocytes; LN cells gave comparable results.

3. Results

3.1. RTE maturation is IL-7 independent

To determine whether IL-7 drives RTE maturation, given that it provides an important survival and homeostatic factor for naïve T cells [28], we co-administered anti-IL-7 plus anti-IL-7Rα directly to RAG2p-GFP Tg mice for 6 days, and tracked the phenotypic maturation of CD4 and CD8 RTEs as a faithful reflection of their functional maturation [16]. The amount of administered antibody was sufficient to extinguish all peripheral IL-7R signaling, because on the day of analysis, not only were peripheral T cells maximally coated with anti-IL-7Rα, but excess anti-IL-7Rα was detected in the serum (Fig. 1A). In addition, the size of the CD4$^-$CD8$^-$ (double negative) three compartment, an IL-7-dependent stage of thymocyte development (reviewed in [28]), was severely reduced, from 1.6×10^6 in controls to 2×10^5 in treated mice, demonstrating the efficacy of the IL-7R blockade. While the phenotypic maturation of the bulk population of GFP$^+$ RTEs was not altered by IL-7R blockade, we concentrated our analysis of RTE maturation on the GFP^{hi} youngest ~10–25% of the RTE population [5] to identify those RTEs that had entered the periphery after the onset of antibody blockade. Consistent with the diminished IL-7Rα expression that characterizes RTEs [2,19], maturation occurred normally in both CD4 and CD8 GFP^{hi} RTEs in the absence of IL-7R signaling (Fig. 1B), demonstrating that IL-7 is dispensable for maturation. These GFP^{hi} RTEs had begun to undergo post-thymic maturation, as their phenotype was more mature than that of their immediate precursors in the thymus (Fig. 1B). Our short-term IL-7R blockade did not adversely affect T cell survival at the time of analysis, as the numbers of naïve T cells from PBS-treated and IL-7R-blocked mice were comparable. Furthermore, the GFP median fluorescence intensities (MFIs) of RTEs from both groups were comparable, suggesting that antibody inoculation did not alter RTE output at this timepoint. These results were corroborated by data we obtained after adoptive transfer of CD4 RTEs into IL-7 null mice (data not shown), but without introducing the undesirable possibility that, prior to transfer, RTEs received IL-7 signals sufficient to drive their maturation.

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