Published in final edited form as:

Eur J Immunol. 2016 July ; 46(7): 1669–1680. doi:10.1002/eji.201546214.

Distinct IL-7 signaling in recent thymic emigrants versus mature naïve T cells controls T-cell homeostasis

Hye Kyung Kim[1], **Adam T. Waickman**[2], **Ehydel Castro**[1], **Francis A. Flomerfelt**[1], **Nga V. Hawk**[1], **Veena Kapoor**[1], **William G. Telford**[1], **Ronald E. Gress**[1]

[1]Experimental Transplantation and Immunology Branch, Bethesda, MD, USA

[2]Experimental Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

IL-7 is essential for T-cell survival but its availability is limited in vivo. Consequently, all peripheral T cells, including recent thymic emigrants (RTEs) are constantly competing for IL-7 to survive. RTEs are required to replenish TCR diversity and rejuvenate the peripheral T-cell pool. However, it remains unknown how RTEs successfully compete with resident mature T cells for IL-7. Moreover, RTEs express low levels of IL-7 receptors, presumably rendering them even less competitive. Here, we show that, surprisingly, RTEs are more responsive to IL-7 than mature naïve T cells as demonstrated by markedly increased STAT5 phosphorylation upon IL-7 stimulation. Nonetheless, adoptive transfer of RTE cells into lymphopenic host mice resulted in slower IL-7-induced homeostatic proliferation and diminished expansion compared to naïve donor T cells. Mechanistically, we found that IL-7 signaling in RTEs preferentially upregulated expression of Bcl-2, which is anti-apoptotic but also anti-proliferative. In contrast, naïve T cells showed diminished Bcl-2 induction but greater proliferative response to IL-7. Collectively, these data indicate that IL-7 responsiveness in RTE is designed to maximize survival at the expense of reduced proliferation, consistent with RTE serving as a subpopulation of T cells rich in diversity but not in frequency.

Keywords

Apoptosis; Bcl-2; Cytokine; Proliferation; STAT5

Introduction

T cells are quintessential effector cells of the adaptive immune system that are generated in the thymus and then exported to peripheral tissues for immune surveillance [1, 2]. The size

Full correspondence: Dr. Hye Kyung Kim, Experimental Transplantation and Immunology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bldg. 10 CRC, Room 3E-3288, 10 Center, Bethesda, MD 20892, USA, kihye@mail.nih.gov.
Additional correspondence: Dr. Adam T. Waickman, Experimental Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. adam.waickman@nih.gov.

Additional supporting information may be found in the online version of this article at the publisher's web-site

Conflict of interest: The authors declare no commercial or financial conflict of interest.

of the peripheral T-cell pool is controlled by both the influx of newly arriving T cells, known as recent thymic emigrants (RTEs), and the efflux of preexisting T cells by cell death [3–5]. While both the influx and efflux of T cells can vary with age or immune challenges, the size of the T-cell pool remains remarkably constant throughout the lifespan of the organism [6]. The mechanism that maintains peripheral T-cell numbers is known as T-cell homeostasis [4, 5], and is thought to be controlled by the availability of a critical survival cytokine for T cells, namely IL-7 [7–9]. Notably, while IL-7 is a nonredundant survival factor for T cells, T cells themselves do not produce IL-7 [10–13]. Thus, T cells are dependent on exogenous IL-7 to survive that is mostly produced by stromal cells in limited amounts so that peripheral T cells constantly compete for IL-7 [14]. Multiple lines of evidence indicate that IL-7 availability is limited in vivo, and that IL-7 availability constrains peripheral T-cell numbers. Among others, transgenic IL-7 expression or recombinant IL-7 administration result in dramatically increased T-cell numbers in mice [15, 16], while increased IL-7 consumption reduces T-cell numbers in the periphery [17].

Because IL-7 availability is limited, a major question in T-cell homeostasis is to understand how newly arriving RTEs successfully compete with preexisting mature T cells for IL-7. A couple of different views have been put forward to explain this conundrum. According to the classical model [18]. RTEs that enter the periphery occupy a survival niche distinct from preexisting T cells. In this way, RTEs are provided with a 3-week window for survival and maturation before they are placed under the same homeostatic constraints as mature T cells [18]. In support of this idea, thymus engraftment experiments demonstrated a linear increase of RTE cell numbers with increasing numbers of thymus grafts, but without a compensatory decrease in mature T-cell numbers [18]. These results suggest that RTE cells can be maintained independently of IL-7, at least short-term, negating a requirement for IL-7 competition upon initial arrival in peripheral tissues. Alternatively, RTEs were proposed to be less competitive for IL-7 than naïve T cells so that the majority of RTEs would succumb to apoptosis upon arrival in peripheral tissues [19]. Under this scenario, the peripheral T-cell compartment is mostly replenished by homeostatic turnover of preexisting T cells. Indeed, cotransfer of RTE and mature naïve T cells into lymphoreplete host mice demonstrated significantly lower recovery of RTEs from host spleen and LN compared to donor mature naïve T cells [19]. A recent study further suggested that proliferating RTEs preferentially converted into $Foxp3^+$ regulatory T cells and become dependent on IL-2 for survival, circumventing their need for IL-7 [20]. However, how much of such conversion contributes to overall RTE survival is not known.

While these views are not mutually exclusive, these partially conflicting findings also indicate that our understanding of RTE biology is far from complete. Specifically, it remains largely unknown if RTEs are indeed less competitive than mature T cells for IL-7 signaling. Moreover, it is also unclear if any proliferation or survival difference between RTE and mature naïve T cells would be due to differences in IL-7 signaling and whether such differences would still exist under IL-7-sufficient conditions.

To address these questions, here we performed a series of adoptive transfer experiments, and directly assessed IL-7 signaling in RTE and naïve T cells. Surprisingly, we found that RTEs were more effective in IL-7 signaling than naïve T cells, even though RTEs expressed

significantly lower levels of surface IL-7Rα. Increased STAT5 phosphorylation in RTE by IL-7, however, did not result in increased proliferation of RTE. In fact, adoptive transfer of RTE and naïve T cells into *Rag2*-deficient lymphopenic mice demonstrated that naïve T cells proliferated better than RTEs. Thus, even as RTEs responded more efficiently to IL-7, their proliferative potential was significantly diminished. Mechanistically, we found that RTEs preferentially interpreted IL-7 signaling as survival signals, which, however, impeded their proliferative potential. naïve T cells, on the other hand, responded to IL-7 signaling preferentially by proliferation and less by survival. Collectively, distinct IL-7 responses may provide the molecular basis for enhanced survival of RTE compared to naïve T cells, and thus equip RTEs to better survive at the expense of diminished proliferation during T-cell homeostasis.

Results

Homeostatic proliferation of RTE and naïve T cells

To examine the effect of IL-7 signaling in T-cell subpopulations, first we identified RTEs and naïve T cells by *Rag2*-GFP reporter (*Rag2*-GFP-Tg) expression [21, 22]. Because *Rag2* activity ceases upon positive selection in the thymus (Supporting Information Fig. 1) [23], the decay of GFP faithfully reports the age of T cells in *Rag2*-GFP-Tg mice. Not all mature T cells are naïve T cells, and a substantial fraction of GFP-negative T cells correspond to memory phenotype T cells expressing high levels of CD44. Thus, we first gated on $CD44^{low}$ cells to identify naïve T cells, among which T cells that still expressed GFP were considered as RTEs (Fig. 1A).

To examine if distinct T-cell subsets respond differently to a lymphopenic environment, we adoptively transferred pooled LN T cells from *Rag2*-GFP mice into $Rag2^{-/-}$ host and recovered donor cells from lymph nodes after 5 days. Before injection, donor T cells were loaded with cell trace violet (CTV) to monitor proliferation in vivo. CTV works similar to CFSE and is diluted out upon cell division to report donor T-cell proliferation. Unlike CFSE, however, CTV has an excitation/emission wavelength that does not overlap with GFP and thus allows tracking of GFP^+ cell proliferation. In recovered donor cells, we gated on $CD44^{hi}GFP^-$, $CD44^{lo}GFP^-$, and $CD44^{lo}GFP^+$ cells to identify memory, naïve, and RTE cells, respectively. naïve $CD4^+$ T cells were significantly less efficient in lymphopenia-induced homeostatic proliferation compared to memory $CD4^+$ T cells that showed robust proliferation (Fig. 1B). In $CD8^+$ T cells, both naïve and memory cells were rapidly proliferating, demonstrating a difference between $CD4^+$ and $CD8^+$ lineage cells in γc cytokine signaling and lymphopenia-induced homeostatic proliferation [24, 25]. Interestingly, RTE cells were similar to $CD4^+$ T naïve cells as they fail to undergo proliferation as demonstrated by the lack of CTV dilution (Fig. 1B).

GFP has a short half-life [22], and also will be diluted out with cell division. Therefore, we asked whether we failed to identify RTE cells because of loss of GFP expression. To examine this possibility, we electronically sorted RTEs and naïve T cells from *Rag2*-GFP-Tg mice and injected them into separate host mice. Purified $CD8^+$ RTE cells showed vigorous proliferation that was accompanied with increase of surface IL-7Rα expression but gradual dilution of GFP expression (Supporting Information Fig. 2A bottom), indicating that $CD8^+$

RTEs can undergo lymphopenia-induced proliferation just like $CD8^+$ naïve T cells (Fig. 1C). These results indicate that injection of pooled T cells to track proliferation of RTE cells can be misleading, and that cell proliferation needs to be assessed in purified T-cell subsets.

Importantly, injection of purified T-cell subsets revealed that naïve $CD8^+$ T cells were significantly more efficient in proliferation than $CD8^+$ RTEs (Fig. 1D), and that $CD8^+$ RTEs undergo cell proliferation at slower rate than naïve T cells (Fig. 1D). The difference in proliferation was presumably due to a difference in IL-7 response, because both RTEs and naïve donor T cells showed minimal proliferation in *Rag2*$^{-/-}$*Il7*$^{-/-}$ (*Rag2/Il7*-DKO) host mice (Fig. 1E) or in congenic lymphoreplete mice (Supporting Information Fig. 2B). These results demonstrate that IL-7 is the primary driver for lymphopenia-induced proliferation (LIP) in both naïve and RTE $CD8^+$ T cells, and that $CD8^+$ RTE cells are less efficient to proliferate in response to IL-7.

Increased IL-7 availability accelerates cell proliferation

Because RTEs were less efficient than naïve T cells to undergo homeostatic proliferation, we asked whether RTEs are less effective in accessing IL-7 in vivo. To exclude this possibility, we utilized osmotic pumps to administer recombinant IL-7 and increase IL-7 bioavailability in vivo. For both RTEs and naïve T cells, we found that T-cell proliferation was dramatically increased in IL-7 pump installed mice compared to control PBS pump installed mice (Fig. 2A and Supporting Information Fig. 3A). Interestingly, increased IL-7 availability induced strong proliferation even in naïve $CD4^+$ T cells, indicating that the inefficient LIP of naïve $CD4^+$ T cells could possibly due to limited availability of IL-7. Notably, increased IL-7 availability dramatically increased naïve $CD8^+$ T-cell proliferation but to a lesser extent the proliferation of $CD8^+$ RTE cells (Fig. 2B). These results demonstrate that inefficient proliferation of $CD8^+$ RTE cells is not necessarily due to limited availability of IL-7. Because $CD8^+$ RTEs expressed robust levels of IL-7Rα (Fig. 2C), these data also suggest that RTEs should be as competent as naïve T cells for IL-7R signaling. Moreover, we did not find any significant differences in gene expression of molecules in the JAK/STAT signaling pathway that would indicate a disadvantage for RTE cells in IL-7R signaling (Supporting Information Fig. 3B). Nonetheless, $CD8^+$ RTEs displayed diminished proliferation upon IL-7 signaling, so that $CD8^+$ naïve T cell-derived donor T cells numbers greatly exceeded $CD8^+$ RTE-derived donor T-cell numbers (Fig. 2D).

Competitive advantage of naïve T cells over RTE cells

To further examine their differences in proliferative potentials, we sorted RTE cells, mixed them with CD45.1 congenic naïve T cells at 1:1 ratio and injected them into *Rag2*$^{-/-}$ host mice. Five days after injection, we found that donor $CD8^+$ T cells had robustly proliferated while donor $CD4^+$ T cells showed minimal proliferation (Fig. 3A). Importantly, coinjected naïve $CD8^+$ T cells still displayed a significantly higher rate of proliferation compared to coinjected $CD8^+$ RTE cells (Fig. 3B), resulting in a significantly increased naïve/RTE ratio (Fig. 3C). This was not only the case when RTEs and naïve T cells were coinjected into PBS pump installed host mice, but also in IL-7 pump installed host mice (Fig. 3C). Altogether, these results indicate that a cell-intrinsic mechanism impairs RTE cell proliferation, independent of the in vivo availability of IL-7.

IL-7 signaling in naïve and RTE cells

To test if RTEs are less proliferative because they are less efficient in IL-7 signaling, we assessed surface IL-7Rα expression on IL-7-signaled RTE and naïve T cells. Notably, RTEs expressed significantly less surface IL-7Rα than naïve T cells, whereas γc expression was comparable between RTEs and naïve T cells (Supporting Information Fig. 4A). IL-7 induces downregulation of its own receptor [17], so that IL-7 receptor expression is a faithful indicator of IL-7 signaling. When assessing IL-7Rα expression at the indicated time points (Fig. 4A), we found that both RTEs and naïve T cells rapidly downregulated IL-7Rα upon IL-7 stimulation (Fig. 4A), suggesting that IL-7R signaling is intact in RTEs. Because IL-7 can also induce IL-7Rα internalization independently of IL-7 signaling [26, 27], we further examined IL-7 downstream signals in RTEs and naïve T cells. To this end, we stimulated LN T cells from *Rag2*GFP-Tg mice with IL-7, and assessed intracellular phospho-STAT5 (pSTAT5) contents in RTEs and naïve T cells. Interestingly, RTEs were significantly more responsive to IL-7 signaling than naïve T cells as demonstrated by increased STAT5-phosphorylation in both $CD4^+$ and $CD8^+$ RTE cells (Fig. 4B and 4C). These results establish that RTEs are more effective in IL-7 signaling than naïve T cells.

Differential IL-7 signaling in RTEs and naïve T cells

The thymus is a prime source of in vivo IL-7 [10–13]. Thus, presumably, thymus-derived RTEs have been recently exposed to IL-7 while naïve T cells have stayed for a prolonged time in the IL-7-poor periphery. Because RTEs and naïve T cells have a distinct history of IL-7 signaling in vivo, we considered the possibility that RTE cells are better IL-7 responders because they were most recently stimulated in an IL-7-rich environment in the thymus. To eliminate differences in prior IL-7 signaling, we prestimulated RTEs and naïve T cells with IL-7 so that all cells were exposed to IL-7 before testing IL-7 signaling. To this end, first, we prestimulated LN T cells for 4 h with IL-7. Prolonged IL-7 stimulation negated any difference in IL-7-induced STAT5 phosphorylation between RTEs and naïve T cells, and we observed robust IL-7 signaling in both RTEs and naïve T cells to equal levels (Fig. 5A). Next, we removed exogenous IL-7 by repeated washing and then rested prestimulated T cells in prewarmed medium for two hours. Finally, we restimulated the cells with IL-7 and assessed their intracellular pSTAT5 contents (Fig. 5B). Even under these conditions, RTEs were still more effective in IL-7 signaling, displaying significantly increased STAT5 phosphorylation upon restimulation (Fig. 5B). naïve T cells, on the other hand, were more refractory to IL-7 restimulation and remained less effective. Why RTEs can recover more efficiently from desensitizing IL-7 signaling is not known and remains to be investigated.

To further examine any differences in IL-7 receptor signaling, we also assessed Akt and mTOR phosphorylation in IL-7-signaled RTEs and mature naïve T cells. Surprisingly and in contrast to IL-7-induced STAT5 phosphorylation, we found that RTEs were significantly less efficient in Akt and mTOR phosphorylation downstream of IL-7 (Fig. 5C). These results further illustrate cell intrinsic differences of IL-7R signaling in RTE versus naïve T cells.

Anti-apoptotic Bcl-2 promotes survival but dampens proliferation

Because RTEs were highly responsive to IL-7 signaling, we wished to examine if expression of downstream effector molecules were also increased in RTEs. Bcl-2 is a cytosolic protein

that protects from programmed cell death and that is induced by IL-7 to maintain naïve T-cell homeostasis [28, 29]. Indeed, we found a significant and selective increase of Bcl-2 protein expression in freshly isolated RTE cells compared to naïve T cells, which agrees with the role of IL-7 in RTE survival (Fig. 6A) [30,31]. Such an increase was specific to Bcl-2 because expression of other anti-apoptotic genes, such as Mcl-1 and Bc1-x_L, were not affected (Supporting Information Fig. 4B). Moreover, in vitro IL-7 stimulation dramatically induced Bcl-2 protein expression in RTE cells, while naïve T cells upregulated Bcl-2 to a significantly lesser degree (Fig. 6B and Supporting Information Fig. 4C). Importantly, RTEs were more resistant to apoptosis and survived even in the absence of exogenous IL-7 (Fig. 6C), suggesting that prior IL-7 signaling in vivo was sufficient to protect RTEs from programmed cell death during overnight medium culture.

Notably, Bcl-2 is not only anti-apoptotic but also antiproliferative [32–34]. Thus, we asked if increased Bcl-2 expression could inhibit proliferation in RTE cells. To directly test this, we adoptively transferred naïve T cells from Bcl-2 transgenic mice (Bcl2-Tg) and WT CD45.1 congenic mice into *Rag2*$^{-/-}$ host mice. Five days after injection, donor cells were recovered from LN and assessed for cell recovery and CTV dilution. Strikingly, we found a significantly skewed and preferential recovery of WT cells over Bcl2-Tg donor T cells (Supporting Information Fig. 5A) that was associated with reduced proliferation of Bcl2-Tg cells compared to WT donor T cells (Fig. 6D). Collectively, these results imply that increased Bcl-2 expression promotes survival but dampens their proliferation. naïve T cells, on the other hand, were less effective in upregulating Bcl-2 by IL-7 signaling, which however, endowed them with greater proliferative potential.

To directly demonstrate this, we purified RTEs from WT (CD45.1) and Bcl2-Tg (CD45.2) mice and adoptively transferred them into lymphopenic host mice (Fig. 6E). Strikingly, increased Bcl-2 expression significantly suppressed proliferation and cell recovery of RTEs from host mice, indicating that Bcl-2 imposes a proliferative disadvantage. Such an effect was not restricted to RTE but also observed for naïve T cells, because cotransfer experiments with WT and Bcl2-Tg naïve T cells also resulted in a preferential proliferation and accumulation of WT naïve T cells compared to Bcl2-Tg naïve T cells (Supporting Information Fig. 5B). Notably, Bcl2-Tg and WT cells expressed comparable levels of surface γc and IL-7Rα and also showed similar kinetics of cytokine receptor expression during proliferation (Supporting Information Fig. 6A and B), suggesting that cytokine receptor expression itself is not the cause for the distinct proliferative outcome of RTE and naïve T cells. Instead, these data suggest that differential response to IL-7 receptor signaling induces distinct proliferation of RTEs and naïve T cells to control T-cell homeostasis.

Discussion

RTEs are critical for T-cell homeostasis as they enrich the T-cell repertoire and ensure clonal diversity of the peripheral T-cell pool [3]. Importantly, both RTEs and mature naïve T cells require IL-7 for survival so that they inevitably compete for a single homeostatic cytokine. But, how these two populations coexist and can be maintained in face of competition has remained largely unknown. Here, we report previously unappreciated differences in IL-7

signaling between RTEs and naïve T cells that permit sharing of a limited survival resource to maximize the diversity of the peripheral T-cell pool.

Conventionally, RTEs are considered less efficient than naïve T cells in competing for IL-7 in vivo because they express lower levels of IL-7 receptor than mature naïve T cells [3]. If so, RTEs would be preferentially lost and preexisting mature naïve T cells retained, which however is counterintuitive considering the large efforts of the thymus to generate RTEs [19, 35]. To explain the significant presence of RTEs in peripheral tissues, several models have been proposed. A continuous influx of RTEs from the thymus into the periphery that replaces lost RTE cells [36], occupation of distinct survival niches by newly arriving RTEs and pre-existing T cells [18], or IL-7 dependent expansion of RTE [30, 37] represent some of these possibilities.

Here, we propose an alternative solution to this problem that reconciles contradictory findings from previous studies. Our model is based on the surprising finding that RTEs were more efficient than naïve T cells in IL-7 signaling even as they expressed significantly lower levels of IL-7Rα than naïve T cells. Specifically, IL-7 signaling in RTEs induced significantly higher levels of STAT5 phosphorylation than in naïve T cells. Paradoxically, RTEs showed diminished IL-7-dependent proliferation upon adoptive transfer into lymphopenic mice. We found cell intrinsic differences in RTEs versus mature naïve T cells in response to IL-7 signaling in that RTEs preferentially induced survival while naïve T cells chose to proliferate. Notably, IL-7 signaling induced high levels of Bcl-2 expression in RTEs whereas it induced only low levels of Bcl-2 in naïve T cells. Thus, RTEs are programmed to survive, whereas naïve T cells are programmed to expand upon homeostatic IL-7 signaling. These results unveil a novel mechanism as to how RTEs and naïve T cells can coexist under homeostatic conditions where IL-7 availability is limited.

The classical concept of homeostasis is fairly limited to the maintenance of a constant number of cells. However, T cells are clonal and they are comprised of a highly diverse population with unique TCR specificities [38]. Therefore, T-cell homeostasis not only requires sustaining normal cell numbers but also the maintenance of a diverse and polyclonal repertoire. Initially, maintaining the T-cell compartment had been mostly attributed to proliferation of activated/memory phenotype T cells [39, 40]. However, follow-up studies revealed that naïve T cells underwent phenotypic conversion into activated/memory phenotype T cells during homeostatic proliferation, demonstrating that the size of the T-cell pool is controlled by both naïve and memory T cells [41–43]. In contrast to naïve T cells, the contribution of RTEs to the peripheral T-cell pools has remained less certain. The classical “niche” concept proposed that RTEs are exempt from peripheral T-cell homeostasis because they occupy a different survival niche from preexisting T cells. Engraftment of multiple thymic lobes induced an increase in total RTE cell numbers that correlated with the number of lobes and followed a trend predicted by the niche model of homeostasis [44]. Accordingly, initial seeding of RTEs occupy a different niche than preexisting naïve T cells. However, once RTEs become more mature, they will enter the naïve T-cell pool and become subject to IL-7-dependent homeostasis [45]. The identity of the survival niche for RTE, which is supposed to be distinct from the niche for mature naïve T cells, has not been reported. Our data now propose that RTEs and naïve T cells do not differ in their survival

requirements, and that both cells require IL-7 for survival. However, they effectively share this limited resource because newly generated RTE cells utilize IL-7 signaling to survive and preserve the new repertoire, while naïve mature T cells utilize IL-7 signaling to expand and maintain size of the T-cell pool. Collectively, there is a division of tasks in maintaining the two aspects of T-cell homeostasis, in that RTEs contribute to repertoire diversity while preexisting mature T cells control cell numbers.

RTEs are functionally and phenotypically distinct from mature naïve T cells [3]. Phenotypically, RTEs express higher levels of CD24 and TCR while expressing lower levels of Qa-2, CD28, and IL-7Rα than mature T cells [3]. Because of their low level of surface IL-7Rα, RTE have been considered less responsive to IL-7 than naïve T cells [3]. The amount of IL-7Rα, however, is determined by multiple factors and does not necessarily correlate with IL-7 signaling capability. For example, tonic TCR engagement desensitizes IL-7 signaling, resulting in increased surface IL-7 receptor expression but reduced IL-7 responsiveness [46, 47]. Activating TCR signals, on the other hand, suppress IL-7Rα transcription and surface expression [48–50]. Also, cytosolic inhibitory molecules such as suppressor of cytokine signaling and protein inhibitor of activated STAT can suppress IL-7 signaling independently of surface IL-7Rα levels [51, 52]. Therefore, IL-7 signaling needs to be directly assessed in individual cells and should not be presumed to correlate with IL-7Rα expression levels.

The use of GFP as a molecular clock to identify RTE revolutionized the field of RTE biology. Using *Rag2*-GFP reporter mice, RTE can be identified without surgical manipulations, administration of dyes or DNA labeling reagents and also at single cell levels, which was previously not possible with assays such as T-cell receptor excision circle analysis [53]. Such feature allowed us to establish a protocol that permitted analysis of intracellular signaling events at single cell levels in RTE. We identified RTE cells by GFP expression, and IL-7 induced STAT activation was simultaneously assessed by intracellular staining with anti-pSTAT5 antibodies. The results were unambiguous, and they demonstrated that in contrast to the prevailing view, RTE cells were more effective in IL-7 signaling than mature naïve T cells.

Collectively, the current study unveiled a cell intrinsic mechanism that confers distinct IL-7 response in RTEs and naïve T cells to maximize the use of a homeostatic cytokine that is only available in limited amount. By selective induction of survival in RTEs and preferential proliferation of naïve T cells, the distinct IL-7 signaling effects in RTE and naïve T cells ensures effective homeostasis of both the size and diversity of the peripheral T-cell pool.

Materials and methods

Mice

C57BL/6 (CD45.2) mice and CD45.1 congenic mice were obtained from the FNLCR, Frederick, MD. *Rag2*$^{-/-}$ mice were purchased from Jackson. Bcl-2-transgenic mice and *Rag2*$^{-/-}$*Il7*$^{-/-}$ mice were provided by Hyun Park, NIH. *Rag2*-GFP-Tg mice were previously described [21]. Animal experiments were approved by the NCI Animal Care and Use Committee, and all mice were cared for in accordance with NIH guidelines.

Flow cytometry

Cells were harvested from the thymus, spleen, and lymph nodes. Data were acquired using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo. Live cells were gated using forward scatter exclusion of dead cells stained with propidium iodide. RTE and naïve T-cell subpopulations were electronically sorted using a FACSAria II (BD Biosciences) cell sorter, based on their GFP and CD44 expression levels. In brief, single cell suspensions were stained for TCRβ, $CD4^+$, $CD8^+$, and CD44 expression and resuspended in sorting buffer (0.5% BSA in Ca^{2+}/Mg^{2+}-free HBSS) at 20×10^6 cells/mL and filtered through 0.45 μM nylon meshes before passing through the cell sorter. Collected cells were washed once in PBS before further processing for tail vein injection or RNA isolation. The following antibodies were used for staining: TCRβ (H57-597), IL-7Rα (A7R34), CD44 (IM7), $CD4^+$ (GK1.5), CD8α (53-6-7), and isotype control antibodies (eBioscience or BioLegend). Intracellular stainings for pAkt and phosphor-mTOR were performed using kits from eBioscience following the manufacturer's instructions.

In vitro IL-7 stimulation

T cells were stimulated with IL-7 as previously described [17]. In brief, single cell suspensions were adjusted to 5×10^6 cells/mL and stimulated with recombinant IL-7 (PeproTech). pSTAT5 contents were assessed after 30 min upon fixing and permeating cells with paraformaldehyde and acetone/methanol. Alternatively, cells were stimulated for 4 h with IL-7 (10 ng/mL), then removed from IL-7 by extensive washing, rested for 2 h in IL-7-free medium and restimulated with IL-7.

Quantitative RT-PCR and PCR array

Total RNA was isolated from sorted cells using NucleoSpin RNA kits (Machery-Nagel). RNA was reverse transcribed into cDNA by oligo(dT) priming with the Superscript III (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed with a Bio-Rad CFX Connect Real-Time PCR machine and the QuantiTect SYBR Green detection system (Qiagen). Primers sequences are as follows. Hprt (5′-TCATTATGCCGAGGATTTGGA-3′; 5′-CAGAGGGC CACAATGTGATG-3′), Bcl-2 (5′-TGTAAATTGCCGAGAAGAAGGG-3′; 5′-TCCCCGTTGGCATGAGAT-3′), Bcl-x_L (5′-GCGGCTGGGAC ACTTTTG-3′; 5′-ACTTCCGACTGAAGAGTGAGCC-3′), Mcl-1 (5′-AGACGGCCTTCCAGGGC-3′; 5′-CCAGTCCCGTTTCGTCCTT-3′). Array analysis of 84 key genes involved in the JAK/STAT signaling pathway was performed with a mouse RT^{2}Profiler™ PCR array system (PAMM-039Y; Qiagen). PCR Array data were analyzed by the http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php.

CTV labeling and adoptive transfer

Donor cells were electronically sorted from lymphocytes isolated from LN, which were pooled out of inguinal, axillary, cervical, and mesenteric area. Before injection, donor cells were loaded with CTV (Invitrogen) as previously described [54]. A total of 10×10^6 cells were tail-vein injected into *Rag2*$^{-/-}$, *Rag2/Il7*-DKO, or lymphoreplete congenic (CD45.1) host mice. Five days after injection, donor cells were recovered from host lymph nodes for analysis.

ALZET osmotic pump installation

Recombinant human IL-7 (Cytheris Inc.) was administered into host mice using ALZET osmotic pumps (Durect) as previously described [15]. IL-7 was delivered at a rate of 5 μg/day for 5 days.

Active caspase-3 assay

Caspase-3 activity was assessed using sulfo-rhodamine-conjugated DEVD-FMK (CaspGLOW red, BioVision). For each assay, 5 million LN cells were placed into 24-well plates in 1 mL complete culture medium with different concentrations of IL-7. Next day, cells were incubated with fluorescent DEVD-FMK for 45 min, washed and analyzed by flow cytometry.

Statistical analysis

Statistical tests were performed with Prism (GraphPad). Statistical significance was determined with Student's *t*-test. $*p < 0.05$ was considered significant. $**p < 0.01$; $***p < 0.001$. Two-way ANOVA test was used to compare more than three groups of normally distributed data. Error bars indicate SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We thank Dr. Hyun Park for critical review of this manuscript. This study was supported by the Intramural Research Program of the US National Institutes of Health, the National Cancer Institute, and the Center for Cancer Research.

Abbreviations:

CTV	cell trace violet
LIP	lymphopenia-induced proliferation
RTE	recent thymic emigrant

References

1. Edgar JD, T cell immunodeficiency. J. Clin. Pathol 2008 61: 988–993. [PubMed: 18755723]
2. Bluestone JA, Bour-Jordan H, Cheng M and Anderson M, T cells in the control of organ-specific autoimmunity. J. Clin. Invest 2015 125: 2250–2260. [PubMed: 25985270]
3. Fink PJ, The biology of recent thymic emigrants. Anna. Reu. Immunol 2013 31: 31–50.
4. Takada K and Jameson SC, naïve T cell homeostasis: from awareness of space to a sense of place. Nat. Reu. Immunol 2009 9: 823–832.
5. Sprent J and Surh CD, Normal T cell homeostasis: the conversion of naïve cells into memory-phenotype cells. Nat. Immunol 201112: 478–484. [PubMed: 21739670]
6. Almeida AR, Rocha B, Freitas AA and Tanchot C, Homeostasis of T cell numbers: from thymus production to peripheral compartmentalization and the indexation of regulatory T cells. Semin. Immunol 200517: 239–249. [PubMed: 15826829]

7. Marsden VS, Kappler JW and Marrack PC, Homeostasis of the memory T cell pool. Int. Arch. Allergy Immunol 2006139: 63–74. [PubMed: 16319493]
8. Carrette F and Surh CD, IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. Semin. Immunol 2012 24: 209–217. [PubMed: 22551764]
9. Jameson SC, T cell homeostasis: keeping useful T cells alive and live T cells useful. Semin. Immunol 2005 17: 231–237. [PubMed: 15826828]
10. Alves NL, Richard-Le Goff O, Huntington ND, Sousa AP, Ribeiro VS, Bordack A, Vives FL et al., Characterization of the thymic IL-7 niche in vivo. Proc. Natl. Acad. Sci. USA 2009 106: 1512–1517. [PubMed: 19164539]
11. Kim GY, Hong C and Park JH, Seeing is believing: illuminating the source of in vivo interleukin-7. Immune Netw. 2011 11: 1–10. [PubMed: 21494371]
12. Mazzucchelli RI, Warming S, Lawrence SM, Ishii M, Abshari M, Washington AV, Feigenbaum L et al.,Visualization and identification of IL-7 producing cells in reporter mice. PLoS One 2009 4: e7637. [PubMed: 19907640]
13. Repass JF, Laurent MN, Carter C, Reizis B, Bedford MT, Cardenas K, Narang P et al., IL7-hCD25 and IL7-Cre BAC transgenic mouse lines: new tools for analysis of IL-7 expressing cells. Genesis 2009 47: 281–287. [PubMed: 19263498]
14. Fry TJ, Connick E, Falloon J, Lederman MM, Liewehr DJ, Spritzler J, Steinberg SM et al., A potential role for interleukin-7 in T-cell homeostasis. Blood 2001 97: 2983–2990. [PubMed: 11342421]
15. Chu YW, Memon SA, Sharrow SO, Hakim FT, Eckhaus M, Lucas PJ and Gress RE, Exogenous IL-7 increases recent thymic emigrants in peripheral lymphoid tissue without enhanced thymic function. Blood 2004 104: 1110–1119. [PubMed: 15130942]
16. Mertsching E, Burdet C and Ceredig R, IL-7 transgenic mice: analysis of the role of IL-7 in the differentiation of thymocytes in vivo and in vitro. Int. Immunol 1995 7: 401–414. [PubMed: 7794820]
17. Park JH, Yu Q, Erman B, Appelbaum JS, Montoya-Durango D, Grimes HL and Singer A, Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. Immunity 2004 21: 289–302. [PubMed: 15308108]
18. Berzins SP, Boyd RL and Miller JF, The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. J. Exp. Med 1998 187: 1839–1848. [PubMed: 9607924]
19. Houston EG Jr., Higdon LE and Fink PJ, Recent thymic emigrants are preferentially incorporated only into the depleted T-cell pool. Proc. Natl. Acad. Sci. USA 2011 108: 5366–5371. [PubMed: 21402911]
20. Paiva RS, Lino AC, Bergman ML, Caramalho I, Sousa AE, Zelenay S and Demengeot J, Recent thymic emigrants are the preferential precursors of regulatory T cells differentiated in the periphery. Proc. Natl. Acad. Sci. USA 2013 110: 6494–6499. [PubMed: 23576744]
21. Yu W, Nagaoka H, Jankovic M, Misulovin Z, Suh H, Rolink A, Melchers F et al., Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. Nature 1999 400: 682–687. [PubMed: 10458165]
22. McCaughtry TM, Wilken MS and Hogquist KA, Thymic emigration revisited. J. Exp. Med 2007 204: 2513–2520. [PubMed: 17908937]
23. Cooper CJ, Orr MT, McMahan CJ and Fink PJ, T cell receptor revision does not solely target recent thymic emigrants. J. Immunol 2003 171: 226–233. [PubMed: 12817002]
24. Cho JH, Kim HO, Surh CD and Sprent J, T cell receptor-dependent regulation of lipid rafts controls naïve $CD8^+$ T cell homeostasis. Immunity 2010 32: 214–226. [PubMed: 20137986]
25. Guimond M, Veenstra RG, Grindler DJ, Zhang H, Cui Y, Murphy RD, Kim SY et al., Interleukin 7 signaling in dendritic cells regulates the homeostatic proliferation and niche size of $CD4^+$ T cells. Nat. Immunol 2009 10: 149–157. [PubMed: 19136960]
26. Luo H, Wu Z, Qi S, Jin W, Han B and Wu J, Ephrinb1 and Ephrinb2 are associated with interleukin-7 receptor alpha and retard its internalization from the cell surface. J. Biol. Chem 2011 286: 44976–44987. [PubMed: 22069310]

27. Ghazawi FM, Faller EM, Sugden SM, Kakal JA and MacPherson PA, IL-7 downregulates IL-7Ralpha expression in human $CD8^+$ T cells by two independent mechanisms. Immunol. Cell Biol 2013 91: 149–158. [PubMed: 23207282]
28. Jiang Q, Li WQ, Aiello FB, Mazzucchelli R, Asefa B, Khaled AR and Durum SK, Cell biology of IL-7, a key lymphotrophin. Cytokine Growth Factor Rev. 2005 16: 513–533. [PubMed: 15996891]
29. Masse GX, Corcuff E, Decaluwe H, Bommhardt U, Lantz O, Buer J and Di Santo JP, Gamma(c) cytokines provide multiple homeostatic signals to naïve $CD4_{(+)}$ T cells. Eur. J. Immunol 2007 37: 2606–2616. [PubMed: 17683114]
30. Azevedo RI, Soares MV, Barata JT, Tendeiro R, Serra-Caetano A, Victorino RM and Sousa AE, IL-7 sustains CD31 expression in human naïve $CD4_+$ T cells and preferentially expands the CD31+ subset in a PI3K-dependent manner. Blood 2009113: 2999–3007. [PubMed: 19008454]
31. Swainson L, Kinet S, Mongellaz C, Sourisseau M, Henriques T and Taylor N, IL-7-inducedproliferationofrecentthymic emigrants requires activation of the PI3K pathway. Blood 2007 109: 1034–1042. [PubMed: 17023582]
32. Cheng N, Janumyan YM, Didion L, Van Hofwegen C, Yang E and Knudson CM, Bcl-2 inhibition of T-cell proliferation is related to prolonged T-cell survival. Oncogene 2004 23: 3770–3780. [PubMed: 15034548]
33. Linette GP, Li Y, Roth K and Korsmeyer SJ, Cross talk between cell death and cell cycle progression: BCL-2 regulates NFAT-mediated activation. Proc. Natl. Acad. Sci. USA 1996 93: 9545–9552. [PubMed: 8790367]
34. Mazel S, Burtrum D and Petrie HT, Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. J. Exp. Med 1996 183: 2219–2226. [PubMed: 8642331]
35. Hassan J and Reen DJ, Human recent thymic emigrants—identification, expansion, and survival characteristics. J. Immunol 2001 167: 1970–1976. [PubMed: 11489977]
36. den Braber I, Mugwagwa T, Vrisekoop N, Westera L, Mogling R, de Boer AB, Willems N et al., Maintenance of peripheral naïve T cells is sustained by thymus output in mice but not humans. Immunity 2012 36: 288–297. [PubMed: 22365666]
37. Soares MV, Borthwick NJ, Maini MK, Janossy G, Salmon M and Akbar AN, IL-7-dependent extrathymic expansion of $CD45RA_+$ T cells enables preservation of a naïve repertoire. J. Immunol 1998 161: 5909–5917. [PubMed: 9834071]
38. Wiegers GJ, Kaufmann M, Tischner D and Villunger A, Shaping the T-cell repertoire: a matter of life and death. Immunol. Cell Biol 2011 89: 33–39. [PubMed: 21060321]
39. Tanchot C, Lemonnier FA, Perarnau B, Freitas AA and Rocha B, Differential requirements for survival and proliferation of $CD8^+$ naïve or memory T cells. Science 1997 276: 2057–2062. [PubMed: 9197272]
40. Tough DF and Sprent J, Turnover of naïve- and memory-phenotype T cells. J. Exp. Med 1994 179: 1127–1135. [PubMed: 8145034]
41. Cho BK, Rao VP, Ge Q, Eisen HN and Chen J, Homeostasis-stimulated proliferation drives naïve T cells to differentiate directly into memory T cells. J. Exp. Med 2000 192: 549–556. [PubMed: 10952724]
42. Goldrath AW, Bogatzki LY and Bevan MJ, naïve T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. J. Exp. Med 2000 192: 557–564. [PubMed: 10952725]
43. Jaleco S, Swainson L, Dardalhon V, Burjanadze M, Kinet S and Taylor N, Homeostasis of naïve and memory $CD4_+$ T cells: IL-2 and IL-7 differentially regulate the balance between proliferation and Fas-mediated apoptosis. J. Immunol 2003171: 61–68. [PubMed: 12816983]
44. Berzins SP, Godfrey DI, Miller JF and Boyd RL, A central role for thymic emigrants in peripheral T cell homeostasis. Proc. Natl Acad. Sci. USA 1999 96: 9787–9791. [PubMed: 10449772]
45. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI and Surh GD, IL-7 is critical for homeostatic proliferation and survival of naïve T cells. Proc. Natl Acad. Sci. USA 2001 98: 8732–8737. [PubMed: 11447288]

46. Park JH, Adoro S, Lucas PJ, Sarafova SD, Alag AS, Doan LL, Erman B et al., 'Coreceptor tuning': cytokine signals transcriptionally tailor $CD8^+$ coreceptor expression to the self-specificity of the TCR. Nat. Immunol 2007 8:1049–1059. [PubMed: 17873878]

47. Kimura MY, Pobezinsky LA, Guinter TI, Thomas J, Adams A, Park JH, Tai X and Singer A, IL-7 signaling must be intermittent, not continuous, during CD8(+) T cell homeostasis to promote cell survival instead of cell death. Nat. Immunol 2013 14:143–151. [PubMed: 23242416]

48. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh GD and Ahmed R, Selective expression of the interleukin 7 receptor identifies effector $CD8^+$ T cells that give rise to long-lived memory cells. Nat. Immunol 2003 4: 1191–1198. [PubMed: 14625547]

49. Schluns KS, Kieper WC, Jameson SG and Lefrancois L, Interleukin-7 mediates the homeostasis of naïve and memory $CD8^+$ T cells in vivo. Nat. Immunol 20001: 426–432. [PubMed: 11062503]

50. Xue HH, Kovanen PE, Pise-Masison GA, Berg M, Radovich MF, Brady JN and Leonard WJ, IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. Proc. Natl Acad. Sci. USA 2002 99: 13759–13764. [PubMed: 12354940]

51. Shuai K, Regulation of cytokine signaling pathways by PIAS proteins. Cell Res. 2006 16: 196–202. [PubMed: 16474434]

52. Alexander WS, Starr R, Metcalf D, Nicholson SE, Farley A, Elefanty AG, Brysha M et al., Suppressors of cytokine signaling (SOCS): negative regulators of signal transduction, J. Leukoc. Biol 1999 66: 588–592. [PubMed: 10534114]

53. Hazenberg MD, Verschuren MC, Hamann D, Miedema F and van Dongen JJ, T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. J. Mol. Med 2001 79: 631–640. [PubMed: 11715066]

54. Quah BJ and Parish GR, New and improved methods for measuring lymphocyte proliferation in vitro and in vivo using CFSE-like fluorescent dyes. J. Immunol. Methods 2012 379:1–14. [PubMed: 22370428]

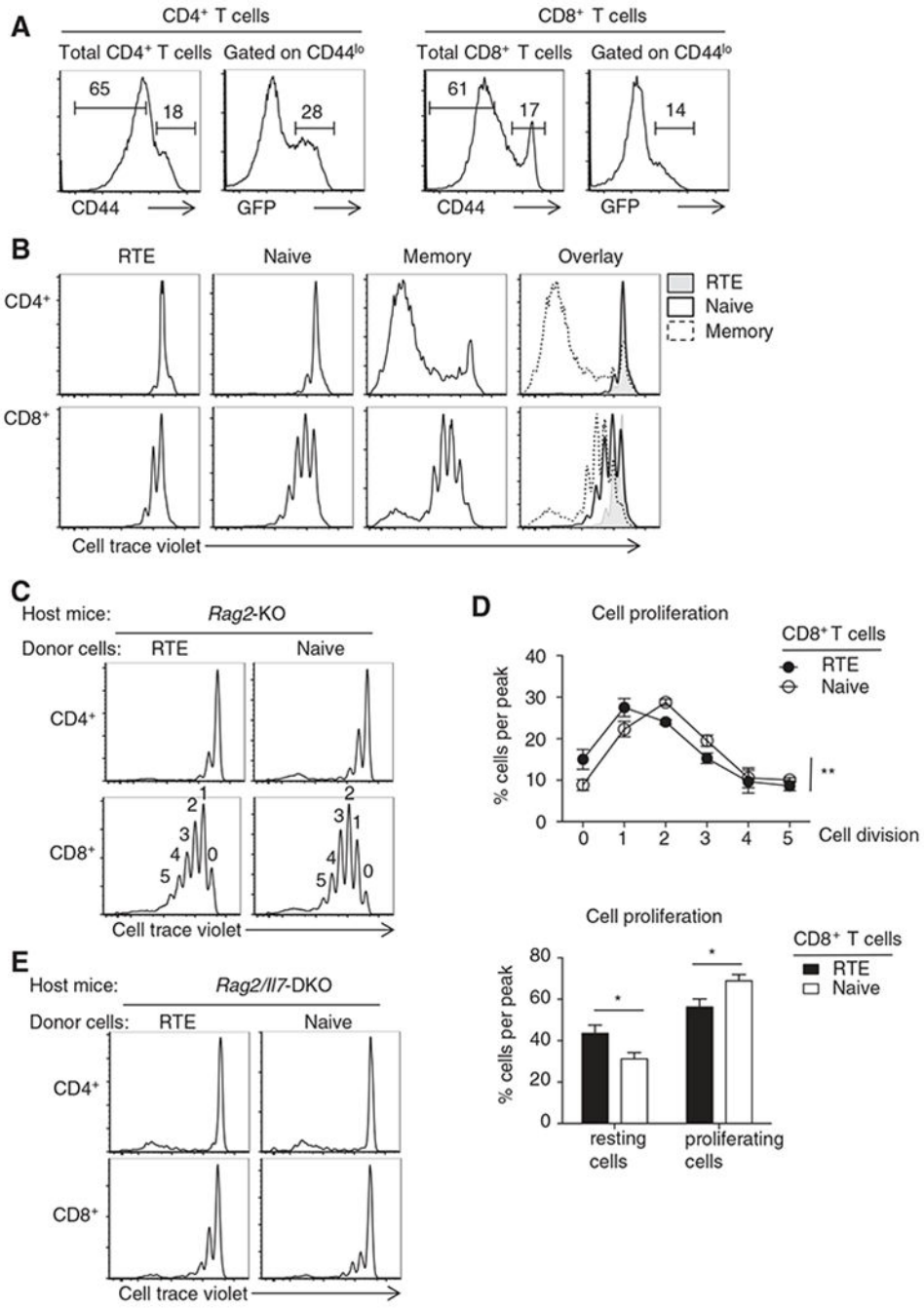

Figure 1.

Recent thymic emigrants are less efficient in homeostatic proliferation than na'ive T cells. (A) Identification of peripheral T-cell subsets. Total $CD4^+$ (left) or $CD8^+$ T cells (right) gated on live cells were assessed for CD44 expression to identify $CD44^{lo}$ naïve cells. RTE were identified as GFP-positive cells among $CD44^{lo}$ naïve T cells. Results are representative of four independent experiments. (B) Homeostatic proliferation of donor total T cells from *Rag2*-GFP-Tg mice were labeled with CellTrace Violet (CTV) and injected into in *Rag2*-KO host mice. After 5 days, donor cells were recovered from LN and assessed for CTV dilution

in the indicated T-cell subsets. Results are representative of two independent experiments. (C) Homeostatic proliferation of sorted RTE and naïve donor T cells. Electronically sorted RTE and GFP-negative $CD44^{lo}$ naïve T cells were labeled with CTV and injected into *Rag2*-KO host mice. After 5 days, donor cells were recovered from spleen and assessed for cell trace violet dilution. Numbers above individual peaks indicate cell division of RTE and naïve $CD8^+$ T cells. Histograms are representative of four independent experiments.(D) Diminished homeostatic proliferation of $CD8^+$ RTE compared to naïve $CD8^+$ T cells. Cell proliferation in *Rag2*-KO host mice was determined by assessing percentage of cells in individual cell trace violet peaks (top). Cells that have undergone no or one division (0, 1) were considered as resting cells, while cells that have undergone more than two divisions (>2) were considered as proliferating cells (bottom). Data are shown as mean ± SEM ($n = 8$ mice) and represent summary of four independent experiments, p values were determined by Student's *t*-test or two-way ANOVA. *$p < 0.05$, **$p < 0.01$.(E) Homeostatic proliferation of sorted RTE and naïve donor T cells in *Rag2/Il7*-DKO hosts. Cell trace violet labeled RTE and GFP-negative $CD44^{lo}$ naïve T cells were injected into *Rag2*-KO host mice. After 5 days, donor cells were recovered from lymph node and assessed for T-cell subset and CTV. Results are representative of two independent experiments with each two host mice per experiment and a total of four *Rag2*-KO host mice.

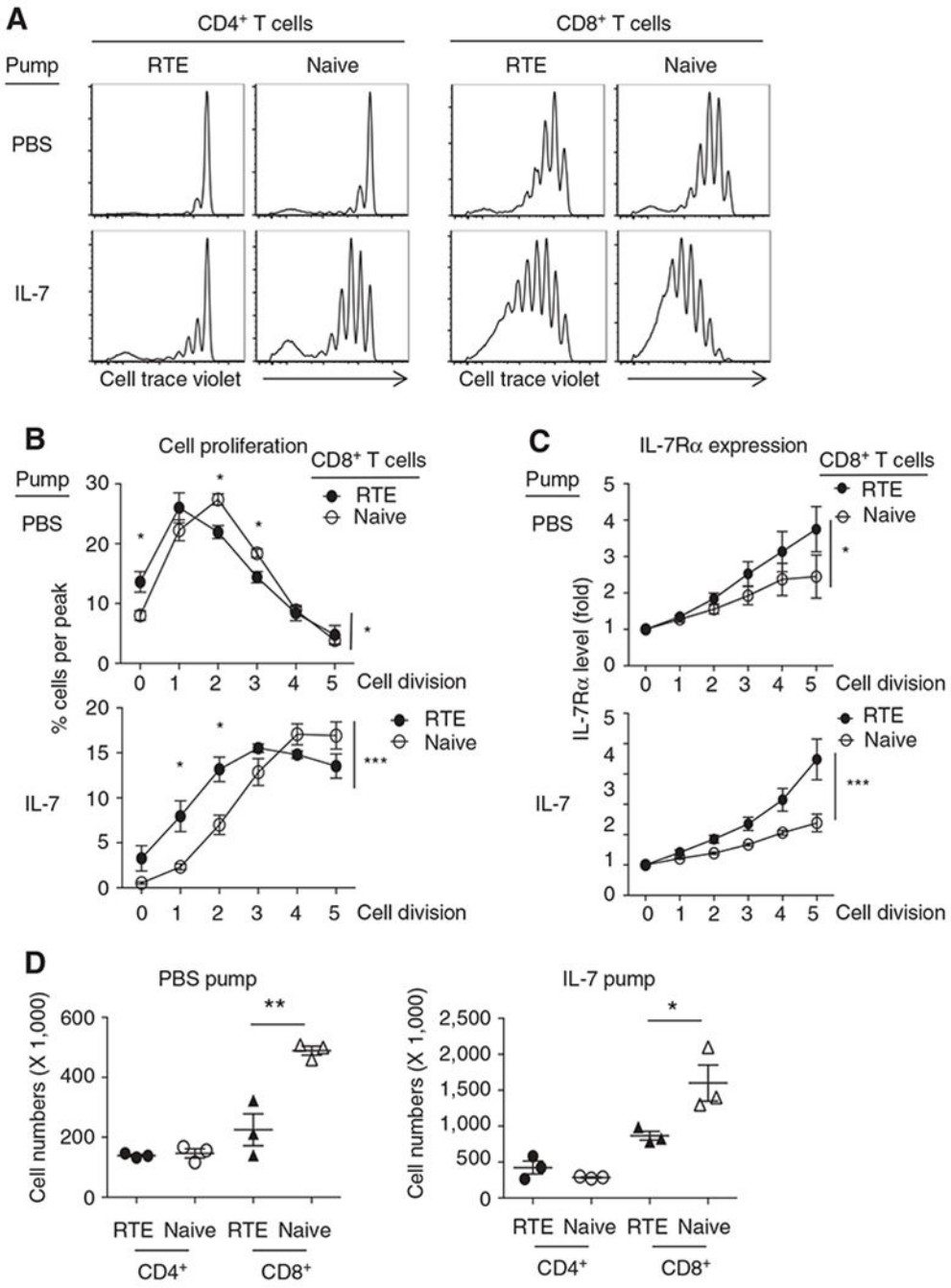

Figure 2.

A cell intrinsic mechanism dampens IL-7-induced homeostatic proliferation of recent thymic emigrants. (A) Donor $CD4^+$ and $CD8^+$ T-cell proliferation in *Rag2*-KO host mice in the presence or absence of exogenous IL-7. Purified RTEs or naïve T cells from *Rag2*-GFP-Tg mice were labeled with CTV and injected separately into *Rag2*-KO host mice. Osmotic pumps with IL-7 or PBS were installed immediately after injection. After 5 days, donor cells were recovered from LN and assessed for CTV dilution. Results are representative of three independent experiments with a total of 12 *Rag2*-KO host mice. (B) Kinetics of $CD8^+$ donor

RTE and naïve T-cell proliferation with or without IL-7 administration. Distribution of proliferated donor cells per cell division was assessed as % cells for each peak of CTV dilution. Results show summary of three independent experiments with a total of 12 *Rag2*-KO host mice. *p* values were determined by Student's t-test and two-way ANOVA. < 0.05. (C) Fold change of IL-7Rα expression on donor RTE and naïve $CD8^+$ T cells upon proliferation, with or without IL-7 administration. Fold change was normalized to undivided cells (0 cell division). Results show summary of three independent experiments with a total of 12 *Rag2*-KO host mice. *p* values were determined by two-way ANOVA. **p* < 0.05, ****p* ρ < 0.001. (D) Donor T-cell numbers after 5 days of homeostatic proliferation in the absence (PBS pump) or presence of IL-7 administration (IL-7 pump). *p* values were determined by Student's t-test. **p* < 0.05, ***p* < 0.01.

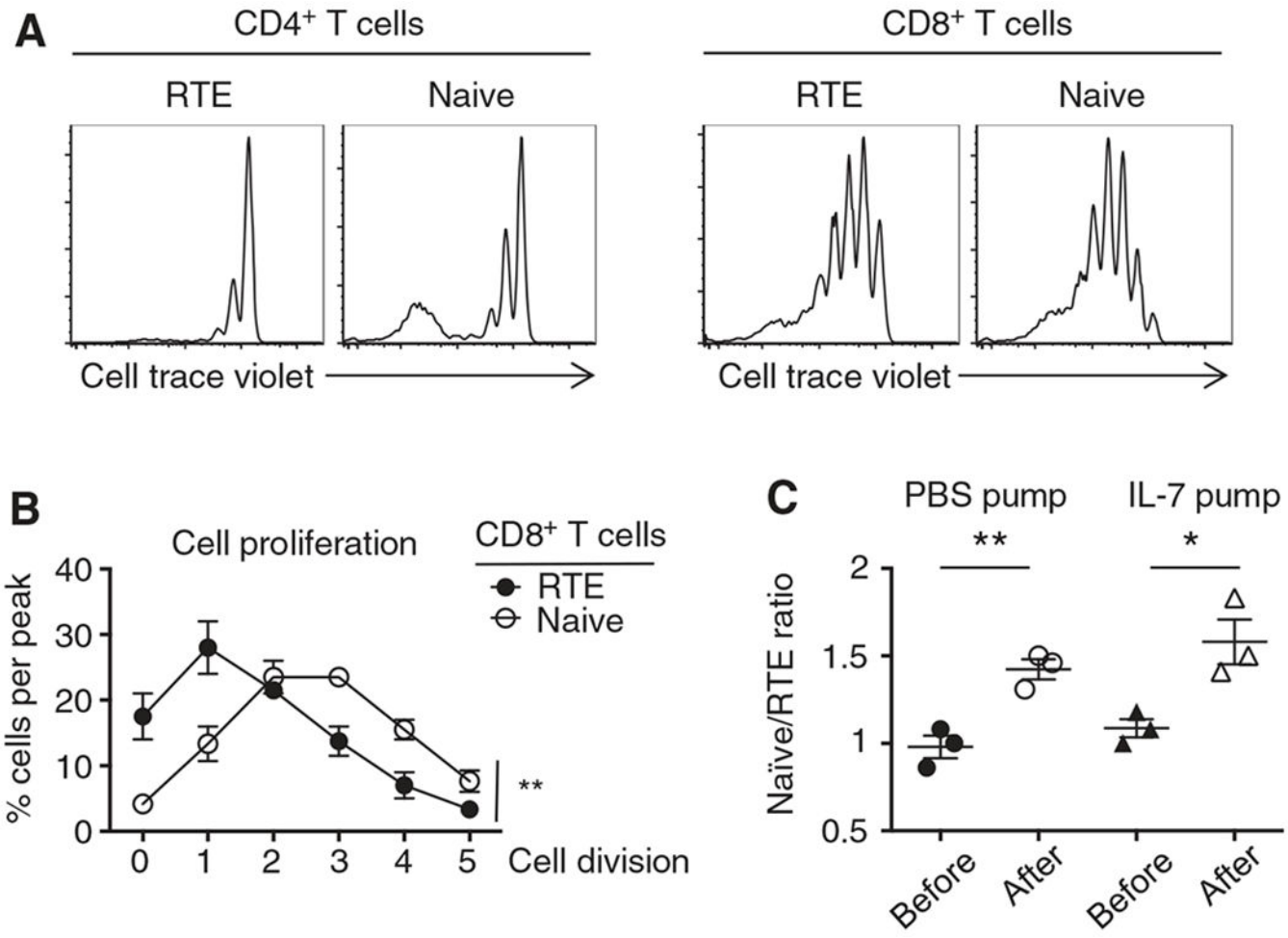

Figure 3.

Competitive advantage of naïve T cells over RTEs during lymphopenia-induced homeostatic proliferation. (A) Donor RTE and naïve T-cell proliferation in *Rag2*-KO host mice. Purified RTEs from CD45.2 *Rag2*-GFP-Tg mice or naïve T cells from CD45.1 congenic mice were labeled with CTV, mixed at 1:1 ratio and coinjected into *Rag2*-KO host mice. After 5 days, donor cells were recovered from LN and assessed for CTV dilution. Results are representative of two independent experiments with a total of 2 *Rag2*-KO host mice. (B) Proliferation kinetics of $CD8^+$ donor RTE and naïve T cells. Distribution of proliferated donor cells per cell division was assessed as % cells for each peak of CTV dilution. Results show summary of two independent experiments with a total of 2 *Rag2*-KO host mice, *p* values were determined by two-way ANOVA. $^{**}p < 0.01$. (C) Donor cell ratios before and after adoptive transfer. Purified RTE from CD45.2 *Rag2*-GFP-Tg mice or naïve T cells from CD45.1 congenic mice were labeled with CTV and mixed at 1:1 ratio. Donor cells were injected into *Rag2*-KO host mice implanted with PBS or IL-7 filled Alzet osmotic pumps. After 5 days, donor cells were recovered from LN, and donor cells numbers from RTE or naïve cell origin were determined. Ratios show results from three independent experiments, *p* values were determined by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$.

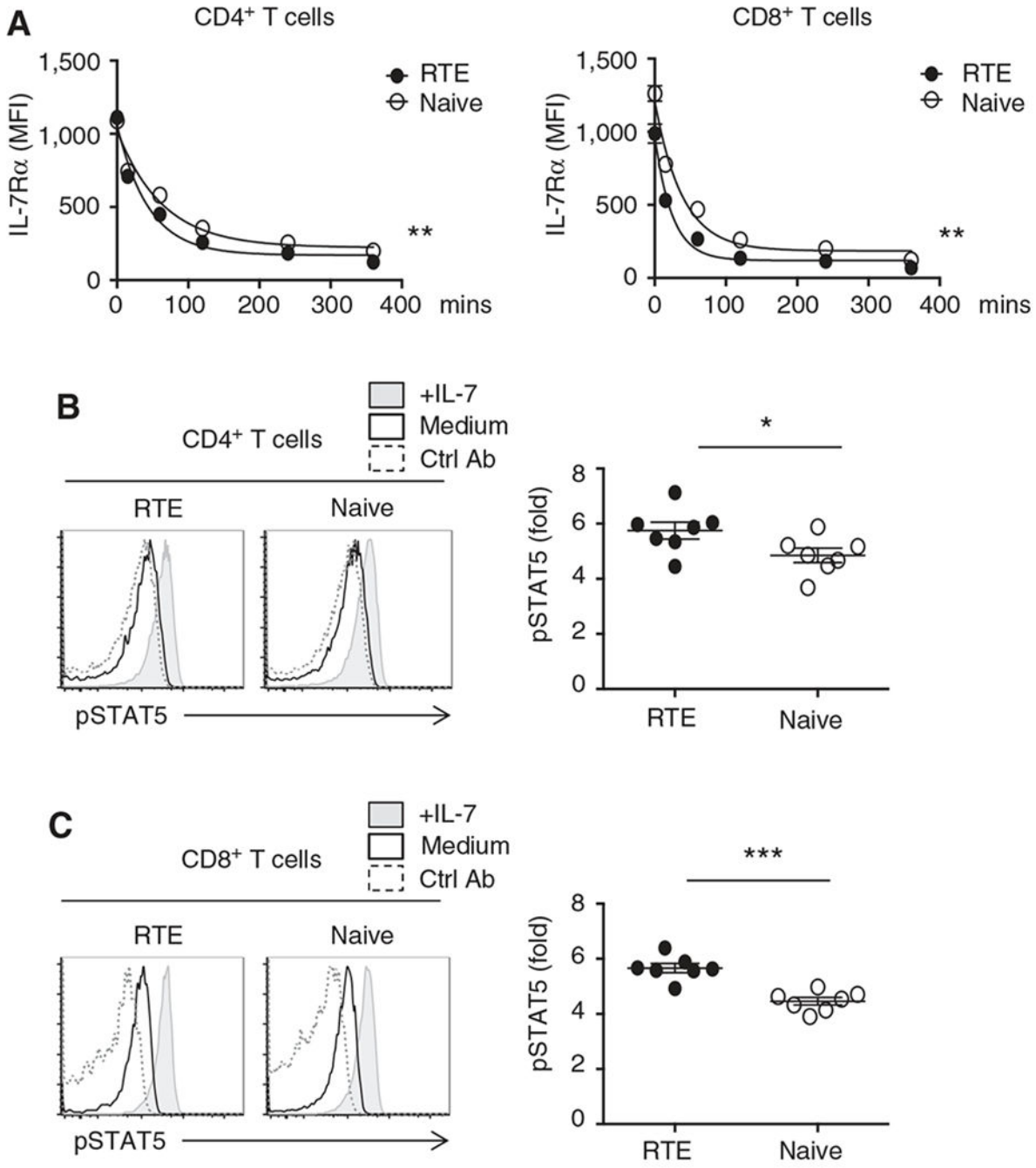

Figure 4.

Enhanced IL-7 responsiveness in recent thymic emigrants. (A) Kinetic of IL-7Rα downregulation on RTE and naïve T cells. LN T cells from *Rag2*-GFP-Tg mice were stimulated with IL-7 (10 ng/mL) and assessed for surface IL-7Rα downregulation on gated RTE and naïve $CD4^+$ and $CD8^+$ T cells. Surface IL-7Rα expression was determined in mean fluorescence intensity (MFI). *p* values were determined by Student's *t*-test. $^{**}p < 0.01$. (B) IL-7 signaling in naïve and RTE $CD4^+$ T cells. Intracellular pSTAT5 level was determined after 30 min of IL-7-stimulation (10 ng/mL) in naïve and RTE $CD4^+$ T cells from *Rag2*-

GFP-Tg mice (left) and is shown as fold increase over medium (right). Each circle represents one independent experiment, p values were determined by Student's t-test. *$p < 0.05$. (C) IL-7 signaling in naïve and RTE $CD8^+$ T cells. Intracellular pSTAT5 level was determined after 30 min of IL-7-stimulation (10 ng/mL) in naïve and RTE $CD8^+$ T cells from *Rag2*-GFP-Tg mice (left) and is shown as fold increase over medium (right). Each circle represents one independent experiment. p values were determined by Student's t-test. ***$p < 0.001$.

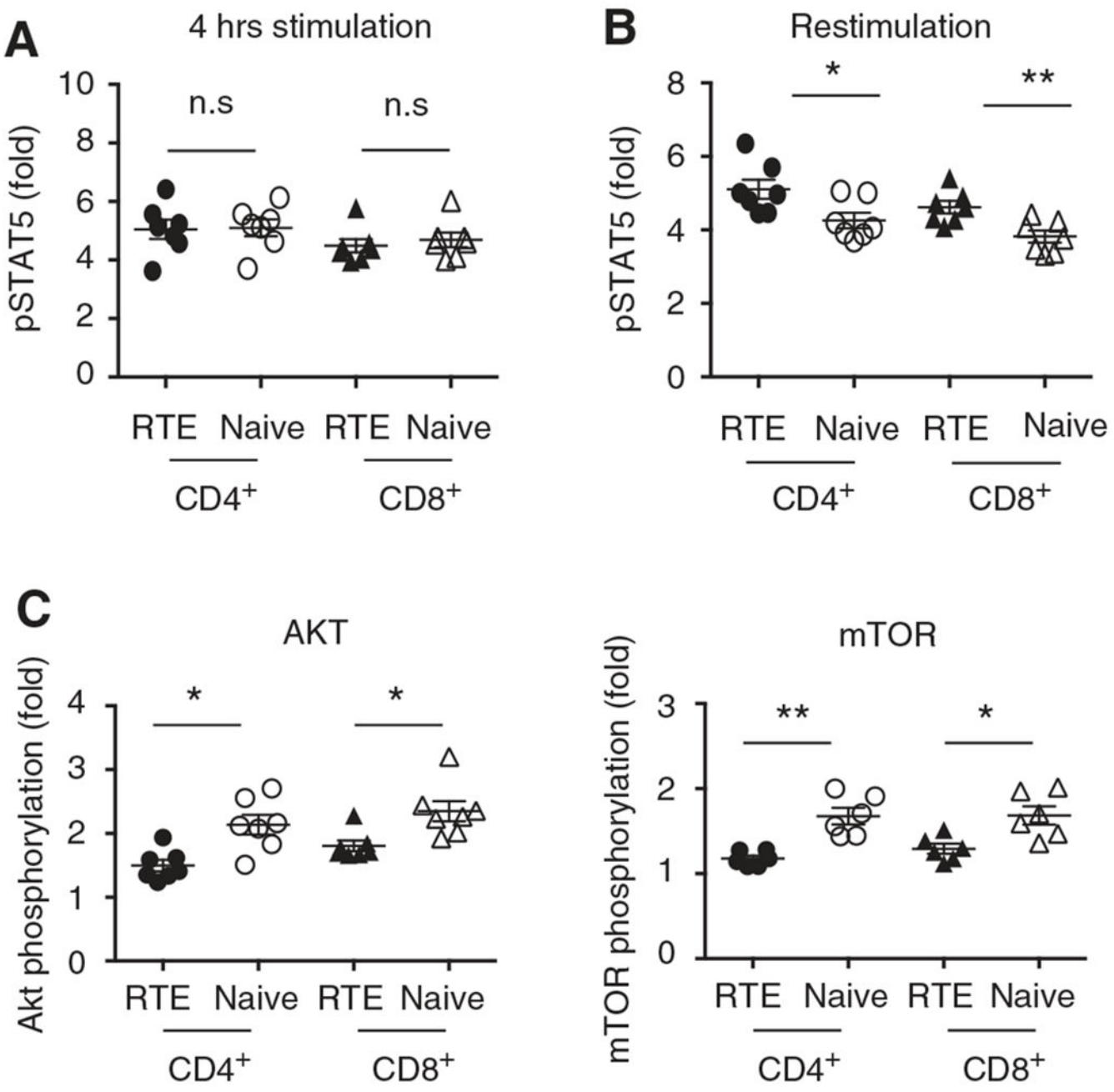

Figure 5.

Differential effects of IL-7 signaling in RTE and naïve T cells. (A) Intracellular pSTAT5 level was determined in *Rag2*-GFP-Tg RTE and naïve T cells stimulated continuously for 4 h, and shown as fold increase over medium. Each circle represents one independent experiment. (B) Intracellular pSTAT5 level was determined upon IL-7 restimulation in RTE and naïve T cells that had been rested for 2 h after IL-7 prestimulation for 4 h. Each circle represents one independent experiment. (C) Phosphorylated Akt and mTOR contents were determined in fixed and permeabilized RTE and naïve T cells that were stimulated with IL-7

(10 ng/mL) for 16 h. Each circle represents one independent experiment, p values were determined by Student's t-test. *$p < 0.05$, **$p < 0.01$.

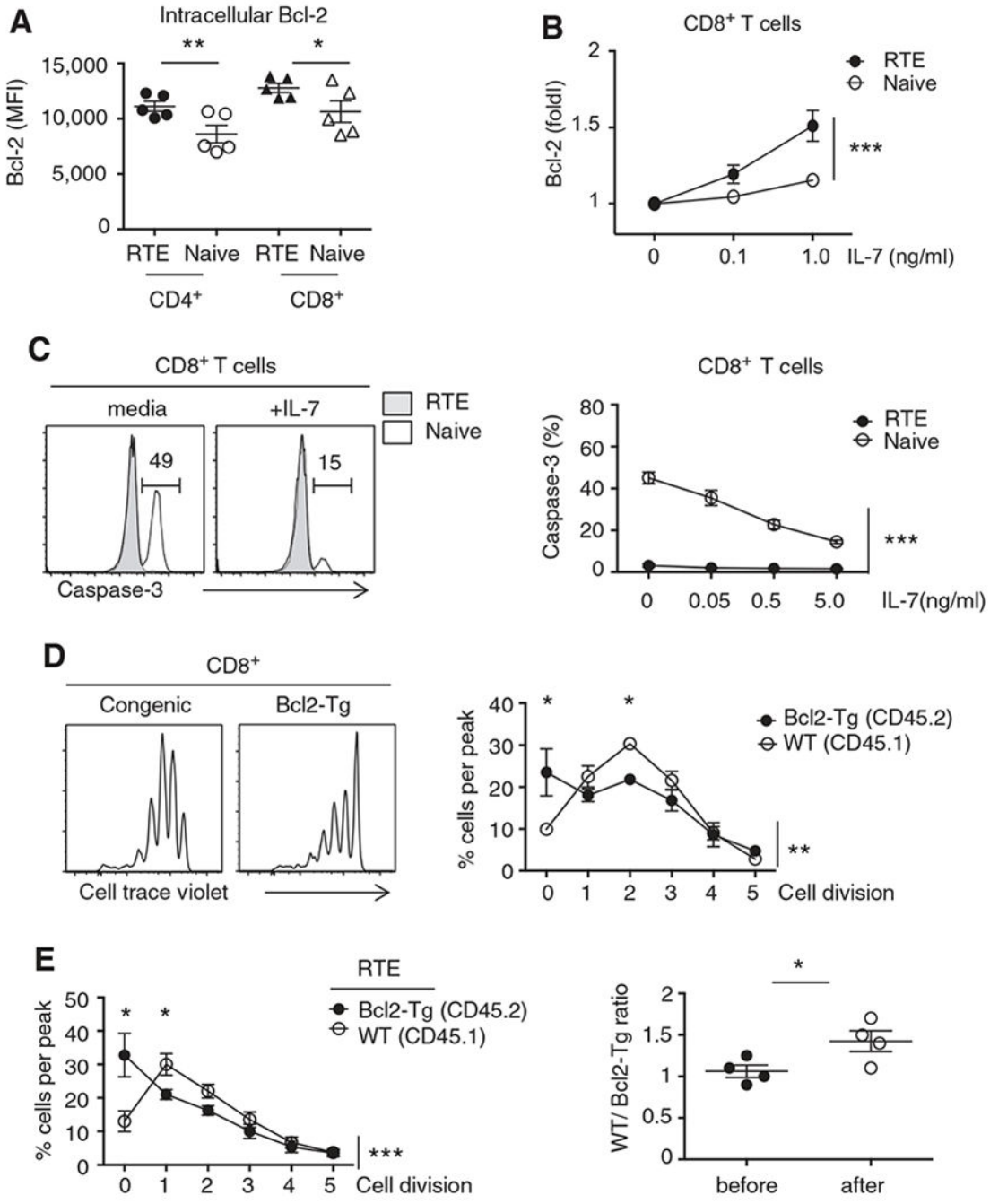

Figure 6.

Increased anti-apoptotic activity in RTE compared to naïve T cells upon IL-7 signaling. (A) Freshly isolated LN T cells from *Rag2*-GFP-Tg mice were assessed for intracellular Bcl-2 protein expression on RTE and naïve T cells. Results are the summary of five independent experiments. p values were determined by Student's t-test. < 0.05, $^{**}p < 0.01$. (B) Intracellular Bcl-2 was assessed after overnight stimulation with the indicated concentration of IL-7 in RTE and naïve T cells from *Rag2*-GFP-Tg mice. Bcl-2 expression was quantified as MFI and was normalized to levels in medium treated cells, which was set to 1. Results are

the summary of five independent experiments. *p* values were determined by two-way ANOVA. ***$p < 0.001$. (C) Increased anti-apoptotic activity in RTE cells. Apoptosis was assessed by caspase-3 activity (CaspGLOW Red) in *Rag2*-GFP-Tg T cells stimulated overnight in medium or IL-7 (left). Graph shows decrease in active Caspase-3 content upon increasing concentration of IL-7 (right). Results are representative of four independent experiments. *p* values were determined by two-way ANOVA. ***$p < 0.001$. (D) Bcl-2 impairs donor cells proliferation in *Rag2*-KO host mice. Distinct kinetics of WT and Bcl2-Tg donor T-cell proliferation in *Rag2*-KO host mice as shown by CTV dilution (left). Distribution of proliferated donor cells per cell division was assessed as % cells for each peak of CTV dilution (right). Results show summary of three independent experiments with a total of three host mice. *p* values were determined by Student's *t*-test and two-way ANOVA. *$p < 0.05$, **$p < 0.01$. (E) Competitive homeostatic proliferation of WT and Bcl2-Tg RTE cells. Electronically sorted RTE from WT and Bcl2-Tg mice were adoptively transferred into *Rag2*-KO host mice and assessed for proliferation. Distribution of proliferated donor cells per cell division was assessed as % cells for each peak of CTV dilution (left). Recovery ratio of WT versus Bcl2-Tg donor origin cells were assessed before and after adoptive transfers (right). Results show summary of four independent experiments with a total of four host mice. *p* values were determined by Student's *t*-test and two-way ANOVA. *$p < 0.05$, ***$p < 0.001$.