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The abundance and availability of cytokine receptor IL-2Rβ (CD122) constrain the lymphopenia-induced homeostatic proliferation of naïve CD4 T cells

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Abstract

Lymphopenia-induced homeostatic proliferation (LIP) is a critical mechanism for restoring T cell immunity upon lymphodepleting insults or infections. LIP is primarily driven by homeostatic cytokines, such as IL-7 and IL-15, but not all T cells respond with the same efficiency to homeostatic proliferative cues. While CD8 T cells vigorously proliferate under lymphopenic conditions, naïve CD4 T cells are substantially impaired in their response to homeostatic cytokines, and they fail to fully expand. Here, we show that the availability of IL-2Rβ (CD122), which is a receptor subunit shared by IL-2 and IL-15, affects both the cytokine responsiveness and the LIP of naïve CD4 T cells in the mouse. The enumeration of surface IL-2Rβ molecules on murine naïve CD4 and naïve CD8 T cells revealed a five-fold difference in IL-2Rβ abundance. Notably, it was the limited availability of IL-2Rβ that impaired CD4 T cell responsiveness to IL-15 and suppressed their LIP. As such, forced IL-2Rβ expression on CD4 T cells by transgenesis bestowed IL-15 responsiveness onto naïve CD4 T cells, which thus acquired the ability to undergo robust LIP. Collectively, these results identify IL-2Rβ availability as a new regulatory mechanism to control cytokine responsiveness and the homeostatic proliferation of murine CD4 T cells.

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Author contributions

CH and JP conceived the project. CH, YJ, HRK, and HKK performed the experiments. HRK, HKK, YJ, RG, CH, and JP analyzed the data. CH and JP wrote the manuscript.

Disclosure

The authors declare no competing interests.

Keywords

Adoptive transfer; Cytokines; Homeostasis; IL-15; Proliferation

Introduction

Homeostatic proliferation (HP) is a powerful mechanism that maintains or restores the size and diversity of the peripheral T cell pool (1–3). In contrast to antigen-driven proliferation which is triggered by agonistic TCR stimulation, HP is mostly driven by cytokine receptor signaling together with tonic TCR signaling (4). It is understood that homeostatic cytokines such as interleukin-7 (IL-7) and interleukin-15 (IL-15) play critical roles in the process of HP (3, 5). Because T cells only consume and do not produce IL-7 or IL-15 (6, 7), the availability of homeostatic cytokines is primarily controlled by the amount of cytokines produced by non-T cells and by the rate in which T cells consume these cytokines (8, 9). Consequently, an increase in T cell numbers would result in decreased availability of homeostatic cytokines because the consumption of homeostatic cytokines would be increased. However, a decrease in T cell numbers would conversely increase the *in vivo* availability of IL-7 and IL-15, resulting in the expansion of the peripheral T cell pool (6, 8, 10, 11). In extreme cases, in which T cells are abolished, such as in chronic lymphopenic *Rag*-deficient mice or upon acute loss of T cells by sublethal irradiation, the amount of homeostatic cytokines is elevated to a level that they can initiate and drive the proliferation of adoptively transferred T cells (1, 3, 4). This event is commonly referred to as lymphopenia-induced homeostatic proliferation (LIP) (2, 12), and LIP forms the molecular basis for the homeostatic maintenance of T cell immunity upon lymphodepleting insults by viral infections or cytoreductive regimens in clinical settings (13–15).

Notably, CD4 and CD8 T cells are distinct in their proliferative response to a lymphopenic environment; that is, CD8 T cells are highly effective in LIP and proliferate vigorously, whereas naïve CD4 T cells are significantly less efficient and poorly expand upon adoptive transfer into lymphopenic host mice (10, 16). This difference in CD4 and CD8 T cell expansion has also been documented in humans, where CD4 T cell reconstitution significantly lags behind CD8 T cell reconstitution in allogeneic stem cell transplants, for example (17, 18). While the function of the thymus, the thymic output, and restoring the peripheral lymphoid niche all play important roles in post-transplant immune reconstitution (19), it remains unclear whether T cell intrinsic or extrinsic factors cause the distinct proliferative response in CD4 and CD8 T cells. Multiple scenarios have been proposed to explain the underlying mechanism behind the differences in CD4 and CD8 T cell response (10, 20, 21). However, the common thread that runs through these models indicates that differences in cytokine responsiveness or utilization may be major factors that account for the discrepancies (17, 22, 23).

Both IL-7 and IL-15 are members of a family of cytokines that share the common γ-chain receptor (γc) for ligand binding and signaling and are collectively known as γc family cytokines (24). While both IL-7 and IL-15 utilize γc and induce the phosphorylation of STAT5 and PI-3 kinase, they also display distinct effects on T cell function. IL-7 utilizes the

proprietary IL-7Rα to transduce critical survival signals for T cells (25).While all T cells express the IL-7 receptor, IL-7 signaling itself is insufficient to drive T cell proliferation (4). IL-15, on the other hand, is a potent driver of proliferation, but curiously, it acts only on CD8 T cells, not on naïve CD4 T cells (7, 20). This difference in IL-15 responsiveness has been previously proposed to underlie the distinct homeostatic and effector functions of CD8 and CD4 T cells (9, 20, 26–28). Mechanistically, it has been proposed that differences in membrane lipid raft content in CD4 and CD8 T cells account for the weak proliferative potential of CD4 T cells (20). According to this model, larger amounts of the ganglioside GM1 are found in CD8 T cell plasma membranes than in CD4 T cell membranes. GM1-enriched microdomains, on the other hand, equip CD8 T cells to respond effectively to IL-15 which depends on the IL-2Rβ cytokine receptor for signaling (20).

Here, we re-examined the mechanism of LIP by hypothesizing that, independent of lipid raft content, the availability of cytokine receptors serves as a parameter to control cytokine responsiveness and the homeostatic proliferation of T cells. By employing IL-2Rβ-transgenic T cells, we found that IL-15 responsiveness is primarily controlled by the availability of IL-2Rβ, which is a receptor subunit shared by IL-2 and IL-15. Specifically, we identified that the limited availability of IL-2Rβ is the determining factor in constraining the LIP of naïve CD4 T cells. These results reveal a newly identified regulatory mechanism of T cell homeostasis that is controlled by tuning the abundance and availability of homeostatic cytokine receptors.

Materials and Methods

Mice

C57BL/6 (B6) and congenic C57BL/6.SJL (CD45.2) mice of both sexes were obtained from the Charles River Laboratories (Frederick, MD). IL-2Rβ-deficient (IL-2Rβ$^{-/-}$) and RAG2$^{-/-}$ mice were purchased from the Jackson Laboratory. Foxp3-EGFP reporter mice were kindly provided by Dr. V.K. Kuchroo (Harvard University) (29). Animal experiments were performed with 6–12-week old mice of both sexes. Mice overexpressing IL-2Rβ driven by the human *CD2* promoter (IL-2RβTg) have been previously described (30). Animal experiments were approved by the NCI Animal Care and Use Committee. All mice were maintained in accordance with NIH guidelines.

Assessing intracellular Bcl-2 and pSTAT5 levels

CD44^{lo} naïve lymph node (LN) T cells from WT and IL-2RβTg mice were stimulated with IL-2, IL-7 or IL-15 (PeproTech) for 3 or 4 days and assessed for intracellular Bcl-2 contents using a Foxp3 staining kit in accordance with the manufacturer's instructions (eBioscience, ThermoFisher). To determine the extent of cytokine-induced STAT5 phosphorylation, LN T cells from WT, IL-2Rβ$^{-/-}$ and IL-2RβTg mice were treated with IL-2, IL-7 or IL-15 for 30 minutes, and phospho-STAT5 (pSTAT5) levels were determined by methanol/acetone fixation and intracellular staining as previously described (30).

Flow cytometry

Flow cytometry data were acquired with a LSR Fortessa, X-20, or LSR II flow cytometer (BD Biosciences). Live cells were identified by using forward scatter exclusion of dead cells stained with propidium iodide. Cell fixation and permeabilization were performed using a Foxp3 Transcription Factor Staining Buffer kit according to the manufacturer's instructions (Thermo Fisher eBioscience). Antibodies with the following specificities were used for staining: HSA (30-F1), Foxp3 (FJK-16), TCRβ (H57–597), IL-7Rα (A7R34), CD45.1 (Ly5.1; A20), CD45.2 (Ly5.2; 104), CD44 (IM7), NK1.1 (PK136), and NKp46 (29A1.4) from Thermo Fisher eBioscience; γc (4G3), CD4 (GK1.5 and RM4.5), CD25 (PC61.5), pSTAT5 (pY694), CD62L (MEL-14), CD122 (TMβ1), CD8α (53–6–7), and CD5 (53–7.3) from BD Biosciences (San Jose, CA); and Bcl-2 (BCL/10C4), Helios (22F6) from BioLegend (San Diego, CA). For Foxp3 and Helios staining, the Foxp3 intracellular staining buffer set was used according to the manufacturer's instructions (Thermo Fisher eBioscience). GM1 content in membrane lipid rafts was visualized with recombinant cholera toxin subunit B conjugated to AlexaFluor-647 (Sigma C34778). TCR Vβ distribution in CD4 T cells was assessed using anti-mouse TCR Vβ screening panel antibody kits (BD Pharmingen).

Intracellular phospho-CD3ζ levels

To assess intracellular phospho-CD3ζ (CD247) contents, LN cells were resuspended at 4×10^6 cells/mL in serum-free media and either analyzed freshly or after stimulation with phorbol myristate acetate (PMA, 25 ng/mL) for 20 minutes at 37°C. The cells were then fixed and permeabilized using the IC fixation kit (Thermo Fisher eBioscience) and stained with the fluorescence-conjugated anti-mouse phospho-CD3ζ (Tyr142) antibody (3ZBR4S, Thermo Fisher eBioscience) according to the manufacturer's recommendations.

In vitro *T cell proliferation assay*

Naïve $CD44^{lo}$ LN T cells were labeled with Cell Trace Violet (Thermo Fisher) and incubated with recombinant IL-2 (0.5 μg/ml), IL-7 (0.5 μg/ml) or IL-15 (0.5 μg/ml) for 5 days. Cell division was assessed by flow cytometry for Cell Trace Violet dye dilution per the manufacturer's recommended protocol (Thermo Fisher).

In vivo *homeostatic proliferation assays*

CD4 LN and splenic T cells were harvested and isolated from congenic $CD45.1^+$ B6 mice and $CD45.2^+$ IL-2RβTg mice by cell sorting using flow cytometry. $CD45.1^+$ WT and $CD45.2^+$ IL-2RβTg CD4 T cells were mixed at a 1:1 ratio, and a total of 10 million CD4 T cells were adoptively transferred into lymphopenic $RAG2^{-/-}$ host mice by retro-orbital injection. After 5 days, LNs and spleens were harvested from the host mice, and the homeostatic proliferation of donor CD4 T cells was assessed by Cell Trace Violet (Thermo Fisher) fluorescence dye dilution. For anti-IL-2 antibody treatment (α-IL-2), the host mice were preconditioned with an i.p. injection of 150 μg of α-IL-2 mAb clone S4B6–1 and 150 μg of α-IL-2 mAb clone JES6–1A12 (both from BioXCell) (31) 2 days before donor T cell transfer. This α-IL-2 injection regimen was then continued every other day until the mice were analyzed.

For the analysis of Foxp3$^+$ Treg cell survival, CD4 T cells were isolated from the pooled LNs and spleens of Foxp3-GFP reporter mice (29). They were then adoptively transferred into pre-treated (either with α-IL-2 or PBS control) RAG2$^{-/-}$ host mice by retro-orbital injection on day 0, and then harvested and analyzed on day 5.

Surface cytokine receptor quantification by flow cytometry

Naïve CD4 T cells were identified by gating on CD4$^+$, TCRβ$^+$, and CD44^{lo} LN cells. The mean fluorescence intensity (MFI) was calculated by subtracting the MFI value of the isotype control for each cytokine receptor antibody staining. A calibration curve correlating the fluorescence measured by flow cytometry to the number of phycoerythrin (PE) molecules was established using Quantum PE molecules of equivalent soluble fluorochrome calibration beads (Bang Laboratories). The calibration curve was then employed to calculate the number of equivalent soluble fluorochromes corresponding to each corrected MFI value, as previously described (32), and the average number of bound antibodies per cell was determined. The fluorescence intensity measurements and calculations were repeated three times.

Statistics

Statistical differences were analyzed by paired two-tailed Student's *t*-test. The data represent the means ± SEM. *P*-values less than 0.05 were considered significant: **P*<0.05, ** *P* <0.01, *** *P* <0.001. All statistical analyses were performed using GraphPad Prism 7 software.

Results

Distinct responsiveness of naïve CD4 and CD8 T cells to IL-15

To demonstrate differences in their cytokine responsiveness, we stimulated naïve CD4 and CD8 T cells with the homeostatic cytokines IL-7 or IL-15, and then assessed their proliferation *in vitro*. Cell proliferation was visualized by Cell Trace Violet (CTV) dilution of cytokine-stimulated CD4 and CD8 T cells (22). IL-7 stimulation provided potent survival signals to both CD4 and CD8 T cells, as demonstrated by the increased amounts of Bcl-2 (Fig. 1A). However, it failed to induce the proliferation of either CD4 or CD8 T cells (Fig. 1B), a finding that can be explained by the IL-7 signaling-induced downregulation of IL-7Rα expression (Supplementary Fig. 1A, **top**) (25). Because IL-7 stimulation results in the downregulation of its own receptor, IL-7 signaling is rapidly terminated upon IL-7 stimulation (25). Notably, it is the lack of prolonged and persistent IL-7 signaling that interferes with T cell proliferation (4). IL-15 stimulation, on the other hand, induced vigorous proliferation of naïve CD8 T cells (Fig. 1B), confirming its function as a strong proliferative cue for T cells (33, 34). In contrast to CD8 T cells, however, naïve CD4 T cells were refractory to IL-15 stimulation. CD4 T cells did not downregulate IL-7Rα upon IL-15 stimulation (Supplementary Fig. 1A, **bottom**), and they failed to undergo IL-15-induced proliferation (Fig. 1B). These results reaffirm previous observations that naïve CD4 and CD8 T cells differ in their responsiveness to IL-15 (30, 35).

Previously, it was proposed that different levels of lipid raft abundance underlie the distinct homeostatic proliferative potentials of naïve CD4 and CD8 T cells (20). Lipid rafts promote

cytokine receptor signaling (36), and CD8 T cells contain significantly greater amounts of lipid rafts than CD4 T cells (20). Indeed, plasma membrane staining of GM1 ganglioside by fluorescence-conjugated cholera toxin B showed significantly higher GM1 levels in CD8 T cells than in CD4 T cells (Fig. 1C). GM1 is commonly used to report the abundance of membrane lipid rafts (20), and our observation agrees with earlier reports documenting that GM1 levels are higher in CD8 T cells (37). Additionally, memory-phenotype CD8 T cells ($CD44^{hi}$), which are better IL-15 responders than naïve CD8 T cells ($CD44^{lo}$) had significantly greater GM1 levels than naïve CD8 T cells (Supplementary Fig. 1B). Thus, the amount of GM1 correlated with IL-15 responsiveness.

However, memory CD8 T cells not only differ in their GM1 content but also in the expression of IL-2Rβ, reportedly showing higher levels of IL-2Rβ than that of naïve CD8 T cells, a finding we have confirmed (38) (Supplementary Fig. 1B). IL-2Rβ is critical for the binding and signaling of both IL-2 and IL-15 (39), and the abundance of IL-2Rβ proteins determines the magnitude of both IL-2 and IL-15 signaling (40). Consequently, the disparate expression of cytokine receptors may be the direct cause of distinct cytokine responsiveness in naïve CD4 and naïve CD8 T cells. Assessment of the surface cytokine receptor expression on naïve CD4 T cells indicated that IL-2Rβ was minimally expressed, as also previously documented in the seminal study by Sprent and colleagues (Supplementary Fig. 1B and Fig. 1C) (35). In fact, an enumeration of IL-2Rβ molecules on naïve CD4 T cells revealed a remarkable lack of functional IL-2 receptors, *i.e.* IL-2Rβ/γc, on naïve CD4 T cells (Fig. 1D). CD8 T cells, on the other hand, expressed substantial numbers of IL-2Rβ protein (Fig. 1D), as previously reported (35). Collectively, these results suggest a scenario in which the distinct IL-15 responsiveness in CD4 and CD8 T cells may be the result of the differential abundance of IL-2Rβ molecules and not because of – or in addition to – the distinct lipid raft composition of CD4 and CD8 T cells.

Enforced IL-2Rβ expression on CD4 T cells

To determine whether the limited availability of IL-2Rβ constrains IL-15 signaling, we asked if enforced IL-2Rβ expression could confer IL-15 responsiveness to naïve CD4 T cells. To this end, we analyzed IL-2Rβ transgenic mice (IL-2RβTg) that express a murine *Il2rb* cDNA under the control of human *CD2* promoter/enhancer gene regulatory elements (30). Enforced IL-2Rβ expression did not affect overall T cell development in the thymus, so that total thymocyte numbers remained similar to those in the WT mice (Supplementary Fig. 2A). Additionally, the CD4 versus CD8 profiles of both total and mature ($TCR\beta^{hi}$ HSA^{low}) thymocytes did not show significant differences from the profile of the WT controls (Fig. 2A). IL-2Rβ expression, however, was dramatically increased on IL-2RβTg thymocytes, including mature CD4 single-positive (CD4SP) thymocytes which are normally devoid of IL-2Rβ (Fig. 2B). Notably, IL-2Rβ overexpression did not alter T cell development or CD4 versus CD8 lineage choice in the thymus. This finding was consistent for different IL-2RβTg founder lines (Supplementary Fig. 2B **and** 2C). Importantly, the increased abundance of IL-2Rβ did not translate into increased IL-15 signaling *in vivo*. These results agree with the observation that IL-15 expression in the thymus is limited (7), and indicate that IL-15 signaling in thymocytes may be primarily controlled by the availability of the ligand and not that of the receptor.

In marked contrast to the thymus, T cells in peripheral lymphoid tissues were significantly affected by the forced IL-2Rβ expression. An assessment of the CD4 and CD8 profiles of the LN T cells revealed substantial skewing toward CD4 T cells, as visualized by the dramatically decreased CD8 versus CD4 ratio in peripheral T cells (Fig. 2C). This decrease in CD8 T cell frequencies was accompanied by a marked loss in CD8 LN T cell numbers (Fig. 2D) and, more prominently, a decrease in $CD44^{lo}$ naïve CD8 T cell frequency (Supplementary Fig. 2D). Consequently, the naive ($CD44^{lo}$) to memory ($CD44^{hi}$) ratio was substantially decreased in IL-2RβTg LN T cells (Fig. 2E). These results suggested that the increased IL-2Rβ abundance was detrimental to the survival or homeostasis of naïve CD8 T cells. Alternatively, there remains the possibility that enforced IL-2Rβ expression may have triggered ectopic IL-15 signaling in naïve CD8 T cells. IL-15 signaling of naïve CD8 T cell may have driven their differentiation into memory-phenotype or virtual memory CD8 T cells (28, 33), resulting in the loss of naïve CD8 T cells as they entered the memory T cell pool. These two scenarios are not mutually exclusive, and future investigations are required to fully understand the underlying mechanism.

IL-2Rβ expression promotes IL-15 signaling in CD4 T cells

Because we did not observe any significant changes in the number of LN CD4 T cells in the IL-2RβTg mice (Fig. 2D), we next asked whether IL-2Rβ overexpression affects the generation of CD4 T cells that depend on IL-2Rβ expression (41). $Foxp3^+$ $CD4^+$ T regulatory (Treg) cells are potent immune suppressers that are primarily generated in the thymus, but also arise in peripheral tissues (42). IL-2Rβ expression is critical for the generation and homeostasis of $Foxp3^+$ Treg cells (43). However, increased IL-2Rβ availability did not alter the frequency or phenotype of $Foxp3^+$ Treg cells (Fig. 3A) (44). Collectively, despite expressing a markedly increased amount of surface IL-2Rβ proteins (Fig. 3B **and** Supplementary Fig. 3A), IL-2RβTg CD4 T cells remained largely unaffected in terms of their differentiation and homeostasis *in vivo*.

To demonstrate that increased IL-2Rβ expression promotes IL-2R signaling, we stimulated LN T cells of WT and IL-2RβTg mice with IL-15 or IL-2 *in vitro*, and assessed their intracellular phospho-STAT5 content. IL-15 and IL-2 share and require IL-2Rβ for ligand binding and signaling (24, 45). Consequently, stimulation with either IL-15 or IL-2 triggered robust STAT5 phosphorylation in CD8 LN T cells, and this effect was abrogated when IL-2Rβ was genetically deleted (Fig. 3C **and** Supplementary Fig. 3B). On the other hand, WT CD4 T cells were refractory to both IL-15 and IL-2 stimulation (Fig. 3C **and** Supplementary Fig. 3B), demonstrating that naïve CD4 T cells are impaired in their ability to respond to cytokines that utilize the IL-2Rβ chain. The small subset of IL-2-and IL-15-responsive cells among CD4 T cells correspond to $Foxp3^+$ Treg cells, which constitutively express IL-2Rβ (Fig. 3C **and** Supplementary Fig. 3B) (41).

Strikingly, enforced IL-2Rβ expression on CD4 T cells enabled effective IL-15 and IL-2 signaling in these cells, as illustrated in the STAT5 phosphorylation downstream of IL-15 and IL-2 stimulation (Fig. 3C **and** Supplementary Fig. 3B). Such IL-2/IL-15 responsiveness of the IL-2RβTg CD4 T cells was acquired despite a low level of GM1 expression compared with that of CD8 T cells (Fig. 3D). These results dissociate GM1 content as a controlling

factor from IL-2/IL-15 signaling, particularly in cells where IL-2Rβ availability is not limiting.

Lymphopenia-induced proliferation of adoptively transferred CD4 T cells

In vitro cytokine stimulation demonstrated that the increased IL-2Rβ abundance is sufficient to endow CD4 T cells with IL-15 responsiveness (Fig. 3C). Nonetheless, we failed to see substantial changes in the CD4 T cell population *in vivo*, indicating that IL-2R signaling was not altered under steady-state conditions *in vivo*. These results are in line with observations indicating that the amount of homeostatic IL-15 is limiting *in vivo* (7) and that IL-2 production is also scarce and only locally available (46). Collectively, these data suggest that increased IL-2Rβ expression does not promote *in vivo* IL-2/−15 receptor signaling under normal steady-state conditions, presumably because it is the availability of the cytokine, not that of the cytokine receptor, that is limiting for T cells during homeostasis.

However, these data also predict that an increase in IL-2Rβ expression would be advantageous when homeostatic cytokines are not limited but abundant. This is precisely the case in severe T cell lymphopenia in which T cells that consume homeostatic cytokines are absent, and thus, the availability of homeostatic cytokines is dramatically increased (33). To test this prediction, we set up adoptive T cell transfer experiments in which WT and IL-2RβTg naïve CD4 T cells were FACS-sorted and co-injected into *Rag2*-deficient ($RAG2^{-/-}$) lymphopenic host mice and recovered 5 days after adoptive transfer (22). To monitor their proliferation, donor T cells were labeled with Cell Trace Ciolet (CTV) before adoptive transfer, and to distinguish the donor-origin of the injected T cells, we utilized CD45.1 (WT) and CD45.2 (IL-2RβTg) congenic markers. Five days after transfer, we recovered donor T cells from LNs and spleen and found preferential accumulation of IL-2RβTg CD4 T cells (Fig. 4A). It is well established that naïve CD4 T cells undergo inefficient LIP upon adoptive transfer, but the reason remains unclear (10, 27). The analysis of CTV dilution of WT and IL-2RβTg CD4 T cells revealed that IL-2Rβ availability plays a critical role in controlling LIP (Fig. 4B), as indicated by the vigorous proliferation of the IL-2RβTg-derived CD4 T cells but not of the WT-origin CD4 T cells under the same lymphopenic conditions (Fig. 4B). Thus, the increased abundance of IL-2Rβ was sufficient to drive the proliferation of donor CD4 T cells (Fig. 4B), which was also illustrated in the dramatically accelerated kinetics of IL-2RβTg donor T cell proliferation compared to WT cell proliferation (Fig. 4C). Notably, the increased IL-2Rβ availability potentiated the LIP of CD4 T cells without showing an increase in GM-1 content (Supplementary Fig. 3C). In fact, in a competitive environment with co-transferred CD8 T cells, IL-2RβTg CD4 T cells underwent LIP as efficiently as WT CD8 T cells, indicating that the cellular abundance of IL-2Rβ is a significant contributing factor in homeostatic proliferation that acts independently of GM1 levels (Supplementary Fig. 4A).

Additionally, to exclude the possibility that the LIP of IL-2RβTg T cells is driven by TCR and not by homeostatic cytokines, we assessed the TCR Vβ repertoire of WT and IL-2RβTg CD4 LN T cells. Using TCR Vβ screening panel kits (Becton Dickinson), we determined the distribution of 15 different Vβ segments among CD4 T cells, and found that the TCR Vβ repertoire of IL-2RβTg CD4 T cells was polyclonal and comparable to that of WT CD4 LN

T cells (Fig. 4D). Thus, we consider it unlikely that IL-2Rβ overexpression would have altered thymic selection to generate an oligoclonal repertoire with TCR-specifities that would expand and vigorously proliferate under lymphopenic conditions. In agreement with this explanation, the abundance of surface CD5 protein, which is a widely used marker for TCR signaling strength (47), was comparable between WT and IL-2RβTg CD4 T cells (Fig. 4E **and** Supplementary Fig. 4B). In addition, steady-state phosphorylation levels of CD3ζ, which can indicate the strength of tonic TCR signaling, also did not differ between WT and IL-2RβTg cells (48) (Fig. 4F **and** Supplementary Fig. 4C). Altogether, these results further bolster the argument that the increased abundance of IL-2Rβ, and not the quantity or quality of TCR, drives the LIP of naïve IL-2RβTg CD4 T cells.

IL-15-induces the proliferation of IL-2RβTg CD4 T cells

Because IL-2Rβ is shared by IL-2 and IL-15, we also sought to confirm that it is the homeostatic force of IL-15, and not that of IL-2, that drives LIP in IL-2RβTg naïve CD4 T cells. To this end, we treated RAG2$^{-/-}$ host mice with neutralizing anti-IL-2 antibodies during the course of 5-day LIP and assessed the proliferation of IL-2RβTg CD4 donor T cells (Supplementary Fig. 4D). We first confirmed the bioactivity of the anti-IL-2 antibodies by setting up adoptive transfer experiments where Foxp3^{+} donor T cells were transferred into RAG2$^{-/-}$host mice (Fig. 5A). Foxp3^{+} Treg cells depend on IL-2 signaling for their survival (43, 49); therefore IL-2 neutralization was expected to result in a specific loss of Foxp3^{+} cells. Indeed, the adoptive transfer of CD4 LN T cells from Foxp3-EGFP reporter mice led to a substantial decrease in Foxp3^{+}CD25^{+} Treg cell frequency in the host mice treated with anti-IL-2 antibodies (Fig. 5A). The same anti-IL-2 antibody treatment, however, did not impair the LIP of IL-2RβTg CD4 T cells (Fig. 5B), indicating that the homeostatic proliferation of IL-2RβTg cells is independent of IL-2.

To directly demonstrate that IL-15 drives the proliferation of IL-2RβTg CD4 T cells, we stimulated and cultured naïve CD4 T cells *in vitro* with recombinant IL-15 proteins. Here, we found that IL-15 can indeed trigger naïve CD4 T cell proliferation, but the IL-15-driven proliferation was specific to the IL-2RβTg T cells because WT CD4 T cells remained refractory to IL-15 stimulation (Fig. 5C). Collectively, these results demonstrate a previously unappreciated role of IL-2Rβ availability in constraining the IL-15/IL-2 cytokine responsiveness of naïve CD4 T cells and in limiting the homeostatic potential of CD4 T cells under lymphopenic conditions.

Discussion

Naïve CD4 T cells and CD8 T cells are distinct in their homeostatic proliferative potential, so that naïve CD4 T cells poorly expand whereas CD8 T cell vigorously proliferate under lymphopenic conditions. Multiple mechanisms have been proposed to explain this difference, and they may act redundantly or additively (10, 20, 21, 27, 50). Our current study reports a role for the IL-2Rβ-chain cytokine receptor in constraining lymphopenia-induced proliferation (LIP) of CD4 T cells, thus unveiling a previously unappreciated aspect of IL-2/IL-15 signaling in controlling homeostatic proliferation. Notably, quantification assays documented that IL-2Rβ protein abundance in CD4 and CD8 naïve T cells was dramatically

different, resulting in their distinct proliferative responses to homeostatic IL-15 signaling. Enforced IL-2Rβ expression on CD4 T cells, however, reversed this defect, and naïve CD4 T cells underwent efficient homeostatic expansion when they were adoptively transferred into lymphopenic host mice. Hence, limiting the abundance and availability of IL-2Rβ is a previously unappreciated mechanism to control the proliferation and expansion of naïve CD4 T cells under lymphopenic settings.

IL-2Rβ is a receptor subunit shared by IL-2 and IL-15, and it is essential for the ligand-binding and signal transduction of both cytokines (45). During T cell development in the thymus, IL-2Rβ is mostly absent on immature thymocytes, but it is present on post-selection thymocytes, primarily *i*NKT cells, CD8 T cells, and on Foxp3$^+$ Treg cells (43, 51). The molecular mechanisms that control IL-2Rβ expression have been extensively investigated (45), and multiple transcription factors, such as Ets1, T-bet, and eomesodermin, were found to induce its expression on T cells (52, 53). The cellular basis of the disparate IL-2Rβ expression observed for CD4 and CD8 naïve T cells, however, remains unclear. It is also unknown whether IL-2Rβ expression on naïve CD4 T cells would be detrimental and whether IL-2Rβ expression must be suppressed on naïve CD4 T cells to avoid undesired perturbations in T cell immunity. Hypothetically, ectopic expression of IL-2Rβ on naïve CD4 T cells can be both immune activating and immunosuppressing. IL-2Rβ may be immune activating because IL-2Rβ not only binds IL-15 but also IL-2. Thus, together with γc, IL-2Rβ transduces IL-2 signaling to trigger T cell activation and proliferation (45). Normally, CD4 T cells do not express IL-2Rβ, and consequently, they cannot respond to IL-2 unless stimulated by TCR, which in turn induces IL-2Rβ expression and confers IL-2 responsiveness (26). Thus, enforced IL-2Rβ expression on resting CD4 T cells potentially triggers IL-2 signaling in antigen-inexperienced T cells and could induce indiscriminate activation of CD4 T cells resulting in autoimmunity or uncontrolled inflammation (54). However, IL-2RβTg mice did not show signs of aberrant immune activation, as they contained normal fractions of naïve and memory CD4 T cells, as determined by CD44 and CD62L expression. Furthermore, peripheral CD4 T cell numbers did not significantly differ in the WT and IL-2RβTg mice, indicating that enforced IL-2Rβ expression did not promote expansion of the CD4 T cell pool. Collectively, these results do not support a scenario in which IL-2Rβ expression must be repressed on naïve CD4 T cells to avoid IL-2 signaling and to induce T cell activation, independent of TCR stimulation.

Alternatively, we also considered the possibility that IL-2Rβ is excluded from naïve CD4 T cells because it can amplify the generation of Foxp3$^+$ Treg cells, thus inducing undesired immune suppression. IL-2 signaling is a non-redundant requirement for the development of Foxp3$^+$ Treg cells in the thymus (45, 55), and increased IL-2Rβ availability may promote the IL-2-driven differentiation and expansion of Foxp3$^+$ CD4 T cells. Normally, IL-2Rβ expression is upregulated only upon strong agonistic TCR signaling during CD4 T cell development, and IL-2 signaling is necessary to protect Foxp3$^+$ CD4 T cells from cell death (56). Enforced IL-2Rβ expression may promote the selection and survival of developing Foxp3$^+$ Treg cells, and the exclusion of IL-2Rβ from conventional CD4 T cells may prevent the premature or increased IL-2 signaling of agonistically signaled T cells. However, we did not observe any noticeable changes in the frequency or number of Foxp3$^+$ Treg cells in IL-2RβTg mice, invalidating a scenario in which IL-2Rβ expression would be suppressed to

constrain Treg cell generation. Collectively, these results advocate against a role for IL-2Rβ to alter CD4 T cell function under steady-state conditions but imply that IL-2Rβ availability may be a critical factor under immune challenging conditions.

In this regard, lymphopenia represents a major challenge to the immune system. Under T-lymphopenic conditions, homeostatic mechanisms remediate the loss of T cells by increasing thymic output and triggering cell proliferation. Cytokines play critical roles in these processes, and particularly, the homeostatic cytokines IL-7 and IL-15 drive the homeostatic proliferation of peripheral T cells. However, it is not clear why CD4 and CD8 T cells differ in their responsiveness to the same homeostatic cytokines such that CD8 T cells, but not CD4 T cells, vigorously expand and reconstitute the T cell pool (12, 57). All CD4 T cells express the CD4 lineage-specifying transcription factor ThPOK which is absent in CD8 T cells (58). We previously documented that ThPOK induced the expression of Suppressor Of Cytokine Signaling-1 (SOCS-1) which is a potent inhibitor of cytokine signaling (59). Thus, we consider it likely that homeostatic cytokine signaling is suppressed in CD4 T cells because of their selective expression of ThPOK. However, ThPOK-deficiency abolishes the generation of CD4 T cells; therefore, it is difficult to assess a direct role for ThPOK in suppressing the LIP of CD4 T cells. Also, it was previously suggested that differences in plasma membrane lipid raft compositions may explain such differences (20). Because IL-2RβTg and WT CD4 T cells contain similar levels of GM1, and because ectopic expression of IL-2Rβ is sufficient to facilitate CD4 T cell proliferation, these data also demonstrate that the requirement for lipid rafts can be abrogated-when IL-2Rβ is available at sufficient amounts.

Cytokines control virtually every aspect of T cell immunity, including the development, survival, and effector function of T cells. Here, we document an unappreciated effect of controlling IL-2Rβ expression as a driver of homeostatic proliferation of CD4 T cells, presumably by controlling accessibility to IL-15 signaling. In contrast to that in CD8 T cells, the role of IL-15 in CD4 T cells remains mostly uncharted and only recently started getting addressed (30, 60). Homeostatic restoration of the CD4 T cell pool is critical for immune reconstitution upon lymphoablative regimens or T cell-depleting diseases. Our current findings provide new insights into the molecular basis of CD4 T cell proliferation and the utilization of IL-2Rβ expression in controlling this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key points

- Naïve CD4 T cells are impaired in lymphopenia-induced proliferation (LIP).
- Limited availability of IL-2Rβ impedes homeostatic IL-15 signaling in CD4 T cells.
- Forced IL-2Rβ expression permits effective LIP of naive CD4 T cells.

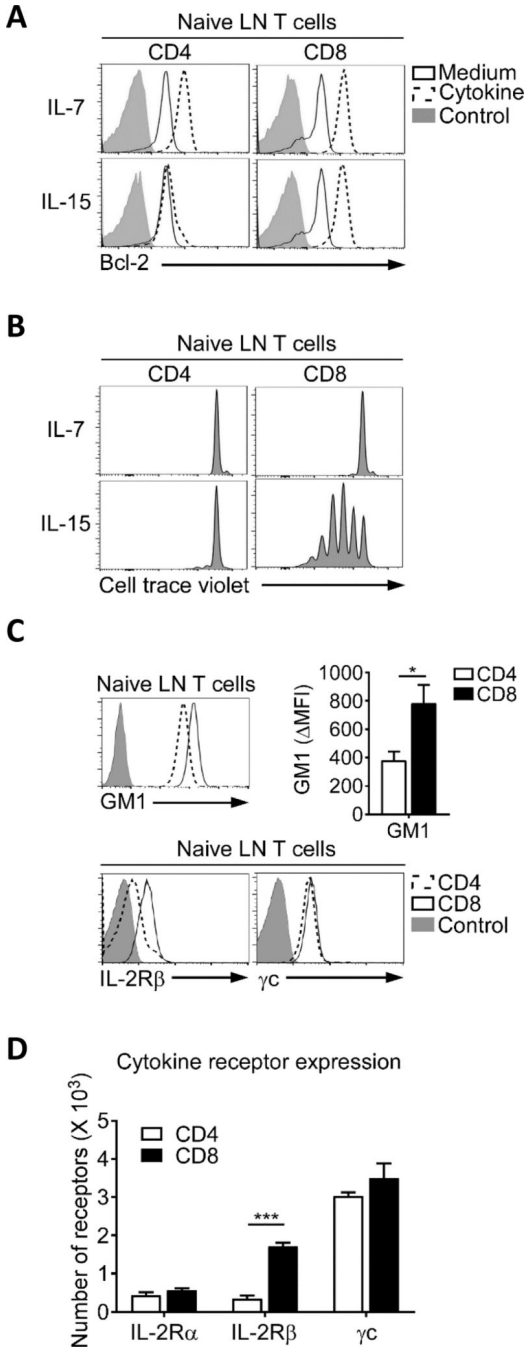

Figure 1. Cytokine-driven proliferation of naïve CD4 and CD8 T cells.

A. Intracellular Bcl-2 contents were assessed in naïve CD4 and CD8 T cells that were treated with IL-7 (10 ng/ml) or IL-15 (100 ng/ml) *in vitro* for 3 days. Results are representative of 7 independent experiments.

B. FACS-purified naïve CD4 and CD8 LN T cells were labeled with Cell Trace Violet, and stimulated with either recombinant IL-7 (0.5 μg/ml) or recombinant IL-15 (0.5 μg/ml) *in vitro* for 5 days. Results are representative of 3 independent experiments.

C. Freshly isolated CD4 and CD8 LN T cells were assessed for GM1 content, as visualized (histogram) and quantitated (bar graph) by cholera toxin B staining (top), and analyzed for IL-2Rβ and γc cytokine receptor expression (bottom). Results are representative of 3 independent experiments.

D. Enumeration of the IL-2Rα, IL-2Rβ and γc receptors on naïve CD4 and CD8 T cells using Quantum MESF beads (Bang Laboratories) and flow cytometry. Results show summary of 3 independent experiments.

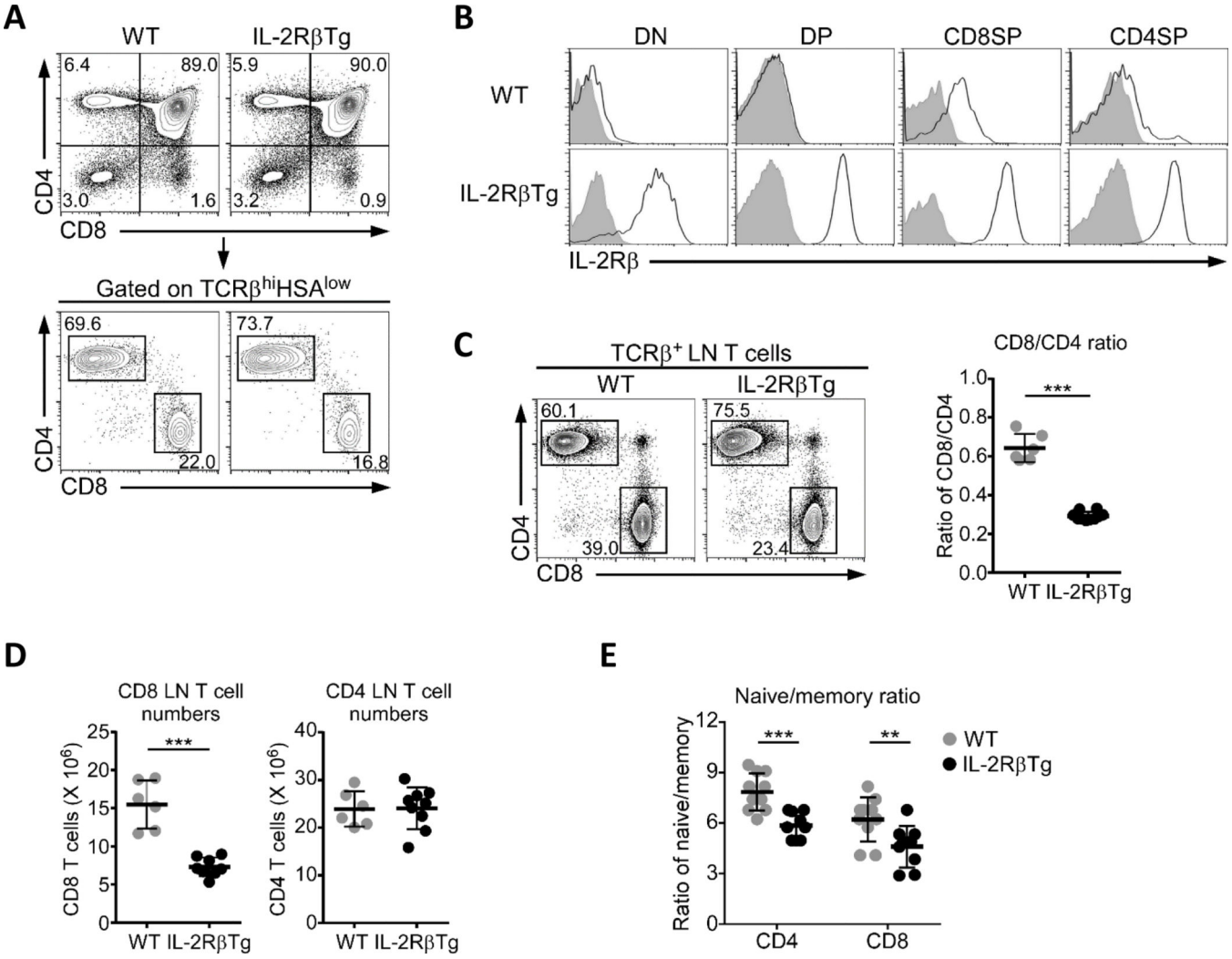

Figure 2. T cell development and differentiation in IL-2RβTg mice.

A. CD4 versus CD8 profiles of total thymocytes (top) and TCRβ^{hi}HSA^{low} mature thymocytes (bottom) of WT and IL-2RβTg mice. Results are representative of 5 independent experiments.

B. IL-2Rβ expression on WT and IL-2RβTg thymocyte subsets as defined by CD4 and CD8 coreceptor expression. Data are representative of 5 independent experiments.

C. CD4 versus CD8 profiles of TCRβ^{+} LN T cells of WT and IL-2RβTg mice (left). Ratio of CD8 versus CD4 T cells in WT and IL-2RβTg was compiled from 5 independent experiments (right).

D. CD8 and CD4 LN T cell numbers in WT and IL-2RβTg mice. Data show summary of 5 independent experiments with a total of 6 WT and 9 IL-2RβTg mice.

E. Naïve and memory T cell subsets were identified among CD4 and CD8 LN T cells of WT and IL-2RβTg mice, and the ratios of naïve/memory cells were determined. Data are representative of 5 independent experiments.

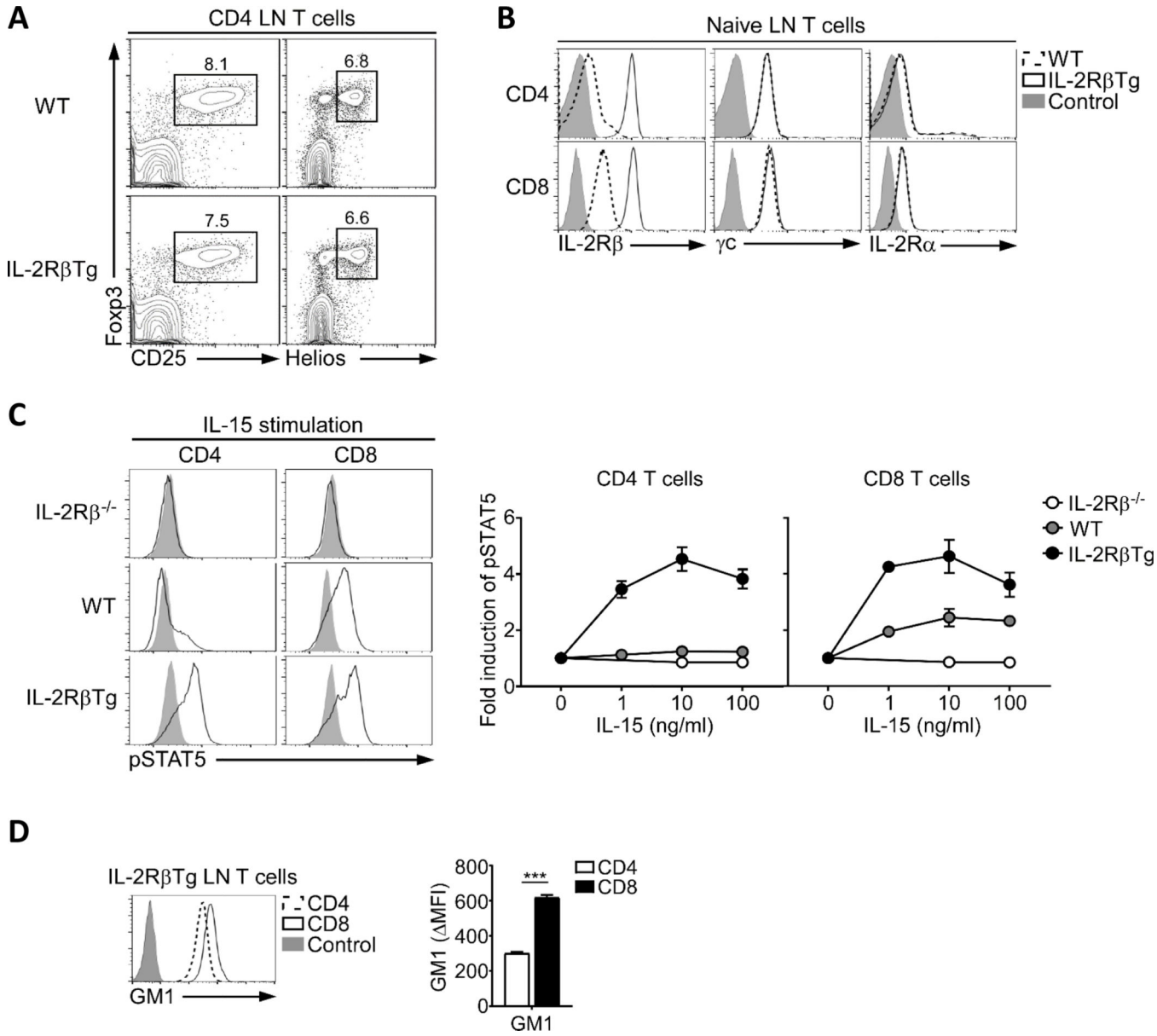

Figure 3. Increased IL-2Rβ availability promotes IL-15 signaling in CD4 T cells.

A. Foxp3^{+} CD25^{+} Treg cell frequencies among WT and IL-2RβTg CD4 LN T cells. Treg cells were identified by Foxp3 versus CD25 and Foxp3 versus Helios expression. Data are representative of 3 independent experiments.

B. IL-2 receptor subunit expression on CD4 and CD8 LN T cells of WT and IL-2RβTg mice. Data are representative of 5 independent experiments.

C. IL-15-induced STAT5 phosphorylation was determined in CD8 and CD4 T cells of WT and IL-2RβTg mice by assessing the intracellular pSTAT5 levels after IL-15 stimulation for 30 min. IL-2Rβ$^{-/-}$ T cells were used as a specificity control (left). Line graphs show fold differences in IL-15-induced STAT5 phosphorylation compared with the untreated control cells (right). Data are representative and show summary of 6 independent experiments.

D. GM1 levels in CD8 and CD4 T cells of IL-2RβTg mice. Histogram is representative (left), and the graph shows summary of GM1 levels (ΔMFI) from 3 independent experiments (right).

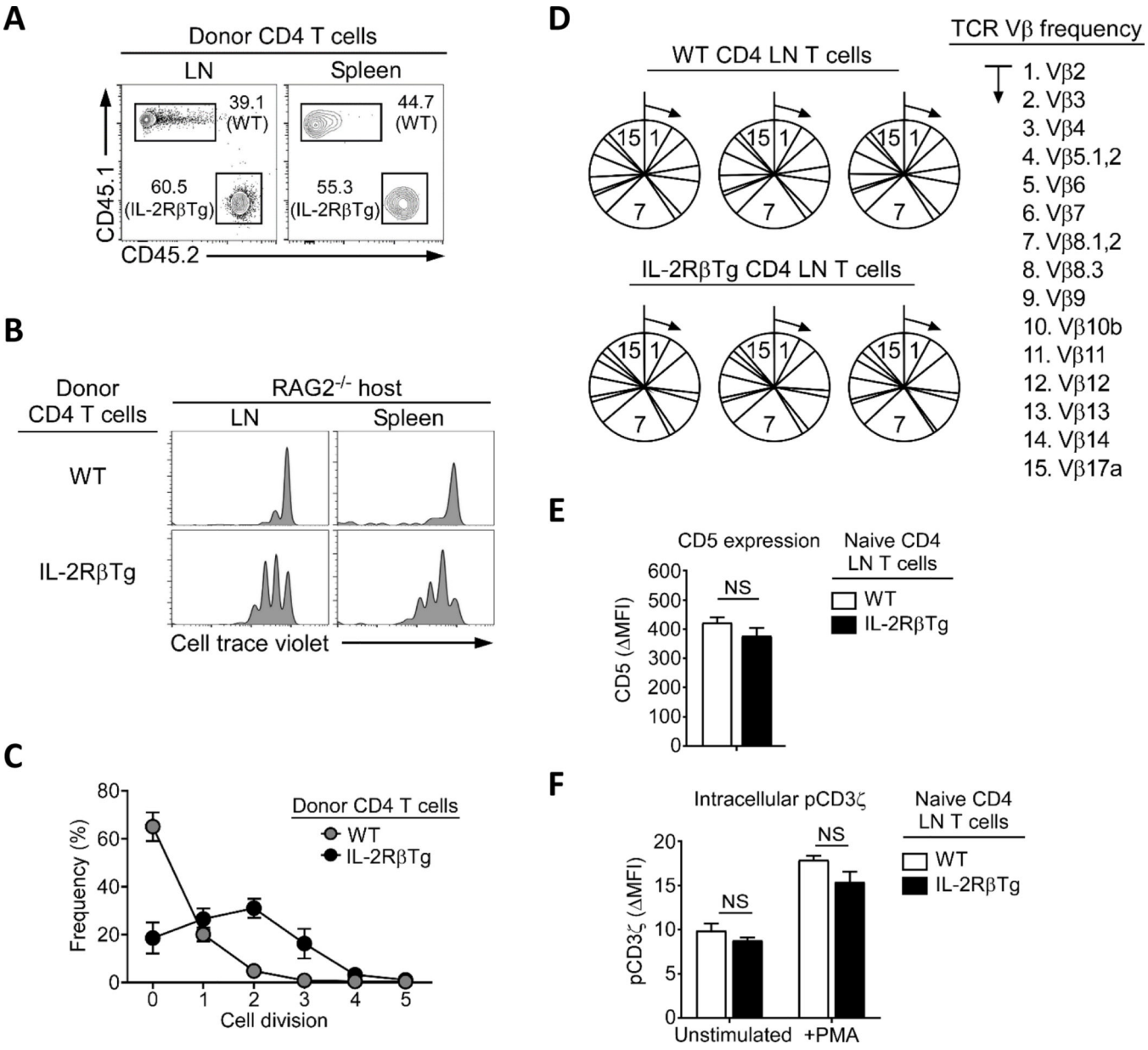

Figure 4. Lymphopenia-induced proliferation of naïve donor CD4 and CD8 T cells.

A. CD45.1 versus CD45.2 profiles of CD45.1$^+$ WT and CD45.2$^+$ IL-2RβTg donor T cells that were mixed at a 1:1 ratio and adoptively transferred into RAG2$^{-/-}$ host mice. Donor T cells were recovered from host LN and spleen 5 days after injection and analyzed by flow cytometry. Data are representative of 4 independent experiments.

B. Cell Trace Violet dilution of the adoptively transferred CD4 donor T cells from CD45.1$^+$ WT and CD45.2$^+$ IL-2RβTg mice, mixed at a 1:1 ratio. Donor origin was identified using CD45.1/2 congenic markers, and cell proliferation was determined for the donor T cells recovered from the LNs and spleen. Data are representative of 4 independent experiments.

C. Frequency of cell division was determined from 1:1 mixed WT and IL-2RβTg naïve CD4$^+$ donor T cells 5 days after adoptive transfer. Undivided T cells are identified as “0” division. Data are representative of 3 independent experiments.

D. TCR Vβ usage in WT and IL-2RβTg CD4 T cells was assessed using 15 different anti-Vβ specific antibodies as indicated. Pie charts show the frequencies of individual TCR Vβ segements among $TCR\beta^+$ CD4 T cells, which was set to 100%. Data show summary from 3 WT and 3 IL-2RβTg mice.

E. Cell surface expression of CD5 was quantified on freshly isolated WT and IL-2RβTg CD4 LN T cells from inguinal LNs. Data show summary of 2 independent experiments with a total of 3 WT and 3 IL-2RβTg mice.

F. Intracellular pCD3ζ levels were quantified in freshly isolated WT and IL-2RβTg CD4 LN T cells. Data show summary of 2 independent experiments with a total of 3 WT and 3 IL-2RβTg mice.

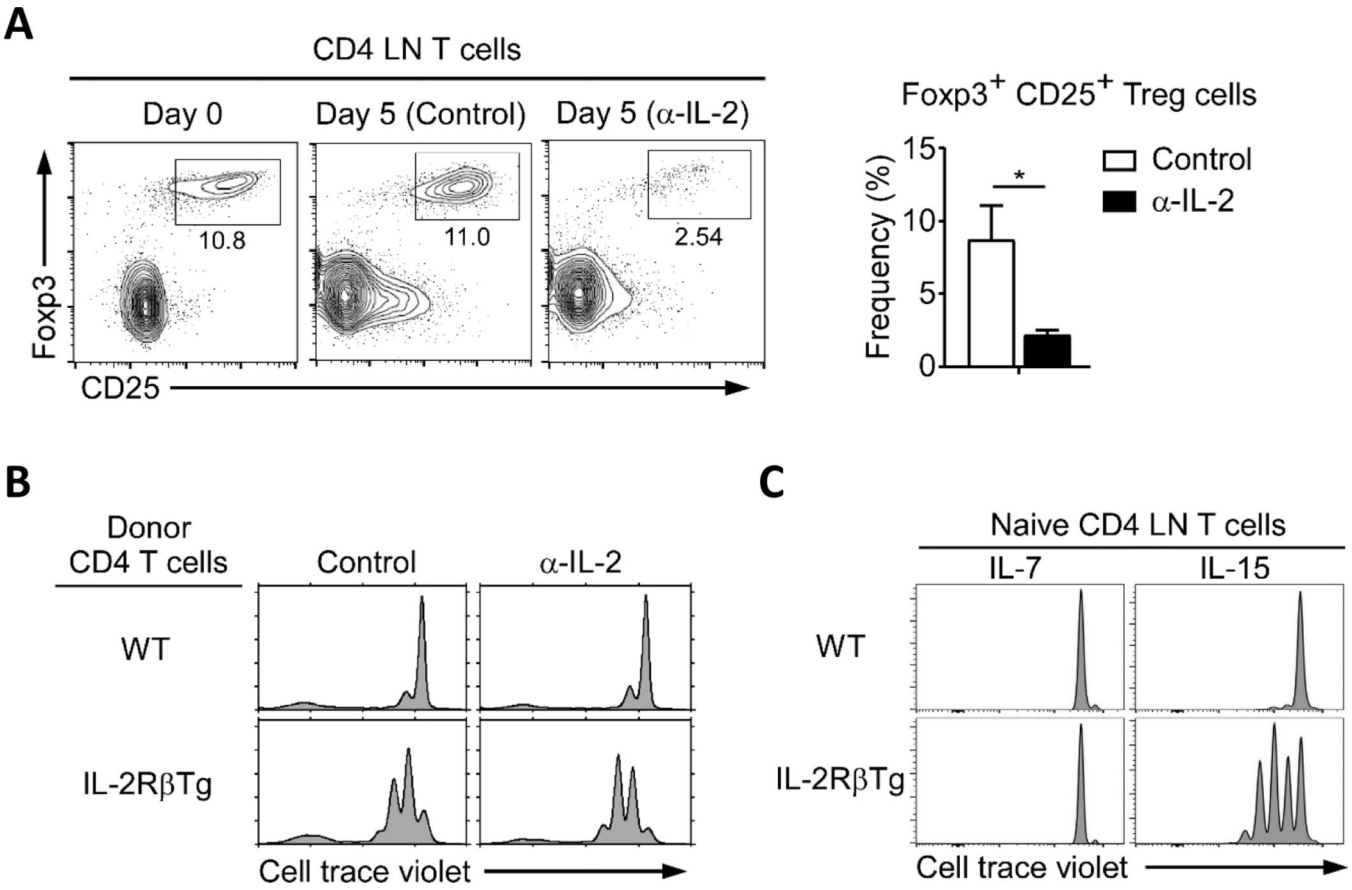

Figure 5. IL-15 drives proliferation of naïve IL-2RβTg CD4 T cells.

A. Frequency of adoptively transferred $Foxp3^{+}CD25^{+}$ Treg cells in $RAG2^{-/-}$ host mice injected with neutralizing anti-IL-2 antibodies (α-IL-2) or vehicle control (control). Donor CD4 T cells were recovered from host mice after 5 days, and assessed for the frequency of Treg cells among CD4 T cells. Contour plots are representative (left), and the bar graph is summary of 3 independent experiments (right).

B. Cell Trace Violet dilution of adoptively transferred WT and IL-2RβTg CD4 donor T cells upon IL-2 neutralization. Results are representative of 2 independent experiments with a total of 3 $RAG2^{-/-}$ host mice that were injected with anti-IL-2 antibodies (α-IL-2) and with 2 $RAG2^{-/-}$ host mice that were injected with vehicle control (control), after adoptive transfer of 1:1 mixed WT and IL-2RβTg CD4 T cells. LNs of host mice were harvested 5 days after T cell transfer and analyzed for congenic markers and Cell Trace Violet dilution.

C. FACS-purified naïve CD4 LN T cells from WT or IL-2RβTg mice were labeled with Cell Trace Violet, and stimulated with either recombinant IL-7 (0.5 μg/ml) or recombinant IL-15 (0.5 μg/ml) *in vitro* for 5 days. Results are representative of 3 independent experiments.