Regulatory T Cell Development

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IL-2, -7, and -15, but Not Thymic Stromal Lymphopoietin, Redundantly Govern CD4\(^+\)Foxp3\(^+\) Regulatory T Cell Development

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Common γ chain (γc)-receptor dependent cytokines are required for regulatory T cell (Treg) development as γc\(^{-/-}\) mice lack Tregs. However, it is unclear which γc-dependent cytokines are involved in this process. Furthermore, thymic stromal lymphopoietin (TSLP) has also been suggested to play a role in Treg development. In this study, we demonstrate that developing CD4\(^+\)Foxp3\(^+\) Tregs in the thymus express the IL-2Rβ, IL-4Ra, IL-7Ra, IL-15Ra, and IL-21Ra chains, but not the IL-9Ra or TSLPRa chains. Moreover, only IL-2, and to a much lesser degree IL-7 and IL-15, were capable of transducing signals in CD4\(^+\)Foxp3\(^+\) Tregs as determined by monitoring STAT5 phosphorylation. Likewise, IL-2, IL-7, and IL-15, but not TSLP, were capable of inducing the conversion of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) thymic Treg progenitors into CD4\(^+\)Foxp3\(^+\) mature Tregs in vitro. To examine this issue in more detail, we generated IL-2Rβ\(^{-/-}\) × IL-7Ra\(^{-/-}\) and IL-2Rβ\(^{-/-}\) × IL-4Ra\(^{-/-}\) mice. We found that IL-2Rβ\(^{-/-}\) × IL-7Ra\(^{-/-}\) mice were devoid of Tregs thereby recapitulating the phenotype observed in γc\(^{-/-}\) mice; in contrast, the phenotype observed in IL-2Rβ\(^{-/-}\) × IL-4Ra\(^{-/-}\) mice was comparable to that seen in IL-2Rβ\(^{-/-}\) mice. Finally, we observed that Tregs from both IL-2Rβ\(^{-/-}\) and IL-2Rβ\(^{-/-}\) mice show elevated expression of IL-7Ra and IL-15Ra chains. Addition of IL-2 to Tregs from IL-2\(^{-/-}\) mice led to rapid down-regulation of these receptors. Taken together, our results demonstrate that IL-2 plays the predominant role in Treg development, but that in its absence the IL-7Ra and IL-15Ra chains are up-regulated and allow for IL-7 and IL-15 to partially compensate for loss of IL-2.


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4 Abbreviations used in this paper: Treg, regulatory T cell; γc, common gamma chain; p-STAT5, phospho-STAT5; LMC, littermate control; TSLP, thymic stromal lymphopoietin.

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expression of IL-7Ra and IL-15Ra chains is suppressed in mature Tregs; this suppression does not occur in IL-2β-/- or IL-2Rβ-/- mice, demonstrating that IL-7Ra and IL-15Ra down-regulation occurs via an IL-2/IL-2Rβ-dependent signaling pathway. Our findings demonstrate that IL-2, IL-7, and IL-15, are the critical γc-dependent cytokines that are responsible for promoting Treg development. In contrast, developing Tregs do not express the TSLP receptor chain, nor respond to TSLP, by inducing phospho-STAT5 (p-STAT5). Thus, at least in the mouse, TSLP does not appear to play a direct role in Treg development.

**Materials and Methods**

### Mice

IL-2β-/-, IL-2Rβ-/-, IL-7Ra-/-, and IL-4Ra-/- mice were obtained from The Jackson Laboratories. IL-2Rβ-/-, IL-7Ra-/-, and IL-4Ra-/- mice were crossed in our laboratory to obtain IL-2Rβ-/- × IL-7Ra-/- and IL-4Ra-/- × IL-2Rβ-/- mice. Mice used were on a C57BL/6 background with the exception of the IL-4Ra-/- and IL-4Ra-/- × IL-2Rβ-/- mice, which were on a mixed BALB/c × C57BL/6 background, and Foxp3-GFP reporter mice, which were on a mixed C57BL/6 × 129 background. Foxp3-GFP reporter mice were provided by Dr. Sasha Rudensky (University of Washington School of Medicine, Department of Immunology, Seattle, WA).

**Flow cytometry and FACS analysis**

Mice were sacrificed and lymph node, spleen, and thymus were isolated. Five million cells were used per staining condition. Cells were first pretreated with an Ab that blocks Fc receptor binding (Clone 24G2). Cells were subsequently stained with the following Abs from eBioscience: CD4-Alexa 700, CD8-allophycocyanin-Alexa Fluor 750 or CD8-FITC, CD3e-FITC, CD25-PE-Cy7 (PE-Cy 7), or CD25-allophycocyanin. In addition, biotinylated Abs for CD122 (IL2Rβ), CD124 (IL4Ra), CD127 (IL7Rα), and IL21Rα, were obtained from eBioscience. Abs for IL-9Ra, TSLP-Rα, and IL-15Ra were obtained from R&D Systems. Isotype control Abs for TSLP-Rβ and IL-15Ra were obtained from R&D Systems, while isotype controls for IL-9Ra were obtained from eBioscience. Both the IL-9Ra and TSLP-Rα were biotinylated according to the manufacturer’s instructions (Sigma-Aldrich, Cat. no. BTAG-1KT), while the IL-15Ra Ab was purchased in a biotinylated form. Streptavidin allophycocyanin from eBioscience was used as a secondary reagent to reveal staining with biotinylated Abs. Intracellular Foxp3 staining was done after fixation, permeabilization, and overnight incubation at 4°C as described previously (8).

To examine whether IL-2 alters IL-7Ra and IL-15Ra expression on Tregs in IL-2β-/- mice, we purified CD4+ splenocytes from IL-2β-/- mice by MACS beads enrichment (Miltenyi Biotec). Purified cells were then stimulated with IL-2 (100 U/ml) for 4, 8, 12, and 24 h. Cells were then harvested and stained for IL-7Ra, IL-15Ra, and Foxp3 expression and analyzed on an LSR II flow cytometer (BD Biosciences).

**Flow cytometry for p-STAT5**

Single-cell suspensions were generated from isolated spleens and thymus from Foxp3-GFP reporter mice. These cell suspensions were pretreated with an Ab that blocks Fc receptor binding. Cells were then stained for the surface markers CD4, CD8, and CD25. Five million cells were then serum starved in 500 μl of 1× DMEM for 30 min at 37°C, then stimulated with either 100 U/ml of IL-2 (PeproTech), or 50 ng/ml IL-4 (PeproTech), IL-7 (R&D Systems), IL-9 (R&D Systems), TSLP (R&D Systems), IL-15 (R&D Systems), or IL-21 (PeproTech) for 20 min. After stimulation, the cells were washed with 1× DMEM to remove all traces of the supernatant; they were then resuspended in 100 μl of fixation medium from the Caltag Fix and Perm kit and incubated at 37°C for 15 min. Afterward, 1 ml of 4°C 100% methanol was added and the cells were incubated overnight at 4°C in the dark. Intracellular p-STAT5 staining was done using the Caltag Fix and Perm kit and PE-conjugated anti-p-STAT5 (BD Biosciences). Non-stimulated cells were used as negative controls.

**Treg conversion assay**

The conversion assay of Treg progenitors into CD4+Foxp3+ Tregs was conducted as previously described (10). In brief, CD4+CD25+Foxp3+ Treg progenitors were sorted from Foxp3-GFP reporter mice (19) and plated in culture in the presence of the indicated amounts of cytokine. Twenty-four hours later cells were stained for CD4 and CD25 and analyzed for expression of these markers plus Foxp3-GFP using an LSR II flow cytometer.

### Results

To examine the role that different cytokines play in Treg development in the thymus, we first analyzed the expression of IL-2βR, IL-4Ra, IL-7Ra, IL-9Ra, IL-15Ra, IL-21Ra, and TSLP-Rα on CD4+Foxp3+ thymocytes and CD4+Foxp3+ splenocytes. We found four basic patterns of cytokine receptor expression. First, the IL-2βR-chain was selectively expressed on CD4+Foxp3+ thymocytes (Fig. 1). This pattern of expression was maintained in splenic Foxp3+ vs Foxp3- T cells. Second, IL-9Ra and TSLP-Rα were not observed on either Foxp3+ or Foxp3- thymocytes. To confirm that our staining for these receptors was working, we also stained peritoneal B1 B cells and CD19+ pre-B cells, which have previously been reported to express the IL-9Ra and TSLPRa chains,
FIGURE 2. Cytokine stimulation and phospho-STAT5 expression on CD4+Foxp3+ thymocytes and splenocytes. Single-cell suspensions of thymocytes or splenocytes from Foxp3-GFP mice were serum starved for 30 min and then stimulated with IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, or TSLP for 20 min. Cells were then stained with Abs to CD4, CD8, CD25, and phospho-STAT5 as described in the methods section. Shown are histograms of phospho-STAT5 expression in CD4SP thymocytes (left column) and CD4− splenocytes (right column). Gray filled in histograms represent staining of unstimulated Foxp3-GFP+ cells. Solid lines and broken lines represent staining of stimulated Foxp3-GFP+ and Foxp3-GFP− cells, respectively. A representative example of two independent experiments is depicted (n = 3 mice). Similar results were obtained when using CD25 to identify Tregs in C57BL/6 mice (n = 13 mice, data not shown).

respectively (17, 18). We detected IL-9Rα expression on peritoneal B1 B cells; as expected, we also observed TSLPRα expression on pre-B cells in the bone marrow, thereby indicating that our Abs to IL-9Rα and TSLPRα are capable of detecting expression of these receptors (data not shown). Third, IL-4Rα and IL-21Rα were expressed equally on Foxp3+ vs Foxp3− thymocytes and splenic T cells (Fig. 1). Last, we observed a dynamic expression pattern for the IL-7Rα and IL-15Rα chains. Expression of both of these receptors was observed in CD4+Foxp3+ thymocytes, but was significantly reduced in splenic T cells (Fig. 1). Thus multiple γε-dependent cytokine receptors, but not the TSLPRα-chain, are expressed on developing Tregs in the thymus.

To assess whether receptor expression on Tregs correlated with function, we stimulated cells with IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, and TSLP and examined STAT5 activation by intracellular staining for p-STAT5. We focused on STAT5 as previous reports have indicated that STAT5 plays a critical role in Treg development (6, 10, 20). For these studies, we identified Tregs using Foxp3-GFP reporter mice; similar studies were also conducted using CD25 as a marker of Tregs. We identified three distinct response patterns. First, IL-2 induced robust STAT5 activation in CD4+Foxp3+ thymocytes; splenic Tregs remained highly responsive to IL2 stimulation (Fig. 2). Second, IL-7 and IL-15 induced modest STAT5 activation in CD4+Foxp3+ thymocytes. However, these responses were almost completely eliminated in CD4+Foxp3+ splenocytes (Fig. 2). Third, IL-4, IL-9, IL-21, and TSLP did not induce detectable STAT5 activation on CD4+Foxp3+ thymocytes (Fig. 2). Similar results were obtained when gating on CD4+CD25+ T cells, with the exception that under those staining conditions IL-4 led to very weak STAT5 phosphorylation in CD4+Foxp3+ thymocytes and splenocytes (data not shown). A potential caveat with the IL-15 studies is that IL-15 could be presented by the IL-15Rα-chain via trans presentation in vivo (21). It is possible, therefore, that our ex vivo stimulation studies may not have allowed for optimal transpresentation of IL-15 to CD4+Foxp3+ Tregs. Thus, developing thymic Tregs respond to IL-2 and IL-7, and to a lesser degree IL-15.

Previous reports have suggested that TSLP plays a role in Treg development (22). However, TSLPR−/− mice have no reported defects in Treg development or function (23). This latter observation is consistent with our failure to observe TSLPR expression on developing Tregs. A potential caveat with our studies is that TSLPR expression levels may be below the limits of detection by flow cytometry. Likewise, the amount of STAT5 phosphorylation induced by TSLP might be below the level that we can detect by flow cytometry. To explore this in more detail, we examined whether TSLPR mRNA was detectable by RT-PCR in CD4+Foxp3-GFP+ thymocytes. These studies indicated that TSLPR mRNA could be detected at some level in developing Tregs (data not shown). A key question then is whether this results in expression of a receptor capable of inducing biological responses. To address this question, we made use of the recent identification of CD4+CD25+Foxp3+ thymocytes as penultimate Treg progenitors that can be converted into CD4+Foxp3+ mature Tregs following stimulation with IL-2 (10, 11). For these studies, we isolated CD4+CD25+Foxp3-GFP+ Treg progenitors and stimulated them overnight with either IL-2, IL-7, IL-15, or TSLP. The cultured cells were then examined for Foxp3-GFP expression. As shown in Fig. 3, IL-2 induced clear conversion of Treg progenitors into CD4+Foxp3-GFP+ Tregs. IL-7 and IL-15 were also capable of inducing the conversion of Treg progenitors into Foxp3+ Tregs, although they were much less effective than IL-2. In contrast,
TSLP stimulation failed to induce conversion of any Treg progenitors into Foxp3+ Tregs. Thus, even if the TSLPR is expressed at very low levels on developing Tregs, it is incapable of inducing Treg differentiation following stimulation with TSLP.

Our observation that IL-2Rβ and IL-7Rα were the predominant receptors expressed on developing thymocytes suggested that IL-2, IL-7, and IL-15 were most likely the key γc-dependent cytokines that drive Treg development. However, given the expression of the IL-4Rα-chain on developing Tregs, we examined whether IL4-dependent signals also played a role in this process. IL-4Rα+ mice show no decrease in the percentage of Tregs in the thymus relative to littermate control (LMC) mice (Fig. 4). Furthermore, splenic Tregs were also not reduced in IL-4Rα+ mice (Fig. 4). To examine whether IL-2Rβ and IL-4Rα-dependent signals played a redundant role in Treg development, we generated IL-2Rβ−/− × IL-4Rα−/− mice. As previously reported, IL-2Rβ−/− mice exhibited reduced numbers of Tregs in both the thymus and spleen; a further reduction was not observed in IL-2Rβ−/− × IL-4Rα−/− mice (Fig. 4). These findings strongly suggest that IL-4Rα-dependent signals are not required for Treg development. It is important to note here that unlike our previous studies, which used IL-2Rβ−/− mice on the C57BL/6 background, the IL-4Rα−/− and IL-2Rβ−/− × IL-4Rα−/− mice in these experiments are on a mixed C57BL/6 × BALB/c background. We have consistently noticed that the IL-2Rβ−/− mice on the C57BL/6 × BALB/c background mice have a more severe phenotype (i.e., fewer Tregs at an earlier age) than IL-2Rβ−/− on the C57BL/6 background. This results in IL-2Rβ−/− mice on the mixed background having a reduced percentage of Tregs relative to that seen in IL-2Rβ−/− mice on the C57BL/6 background.

Given the expression of both functional IL-7Rα and IL-2Rβ on developing Tregs, we predicted that these two cytokine receptors might both be capable of driving Treg development. Consistent with our previous report, we found that although total numbers of T cells are greatly reduced in IL-7Rα−/− mice, the percentage of Tregs relative to other T cell subsets was not affected (Fig. 5A) (8). Thus, IL7Rα signaling is not required for Treg development. However, it remains possible that IL-2Rβ and IL-7Rα can act redundantly to drive Treg development. To test this possibility, we compared Treg differentiation in IL-7Rα−/− vs IL-2Rβ−/− × IL-7Rα−/− mice. We found that IL-2Rβ−/− × IL-7Rα−/− mice showed a significant decrease in Treg numbers when compared with IL-7Rα−/− mice (p = 0.009, Student’s t test) (Fig. 5, A and B). Importantly, the numbers of Tregs found in IL-2Rβ−/− × IL-7Rα−/− mice (thymus = 195 ± 75; spleen = 686 ± 136) were comparable to that which we observed in age-matched γc−/− mice (thymus = 80 ± 22; spleen = 1500 ± 651) in our previous studies (Fig. 5B) (8). These experiments demonstrate that IL-2Rβ and IL-7Rα-dependent cytokines are the only γc-dependent cytokines required for Treg development.

IL-7Rα and IL-15Rα are expressed at quite low levels on mature splenic Tregs. Thus, it is rather surprising that splenic Tregs are maintained in young IL-2−/− mice. To examine this further, we stained CD4+ Foxp3+ Tregs from LMC and IL-2−/− mice for the expression of IL-7Rα and IL-15Rα. We found that CD4+ Foxp3+ T cells from IL-7Rα−/− and IL-15Rα−/− mice were greatly reduced in thymus and spleen compared with wild type LMC. Splenocytes were isolated from 4- to 5-wk-old LMC, IL-7Rα−/−, and IL-15Rα−/− mice and stained with Abs for CD4, CD8, and Foxp3 to identify splenic Tregs. Shown are flow cytometry plots of thymus (top panel) or spleen cells (bottom panel) gated on CD4+ T cells. A representative example of six independent experiments is depicted.

**FIGURE 4.** Treg development in IL-4Rα−/− and IL-2Rβ−/− × IL-4Rα−/− mice. A, Thymus and spleen were harvested from 4- to 5-wk-old LMC, IL-4Rα−/−, IL-2Rβ−/−, and IL-2Rβ−/− × IL-4Rα−/− mice. Cells were stained with Abs to CD4, CD8, and Foxp3 to identify Tregs. Shown are flow cytometry plots of thymus (top panel) or spleen cells (bottom panel) gated on CD4+ T cells. A representative example of six independent experiments is depicted.

**FIGURE 5.** Treg development in IL-7Rα−/− and IL-2Rβ−/− × IL-7Rα−/− mice. A, Thymus and spleen were harvested from 4- to 5-wk-old LMC, IL-7Rα−/−, and IL-2Rβ−/− × IL-7Rα−/− mice. Cells were stained with Abs to CD4, CD8, and Foxp3 to identify Tregs. Shown are flow cytometry plots of thymus (top panel) or spleen cells (bottom panel) gated on CD4+ T cells. A representative example of six independent experiments is depicted. B, Shown are bar graphs representing total numbers of CD4+Foxp3+ Tregs in the thymus (left panel) and spleen (right panel). Error bars represent SEM; n = 6 IL-7Rα−/− and 6 IL-2Rβ−/− × IL-7Rα−/− mice; p values were calculated using two-tailed Student’s t test.

**FIGURE 6.** Expression of IL-2Rβ, IL-7Rα, and IL-15Rα on CD4+ Foxp3+ Tregs from IL-2−/− and IL-2Rβ−/− mice compared with wild type LMC. Splenocytes were isolated from 4- to 5-wk-old LMC, IL-2−/− and IL-2Rβ−/− mice and stained with Abs for CD4, CD8, and Foxp3 to identify splenic Tregs. Shown are CD4+ Foxp3+ gated cells stained for IL-2Rβ (left panels), IL-7Rα (middle panels), and IL-15Rα (right panels). Gray histograms represent staining of CD4+ Foxp3+ cells with isotype control Ab. Solid lines represent histograms of CD4+ Foxp3+ T cells from IL-2−/− (top panel) or IL-2Rβ−/− (bottom panel) mice; broken lines represent staining of CD4+ Foxp3+ Tregs from wild-type LMC mice. A representative example of three independent experiments is depicted (n = 6 IL-2−/− and 3 IL-2Rβ−/− mice).
Tregs in IL-2−/− mice expressed significantly higher levels of both the IL-7Rα and IL-15Rα chains (Fig. 6). We considered two explanations for these findings. First, it is possible that, in the absence of IL-2, any splenic Tregs that express higher levels of IL-7Rα or IL-15Rα have a competitive advantage and are selectively expanded. Alternatively, it is possible that IL-2/IL-2Rβ-dependent signals actively down-regulate IL-7Rα or IL-15Rα expression. To distinguish between these two possibilities, we stained the few CD4+Foxp3+ Tregs in IL-2Rβ−/− mice for IL-7Rα and IL-15Rα expression. Once again, we observed increased expression of both IL-7Rα and IL-15Rα on Tregs from IL-2Rβ−/− vs LMC mice (Fig. 6). In IL-2Rβ−/− mice, IL-15Rα expression provides no competitive advantage. Thus, this latter finding strongly suggests that IL-15Rα down-regulation, and likely IL-7Rα as well, is due to IL-2Rβ-dependent signals. To investigate this further, we took CD4+ splenocytes from IL-2−/− mice and stimulated those cells with IL-2. We then examined expression of IL-7Rα and IL-15Rα chains on CD4+Foxp3+ cells (Fig. 7). We observed down-regulation of the IL-7Rα-chain as early as 8 h after IL-2 stimulation; both the IL-7Rα and IL-15Rα chains were clearly down regulated after 24 h of IL-2 stimulation. Taken together, these studies indicate that IL-2 dependent signals can negatively regulate IL-7Rα and IL-15Rα expression on CD4+Foxp3+ Tregs.

Discussion

These studies identify IL-2, IL-7, and IL-15 as the sole γc-dependent cytokines required for Treg development in the thymus. Four pieces of data support this conclusion. First, receptors for these three cytokines are expressed on developing Tregs in the thymus. Second, these cells can respond to IL-2 and IL-7 by inducing robust STAT5 activation. The only other γc-dependent cytokine receptors expressed on thymic Tregs are IL-4Rα and IL-21Rα. However, IL-4 and IL-21 did not induce STAT5 activation in CD4+Foxp3+ thymocytes and only induced minimal STAT5 activation in mature CD4+Foxp3+ splenocytes. The role of IL-15 is somewhat more complicated. We observed only weak STAT5 induction following ex vivo stimulation of thymic CD4+Foxp3+ Tregs with IL-15. This may reflect the absence of accessory cells in our ex vivo stimulation cultures that would allow for effective trans presentation of IL-15 to developing Tregs. Third, IL-2 and to a lesser degree IL-7 and IL-15 were capable of inducing the conversion of CD4+CD25+Foxp3+ Treg progenitors into mature Foxp3+ Tregs. In contrast, TSLP was completely ineffective in this assay. Finally, we found that IL-2Rβ−/− × IL-7Rα−/− mice recapitulated the phenotype reported in γc−/− mice which are essentially devoid of Tregs. In contrast, the reduction in thymic Tregs in IL-2Rβ−/− × IL-4Rα−/− mice was no more severe than that seen in IL-2Rβ−/− mice. Taken together with our previous observation that IL-2−/− × IL-15−/− mice have significantly fewer Tregs that IL-2−/− mice, our findings strongly support the conclusion that IL-2, IL-7, and IL-15, but not other γc-dependent cytokines, can contribute to Treg differentiation in the thymus.

We also examined the role of TSLP on Treg differentiation as previous studies have suggested that TSLP plays an important role in human and murine Treg development (17, 22). Our studies rule out a direct role for TSLP in murine Treg differentiation. First, consistent with our earlier observation, IL-7Rα−/− mice show no reduction in Tregs relative to non-Tregs. Second, we could not detect expression of TSLPR on thymic Tregs nor induce STAT5 activation following stimulation of these cells with TSLP. Third TSLP was incapable of inducing the conversion of thymic Treg progenitors into CD4+Foxp3+ Tregs. These findings demonstrate that TSLP cannot play a direct role in Treg development. It remains possible that TSLP plays an indirect role by acting on other cell types that may be involved in promoting Treg differentiation. However, this function is either not unique to TSLP, or not critical, as Tregs clearly develop in mice lacking the IL-7Rα-chain, which is a critical component of the TSLPR.

Although our studies demonstrate that IL-2, IL-7, and IL-15 can redundantly contribute to Treg development and homeostasis, it seems likely that IL-2 is the relevant cytokine in wild-type mice. Specifically, we found that expression of IL-7Rα and IL-15Rα were significantly increased on Tregs in both IL-2−/− and IL-2Rβ−/− mice. Ex vivo stimulation of CD4+Foxp3+ Tregs from IL-2−/− mice with IL-2 led to rapid down-regulation of both of these receptor subunits. Thus, IL-2 plays an important role in rendering CD4+Foxp3+ Tregs uniquely responsive to IL-2-dependent signals in wild-type mice. This most likely serves to link Treg homeostasis directly to effector T cell activation and IL2 secretion. Effector T cell IL2 production appears to be critical for Tregs to expand in step with activated effector T cells and thereby mediate effective suppression. Supporting this conclusion, IL-2−/− mice, but not IL-7−/−, or IL-15−/− mice, show signs of T cell activation and ultimately succumb to lethal multiorgan autoimmune disease. Thus, although IL-7 and IL-15 are capable of sustaining Treg populations in young mice, they are not effective at expanding these cells sufficiently during ongoing immune responses. Finally, these findings also have implications for the use of low-level IL-7Rα expression to identify Tregs (24, 25) as this receptor may be up-regulated under conditions of limited IL-2 availability.

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Disclosures

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