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IL-2R Signaling Is Essential for Functional Maturation of Regulatory T Cells during Thymic Development

Guoyan Cheng,* Aixin Yu,* Michael J. Dee,* and Thomas R. Malek*†

CD4⁺ Foxp³⁺ regulatory T cells (Tregs) are an independent cell lineage, and their developmental progression during thymic development depends on IL-2 signaling. However, the role of IL-2R signaling during thymic Treg development remains only partially understood. The current study assessed the contribution of IL-2 to the expansion and functional programming of developing Tregs. In the absence of IL-2Rβ signaling, predominantly CD4⁺ CD25⁺ Foxp³⁺ T cells were found, and these cells exhibited somewhat lower expression of the proliferative marker Ki67. These immature Tregs, which represent products of failed development, were also found in normal mice and were characterized by markedly lower expression of several Treg functional molecules. Therefore, IL-2R is required for the progression, functional programming, and expansion of Tregs during thymic development. An IL-2R–signaling mutant that lowers STAT5 activation readily supported Treg functional programming, but Treg proliferation remained somewhat impaired. The requirement for IL-2 during thymic Treg expansion was best illustrated in mixed chimeras where the Tregs with mutant IL-2Rs were forced to compete with wild-type Tregs during their development. Tregs with impaired IL-2R signaling were more prevalent in the thymus than spleen in these competitive experiments. The general effectiveness of mutant IL-2Rs to support thymic Treg development is partially accounted for by a heightened capacity of thymic Tregs to respond to IL-2. Overall, our data support a model in which limiting IL-2R signaling is amplified by thymic Tregs to readily support their development and functional programming, whereas these same conditions are not sufficient to support peripheral Treg homeostasis. *The Journal of Immunology, 2013, 190: 1567–1575.

N atural CD4⁺ Foxp³⁺ regulatory T cells (Tregs) are a distinct T cell lineage that develops in the thymus; they are essential to prevent self-reactivity by suppressing peripheral autoreactive T cells that escape thymic negative selection. IL-2R signaling is essential and nonredundant for thymus Treg development and for peripheral Treg homeostasis and competitive fitness (1–6). The central role of IL-2 in Treg biology is exemplified by the uncontrolled hyperactivation of T cells and lethal autoimmunity associated with IL-2–deficient, IL-2Rα–deficient (CD25), and IL-2Rβ–deficient (CD122) mice that typically die by 4–12 wk of age (7–10). Importantly, restoring Tregs in IL-2– or IL-2Rα–deficient mice is sufficient to completely prevent this autoimmunity (11), directly implicating defective Tregs as the main reason for autoimmunity in the absence of IL-2R signaling. The absence of IL-2R signaling in mice results in the development of only CD4⁺ Foxp³⁺ CD25⁻ T cells that may represent immature Tregs (12, 13). However, in addition to defects in Tregs, IL-2Rα–deficient mice do not effectively program CD8⁺ T memory responses (14). Thus, the lack of IL-2 also tempers autoreactive T cells that contribute to somewhat delayed lethal autoimmunity compared with Foxp³-deficient mice.

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Abbreviations used in this article: B6, C57BL/6; DN, double-negative; DP, double-positive; MFI, mean fluorescence intensity; PFA, paraformaldehyde; 2Rβ⁻/−, IL-2Rβ⁻/−; SP, single-positive; Treg, regulatory T cell; WT, wild-type; Y0, IL-2RβWT; Y3, IL-2Rβ341,395/404.

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IL-2 contributes to thymic Treg development through activation of STAT5 (15). Low levels of IL-2 selectively compartmentalized within the thymic microenvironment are sufficient to drive thymic Treg development (16). Indeed, weak IL-2R signaling generated by mutant IL-2Rs, in which key cytoplasmic tyrosine residues of IL-2Rβ were replaced with phenylalanine, readily supports thymic Treg development (17). Thus, IL-2 promotes Treg development through a low IL-2R–signaling threshold, providing a mechanism to yield Tregs even when IL-2 is limiting. Current models are consistent with a two-step developmental process whereby TCR signals during the thymic-selection process lead to induction of CD25 on developing CD4⁺ CD8⁻ thymocytes, and IL-2 provides an instructive signal to these precursor cells to upregulate both Foxp3 and CD25 into mature Tregs (18, 19).

Genome-wide expression analysis comparing peripheral Tregs bearing wild-type (WT) versus impaired IL-2R revealed an over-representation of differentially expressed genes that function for cell growth and death (5, 17), implicating IL-2 as a key regulator of Treg homeostasis. Consistent with this idea, IL-2 favors the expansion of peripheral Tregs that express low levels of the proapoptotic molecule Bim (20). Furthermore, the Treg lineage cells from IL-2⁻/⁻ mice that do not express Bim showed an increase in peripheral CD4⁺ Foxp³⁺ T cells, such that these mice now contained a normal percentage and number of peripheral CD4⁺ Foxp³⁺ T cells, further linking IL-2 to the regulation of peripheral Treg numbers. However, these Bim⁻/⁻ IL-2⁻/⁻ mice still exhibited severe autoimmunity due to impaired Treg function (21). Overall, these and other findings indicate that IL-2R signaling exerts a strong influence on peripheral Treg homeostasis and function that is reflected, in part, by IL-2R–dependent STAT5 regulation of Foxp3 expression (22–26).

Although the involvement of IL-2 in peripheral Tregs is relatively well studied, comparatively little is known about the contribution of IL-2R signaling during thymic Treg development. The above findings for peripheral Tregs raise the possibility that the reduction of Tregs associated with IL-2/IL-2R deficiency might

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reflect impaired IL-2–dependent expansion and/or survival of developing thymic Tregs. However, IL-2 may more broadly instruct Tregs as they develop and may be critical to program Treg function. The current study investigated these possibilities and showed that, in the absence of IL-2Rβ signaling, the major defect in thymic Tregs was related to impaired development of the Treg functional program. Furthermore, competitive in vivo experiments revealed that low IL-2R signaling is much more efficient in promoting thymic Treg development than maintaining peripheral Treg homeostasis.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from The Jackson Laboratory. B6 Rag2<−/−;γc<−/− mice were purchased from Taconic Farms. Congenic CD45.1, congenic Thy1.1, and TCRα−/− B6 mice, all on the C57BL/6 genetic background, were bred and maintained in the animal facility at the University of Miami. C57BL/6 IL-2R<β−/− (2R<β−/−), as well as T cell–specific transgenic B6 IL-2RWT (Y0) and IL-2Rβ<Y341,395,498 (Y3) mice on the 2Rβ<−/− genetic background, were described previously (8, 17). Foxp3/RFP reporter mice were kindly provided by R. Flavell (Yale University, New Haven, CT). All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Miami.

Treg suppression of autoimmunity in 2Rβ−/− mice

Neonatal 2Rβ−/− mice (1–3 d old) received purified CD45.1-congenic CD4+ T cells (2–3 × 10^6) using anti-CD4 magnetic beads (Miltenyi Biotec) by i.v. injection to the superficial vein. Such treatment results in life-long engraftment of donor Tregs and prevents autoimmunity associated with 2Rβ−/− mice (11). These mice are referred to as “cured” 2Rβ−/− in this article.

Abs and FACS

Single-cell suspensions were prepared for flow cytometry as previously described (3). Typically, 300,000 events were collected during analysis. Intracellular staining of Foxp3 was performed according to the manufacturer’s instructions (eBioscience). Bcl-2, Ki67, and CTLA4 (BioLegend) were usually stained in combination with Foxp3, using the protocol for Foxp3 intracellular staining. Surface mAbs to CD4, CD8, CD45, and Thy1.2 were purchased from BioLegend; those to CD23, CD103, CD39, and Klrk1 were purchased from eBiosciences. All results were analyzed using a BD LSR II and Diva software.

p-STAT5 activity

Intracellular FACS staining for p-STAT5 was performed as previously described (D). Typically, 300,000 events were collected during analysis. Intracellular staining of Foxp3 was performed according to the manufacturer’s instructions (eBioscience). Bcl-2, Ki67, and CTLA4 (BioLegend) were usually stained in combination with Foxp3, using the protocol for Foxp3 intracellular staining. Surface mAbs to CD4, CD8, CD45, and Thy1.2 were purchased from BioLegend; those to CD23, CD103, CD39, and Klrk1 were purchased from eBiosciences. All results were analyzed using a BD LSR II and Diva software.

Bone marrow chimeras

Bone marrow chimeras were generated as previously described (3). Briefly, Thy1.1 B6 mice (900 rad), TCRα−/− mice (600 rad), and Rag2−/−;γc<−/− mice (900 rad) received a single dose of total body irradiation 24 h later, these mice received a mixture of the indicated T cell–depleted bone marrow (5 × 10^6 total cells) by i.v. injection into the tail vein. The mice were maintained with drinking water containing gentamicin (1 mg/ml).

Statistical analysis

Data were analyzed using Prism 5.0 by either a one-way ANOVA with the Tukey multiple comparison test when more than two groups were compared or by an unpaired t test when two groups were compared. Significant differences are indicated in the figures.

Results

IL-2 influences the growth of developing Tregs

We previously showed that 2Rβ−/− mice do not develop a normal compartment of Tregs (11). However, polyclonal T cell activation and autoimmunity rapidly ensue in these mice, raising the possibility that Treg development may be indirectly affected by this inflammatory environment. To test this possibility, we compared mainstream T cell development in 3–8-wk-old untreated autoimmune 2Rβ−/− mice and autoimmune-free “cured” 2Rβ−/− mice. The latter mice received Tregs by the transfer of unfractionated syngeneic CD45.1-congenic CD4+ T cells shortly after birth. Past studies established that the Tregs are the key cell population that prevents this autoimmunity (11).

When these mice were compared with control WT B6 mice, the proportion and distribution of double-negative (DN), double-positive (DP), CD4 single-positive (SP), and CD8 SP thymocytes were within the normal range (Fig. 1A). However, thymic cellularity was somewhat, but equivalently, reduced in 2Rβ−/− and “cured” 2Rβ−/− mice (Fig. 1B). This effect might reflect some inefficiency related to lack of expression of IL-2Rβ on lymphoid precursor cells. As expected, Treg development was impaired, with a 2–3-fold reduction in the proportion of CD4+ Foxp3+ T cells from 2Rβ−/− and “cured” 2Rβ−/− mice (Fig. 1C, 1D). However, a substantial fraction of the CD4+ Foxp3+ cells in the thymus of the “cured” 2Rβ−/− mice were CD45.1+ donor cells. When considering only the recipient-derived 2Rβ−/− thymocytes, “cured” 2Rβ−/− mice contained a significantly (p < 0.0001) lower fraction (Fig. 1D) of CD4+ Foxp3+ T cells than found in untreated 2Rβ−/− mice. Because 2Rβ−/− and “cured” 2Rβ−/− mice contain similar numbers of CD4 SP (Fig. 1A, 1B), this difference also represents a proportional decrease in CD4+ Foxp3+ T cell numbers. This finding suggests that factors associated with the accompanying autoimmunity in untreated 2Rβ−/− mice may increase the number of developing CD4+ Foxp3+ cells.

IL-2–induced signaling promotes T cell proliferation (27) and survival (28). Thus, the lower numbers of CD4+ Foxp3+ thymocytes in 2Rβ−/− and “cured” 2Rβ−/− mice might reflect impaired proliferation or survival. By evaluating the proliferation marker Ki67 and the antiapoptotic marker Bcl-2, we found that the expression of Bcl-2 by 2Rβ−/− and host-derived “cured” 2Rβ−/− CD4+ Foxp3+ cells was high and comparable to WT Tregs (Fig. 2A, top panels, Fig. 2B left panel). In contrast, compared with Foxp3+ thymocytes from WT B6 mice, only host-derived 2Rβ−/− CD4+ Foxp3+ cells from “cured” 2Rβ−/− mice exhibited a lower fraction of Ki67+ cells (Fig. 2A, bottom panels, Fig. 2B, right panel).

FIGURE 1. Thymic Treg compartment in the absence of IL-2Rβ signaling. Distribution of DN, DP, SP CD4, and SP CD8 thymocytes (percentage of positive cells are shown in each quadrant) (A) and total thymic cellularity (B) for the indicated mice. Gating strategy (C) and the frequency (D) of Foxp3+ cells in SP CD4 T cells in the thymus of the indicated mice. Data in (B) and (D) are mean ± SD from five to eight mice/group. *p < 0.05, ***p < 0.001.
FACS analysis of CD4+ Foxp3+ thymocytes indicates that the level of whether the Foxp3+ cells were obtained from untreated autoimmune R mice is consistent with the notion that the inflammatory environment associated with autoimmunity in R mice somewhat increased the overall numbers of CD4+ Foxp3+ thymocytes in an IL-2-independent manner. The donor-derived WT Tregs in the thymus of cured 2R mice showed a slightly different pattern, primarily with regard to the detection of a readily measured fraction of Bcl–2lo cells (Fig. 2A); additionally, the mean fluorescence intensity (MFI) of Bcl-2 expression was lower, but this difference was not statistically significant (Fig. 2B).

**Phenotypically distinct CD4+ Foxp3+ thymocytes in mice lacking IL-2Rβ signaling**

FACS analysis of CD4+ Foxp3+ thymocytes indicates that the level of Foxp3 and CD25 was lower in the absence of IL-2Rβ-dependent signaling compared with WT Tregs (Fig. 3A). Quantitative analysis showed a 2-fold reduction in Foxp3 expression by CD4+ Foxp3+ cells from 2R−/− mice and host-derived cured 2R−/− mice (Fig. 3A). In addition, very few CD4+ Foxp3lo thymocytes expressed CD25 in the absence of IL-2Rβ signaling (Fig. 3A). This finding is consistent with a role for IL-2 in upregulating the expression of CD25 (29). More recently, genome-wide expression profiling showed that CD103, which marks activated Tregs, depends on IL-2R signaling (17). Expression of CD103 by CD4+ Foxp3+ thymocytes was also highly reduced in the absence of IL-2R signaling (Fig. 3B). In each of these cases mentioned above, an identical requirement for IL-2Rβ signaling was noted, regardless of whether the Foxp3+ cells were obtained from untreated autoimmune or cured 2Rβ−/− mice, suggesting that these activities are nonredundantly dependent upon IL-2. Because high Foxp3 expression is a determinant checkpoint for Treg lineage commitment (22, 24, 25), these findings also suggest that the developmental progression of Tregs is impaired in the absence of IL-2Rβ signaling. It is also noteworthy that the donor-derived WT Tregs in the thymus of 2R−/− mice expressed a phenotype that is markedly distinct from the host-derived 2Rβ−/− Foxp3hi cells (Fig. 3); these donor cells expressed the highest levels of Foxp3, CD25, and CD103, consistent with their peripheral origin and responsiveness to IL-2, which readily occurs in IL-2-deficient mice (30).

**Functional programming of developing Tregs depends on IL-2Rβ signaling**

A number of molecules have been associated with mediating Treg suppressive function (31). Three of these—CTLA4, CD39, and CD73—are readily measured by FACS analysis. In comparison with WT Tregs, 2Rβ−/− and cured 2Rβ−/− CD4+ Foxp3hi T cells showed a marked and an equivalent reduction in the expression of each of these suppressive mediators (Fig. 4). Thus, IL-2/IL-2R signaling is essential for the functional programming of thymic Tregs. This result also indicates that the uncontrolled autoimmunity associated with untreated 2R−/− mice is likely due to limited production of thymic CD4+ Foxp3lo T cells that do not express normal levels of suppressive molecules.

**WT thymic Foxp3lo CD25− T cells express properties similar to CD4+ Foxp3+ 2Rβ−/− thymocytes**

The preceding experiments indicated that the thymus from 2R−/− and cured 2Rβ−/− mice harbor mostly CD4+ Foxp3lo CD25− T cells with normal expression of Bcl-2 but impaired expression of CD103, CTLA4, CD39, and CD73. When one examines Tregs from the thymus of WT mice, a substantial fraction (20–30%) is harbored mostly CD4+ Foxp3lo CD25neg/lo. Therefore, we tested whether WT Foxp3lo and CD25neg/lo Tregs exhibited properties related to CD4+ Foxp3lo 2R−/− thymocytes. When WT Tregs were gated on CD25− Foxp3+ cells (Fig. 5A), most were Foxp3lo. Reciprocally, when WT Tregs were gated on Foxp3hi cells (Fig. 5B), the large majority were CD25−. Therefore, there is a readily measurable fraction of WT CD4+ Foxp3lo/ CD25− thymocytes. WT CD25− (Fig. 5A) or WT Foxp3lo (Fig. 5B) Tregs each showed reduced expression of CTLA4, CD103, CD39, and CD73 but a similar expression of Bcl-2 and Ki67 compared with CD25+ or Foxp3hi Tregs. This pattern of expression is highly analogous to that of

**FIGURE 2.** IL-2 in the regulation of Treg proliferation and Bcl-2 expression. Representative graphs (A) and expression (B) of Bcl-2 and Ki67 in thymic Tregs from WT and 2Rβ−/− mice and the host and donor compartment of cured 2Rβ−/− mice. The MFI for Bcl-2 is based on the total Tregs in the respective graph. Data are mean ± SD from five to eight mice/group. *p < 0.05. n.s., Not significant.

**FIGURE 3.** IL-2 regulates expression of Foxp3, CD25, and CD103 in thymic Tregs. (A) Representative dot plots and MFI of Foxp3 and percentage of CD25 expression by the indicated Treg populations. (B) Representative graphs and percentage of CD103 expression from the indicated thymic Tregs. Data are mean ± SD from three to eight mice/group. ***p < 0.001. n.s., Not significant.
CD4* Foxp3lo 2Rβ−/− thymocytes and indicates that these CD25− or Foxp3−/hi Tregs are not an aberrant cell population only associated with 2Rβ−/− mice. Collectively, these data demonstrate that CD4* Foxp3lo CD25− thymocytes represent a normal thymocyte population that arises as a consequence of Treg development and suggest that these Tregs in WT mice arise because they have not received a productive IL-2 signal.

The relationship between Foxp3lo CD25− and Foxp3hi CD25hi thymocytes

CD4* CD25* Foxp3− thymocytes contain precursors to Tregs (18). This population was readily found in the thymus of 2Rβ−/− mice (Fig. 6, P1 gate). As thymocytes mature, their expression of CD24 (HSA) is downregulated. Correspondingly, CD4* CD25* Foxp3− cells from WT and 2Rβ−/− mice expressed predominantly high levels of CD24. The CD4* CD25* Foxp3hi cells that dominate the P2 gate were mostly CD24− (Fig. 6), consistent with more mature T cells, although more cells with an immature CD24 phenotype were seen in the thymus of 2Rβ−/− mice. The CD4*

Fig. 4. IL-2 regulates the thymic Treg functional program. Representative graphs and MFI or percentages of CD39, CD73, and CTLA4 in thymic Tregs from WT or 2Rβ−/− mice and the host and donor compartment of “cured” 2Rβ−/− mice. Data are mean ± SD from three to eight mice/group. *p < 0.01, **p < 0.001.

CD4* CD25* CD4+ Foxp3hi thymic Tregs in normal mice. Representative dots plots (left panel) for CD25− versus CD25* (A) or Foxp3lo versus Foxp3hi (B) by thymic Tregs. Expression (middle and right panels) of the indicated markers for CD25− and CD25* (A) or Foxp3lo and Foxp3hi (B) Tregs. Data are mean ± SD from five or six mice/group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. n.s., Not significant.

Fig. 5. Properties of CD25− versus CD25* and Foxp3lo versus Foxp3hi thymic Tregs in normal mice. Representative dots plots (left panel) for CD25− versus CD25* (A) or Foxp3lo versus Foxp3hi (B) by thymic Tregs. Expression (middle and right panels) of the indicated markers for CD25− and CD25* (A) or Foxp3lo and Foxp3hi (B) Tregs. Data are mean ± SD from five or six mice/group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. n.s., Not significant.

The effect of suboptimal IL-2Rβ signaling on Treg development

We recently developed transgenic mice (Y3) on the 2Rβ−/− background whose T cells express Y→F mutations in three tyrosine residues (Y341, Y395, Y498) of IL-2Rβ that are required for normal recruitment of the adaptor Shc and STAT5 as a consequence of the IL-2/IL-2R interaction. Although this mutation leads to weak...
transient IL-2–induced p-STAT5, Y3 mice have no outward problems in Treg thymic development or peripheral homeostasis, as reflected in Treg number or frequency or Foxp3 levels (17). To further define the efficiency of this weak IL-2R signaling during thymic Treg development, CD4+ Foxp3hi versus Foxp3lo thymocytes from WT, Y3, and 2R−/− mice (Fig. 8A) were examined for expression of several molecules dependent on IL-2R signaling (Fig. 8B). Compared with the numerous Foxp3lo and the few Foxp3hi T cells in the thymus of 2R−/− mice, weak IL-2R signaling associated with Y3 IL-2Rβ−/− mice supported increased expression of CD25, CD103, and CTLA4. This effect was most striking for Foxp3hi Tregs and was generally comparable to expression of these molecules by WT Tregs, although CD25 expression by Y3 Foxp3hi Tregs was slightly, but not significantly, lower. Y3 Tregs showed reduced proliferation compared with WT and untreated 2R−/− CD4+ Foxp3hi cells. Because Ki67 expression was also somewhat reduced by the Foxp3hi cells from “cured” 2R−/− Tregs (Fig. 2), this result suggests that weak IL-2R signaling does not fully support proliferation of developing Tregs. In contrast, Bcl-2 levels were comparable for WT, Y3, and 2R−/− Foxp3hi versus Foxp3lo thymocytes. These data further link IL-2R signaling with Foxp3hi Tregs and indicate that functional programming and CD103-activated Tregs are readily supported by Y3 IL-2Rβ.

*p-STAT5 activation by Tregs in vivo*

p-STAT5 activation in vivo was assessed for WT, Y3, and 2R−/− CD4+ Foxp3hi thymocytes by immediately evaluating p-STAT5 expression without the addition of IL-2 (Fig. 9A, 9B). In comparison with CD4+ Foxp3hi 2R−/− T cells, Y3 Tregs expressed substantial p-STAT5, which was nearly comparable to that associated with WT Tregs. The lack of p-STAT5 expression by Foxp3hi cells from 2R−/− mice indicates that the in vivo p-STAT5 associated with WT and Y3 Tregs is primarily the result of IL-2R signaling. As noted previously (17), in vitro stimulation of Foxp3hi thymocytes with IL-2 resulted in most WT and Y3 Tregs to activate p-STAT5, although the percentage and MFI of p-STAT5+ cells were somewhat reduced for Y3 Tregs (Fig. 9A, 9B). Collectively, the nearly normal thymic development, as assessed by CTLA4, CD103, and CD25 expression, associated with Y3 Tregs is accounted for by relatively normal p-STAT5 signaling.

Past work indicated that an equivalent level of IL-2 in vitro activated greater p-STAT5 in thymic Tregs compared with peripheral Tregs from normal mice (17), suggesting that, during thymic development, Tregs potentially generate greater IL-2R signaling and are highly sensitive to an environment that is limiting in IL-2. This trend was also readily apparent when evaluating p-STAT5 activation in vivo. Tregs directly isolated from the thymus of WT mice showed a greater percentage (Fig. 9C) of...
FIGURE 8. Characterization of thymic Tregs with suboptimal IL-2R signaling. Representative dot plots (A) and expression of the indicated molecules (B) by Foxp3$^{lo}$ versus Foxp3$^{hi}$ thymic Tregs from the indicated mice. Data are mean ± SD from three to five mice/group. Statistically significant differences are shown for comparisons between WT and Y3 mice. $^*p < 0.05$.

p-STAT5$^+$ cells compared with Tregs from the spleens, whereas Tregs from Y3 thymus showed a similar percentage of p-STAT5$^+$ cells in the thymus and spleen (Fig. 9C). Importantly, compared with splenic Tregs, the level of p-STAT5 was nearly 2-fold greater for Tregs from the thymus of WT and Y3 mice (Fig. 9D). Thus, in the steady state in vivo, relatively normal p-STAT5 activation is observed with regard to thymic Y3 Tregs, including a higher activation of this IL-2R–associated signaling molecule.

Competitive thymic development and weak IL-2R signaling

Although several properties associated with Y3 Tregs appeared outwardly normal, Y3 Tregs showed somewhat lower proliferation as assessed by Ki67 expression. To more broadly evaluate the efficiency of IL-2R signaling associated with Y3 IL-2R$^b$, mixed bone marrow chimeras were prepared to compare the development of Y3 versus WT Tregs in the same environment (Fig. 10A). Development by WT CD45.1$^+$ B6 Tregs was compared with CD45.2$^+$ Tregs expressing transgenic WT Y0 or Y3 IL-2R$^b$ (on the 2R$^β$−/− genetic background) after a 1:1 mixture of the respective T cell–depleted bone marrow was transferred into lethally irradiated Thy-1.1$^+$ WT or sublethally irradiated Rag 2$^{-/-}$ $γc$ $−/−$ recipients. Y3 mice express levels of transgenic IL-2R$^b$ that is similar to WT B6 mice, whereas those of Y0 mice are ~2-fold higher (17). In other experiments, development of CD45.1$^+$ WT B6 Tregs was compared with CD45.2$^+$ WT B6 Tregs or Y3 Tregs after a 1:1 or 1:4 mixture of the respective T cell–depleted bone marrow was transferred into sublethally irradiated TCRα$−/−$ recipients. The gating strategy used to follow donor thymic- and spleen-derived SP CD4$^+$ Foxp3$^+$ conventional T cells and CD4$^+$ Foxp3$^+$ Tregs is shown in Fig. 10B.

Thymic reconstitution was largely comparable (60–100 × 10$^6$ cells) when recipients were examined 8 wk after bone marrow transfer. The relative representation of donor CD4 SP T cells and Tregs of CD45.2$^+$ Y0, Y3 and B6 origin to control donor WT CD45.1$^+$ origin was evaluated from the thymus and spleen for each recipient. We expected the ratio of experimental CD45.2$^+$/control WT CD45.1$^+$ T cells to be 1 in the thymus when equal numbers of bone marrow cells were transferred, if both bone marrow donors contained an equivalent number of precursor cells. However, these ratios were often <1 when Y0 or Y3 bone marrow was mixed with CD45.1$^+$ WT bone marrow, suggesting that the numbers of progenitors were lower in mice with the 2R$^β$−/− genetic background. Overall, this lower ratio was consistent when examining major thymic subpopulations (i.e., DN, DP, and CD4 and CD8 SP cells; data not shown).

To correct for this effect and assess the efficiency that Y3 Tregs compete with WT CD45.1 Tregs in the thymus and spleen, we considered the relative representation of Y3 Tregs and Y3 conventional CD4$^+$ SP T cells from these tissues (Fig. 10C, left panel). As a control, CD45.2$^+$ B6 WT Tregs competed almost equivalently with CD45.1$^+$ WT Tregs at a 1:1 or 4:1 ratio of donor cells in the thymus based on the relative development of SP CD4$^+$ thymocytes. In contrast, there was a consistent trend in all experiments for a lower relative representation of Y3 Tregs when they developed in the thymus in competition with WT CD45.1$^+$ Tregs. To assess the extent to which constitutive expression of transgenic IL-2R$^b$ might influence this type of competitive experiment, the development of Tregs in the thymus was compared from mice transgenic IL-2R$^b$ expressing transgenic IL-2R$^b$ in the thymus to determine the impact of thymus competition with WT CD45.1$^+$ Tregs. These experiments showed a preference for Tregs expressing the WT Y0 IL-2R$^b$ transgene, consistent with higher levels of this transgenic IL-2R$^b$. Collectively, these data indicate that impaired IL-2R signaling by Y3 thymocytes support Treg development in a competitive setting with WT Tregs, but WT Tregs are somewhat favored. This finding is in striking contrast to the spleen, where nearly the entire Treg pool is derived from WT Tregs.

FIGURE 9. p-STAT5 activity of thymic Tregs. Representative graphs (A) and percentage and MFI (B) of p-STAT5 expression by Tregs from the indicated mice. Data are mean ± range from two mice/group. Percentage (C) and relative MFI (thymus:spleen) (D) of p-STAT5 expression directly ex vivo by WT B6 and Y3 thymic and splenic Tregs. Data are mean ± SD from five mice. $^*p < 0.05$. ns, Not significant.
Thus, impaired IL-2R signaling is much more efficient in maintaining thymic Treg development in comparison with peripheral homeostasis.

The expression of CD25, which depends on IL-2R signaling, by Y3 thymic and peripheral Tregs is comparable to normal WT Tregs in a noncompetitive setting (17). Therefore, we assessed CD25 expression by the various Treg populations as an indication of the level of IL-2R signaling in these mixed chimeras (Fig. 10D). CD25 expression levels of CD45.2+ donor-derived Tregs were normalized to control CD45.1+ WT Tregs. In all cases, the levels of CD25 expressed by Y3 Tregs were decreased. In the thymus, CD25 levels were directly proportional to the overall representation of Y3 Tregs, with greater CD25 expression levels by Y3 Tregs correlating with greater representation of Y3 Tregs. Although very few Y3 Tregs were found in the spleen, the level of CD25 was similar to that in the thymus. This latter finding is consistent with these splenic and thymic Tregs receiving a similar IL-2R signal. This finding further supports the notion that thymic Treg development is generally effective with low IL-2R signaling.

Discussion

Current work indicates that IL-2 provides essential signals during thymic Treg development through activation of STAT5 (15). IL-2 is generally thought to function as a growth/survival signal as Tregs develop in the thymus. This conclusion is based primarily on the finding that the thymus of IL-2−/− or IL-2R−/− mice harbors somewhat lower numbers of CD4+ Foxp3+ Tregs. The detection of CD4+ Foxp3+ Tregs in the periphery of IL-2−/− and IL-2R−/− mice, although at a markedly reduced proportion, has led to the common view that autoimmunity associated with these animals primarily represents a defect in Treg peripheral homeostasis (4). However, recent work showed that when peripheral Treg numbers and proportions were restored to normal levels in IL-2−/− mice by abrogating Bim expression, severe lethal autoimmunity still occurred (21). This effect was associated with impaired Treg function, although another contributing factor may be increased Th17 cells that were not antagonized by the lack of IL-2 (30).

In this study, we directly show that thymic development of CD4+ Foxp3+ Tregs is highly dysregulated in 2R−/− mice. Although IL-2Rβ is also involved in IL-15 signaling, we interpreted this impairment to primarily reflect a role for IL-2, because IL-15 knockout mice exhibit normal Treg production and do not exhibit autoimmunity (36). Our data are consistent with a thymic Treg developmental block in 2R−/− mice that gives rise predominantly to functionally immature CD4+ Foxp3+ CD25− Tregs that express markedly reduced levels of key molecules (i.e., CTLA, CD39, and...
CD73) that mediate suppressive function. Low levels of these molecules were also detected in IL-2−/− Bim−/− mice (21). These findings, coupled with our results, indicate that the impaired function of normal levels of CD4+ Foxp3hi CD25+ T cells in the periphery of these double-knockout mice is the result of impaired functional programming of Tregs during thymic development. Thus, the primary explanation for autoimmunity in the absence of IL-2R signaling is that Tregs produced in the thymus are developmentally blocked and largely nonfunctional.

The detection of primarily CD4+ Foxp3hi CD25− Tregs in the thymus of 2R−/− mice does not represent some aberrant cell populations due to high cytokine production as a consequence of autoimmunity. In this regard, most of the properties associated with these Tregs were also observed when we evaluated thymic host-derived CD4+ Foxp3+ T cells from 2R−/− mice that were rendered autoimmune-free by providing them WT Tregs at birth. In addition, the thymus of WT mice harbors a substantial proportion of CD4+ Foxp3hi CD25hi cells with low expression of CTLA4, CD39, and CD73. Thus, the detection of these Tregs in the thymus of 2R−/− mice likely reflects some block during Treg development.

A simple explanation for the impaired expression of Treg functional molecules in developing Tregs is that their lower levels of Foxp3 are not sufficient to properly induce and reinforce the Treg-suppressive program (25). It is important to note that we also detected a small fraction of Foxp3hi Tregs in the thymus of 2R−/− mice. However, these cells still expressed a low level of CTLA4, even though CTLA4 is a direct target of Foxp3 regulation (37), and an increased proportion of these cells was CD24hi, which is indicative of a more immature thymocyte population. This finding indicates that normal levels of Foxp3 are not sufficient to induce the Treg-suppressive program without IL-2R signaling, as well as that these cells are not equivalent to CD4+ Foxp3hi CD25− Tregs in WT mice. This observation also raises the possibility that IL-2 exerts several important effects during thymic development to yield a mature functional Treg that goes beyond its known role in contributing to the activation of Foxp3 through STAT5 (19).

Our findings also support a role for IL-2 in the expansion of developing Tregs. This conclusion is based on the lower levels of the Kif67 proliferative marker expressed by the developmentally impaired Tregs found in the thymus of “cured” 2R−/− mice. However, these Tregs from untreated 2R−/− mice showed similar Kif67 expression to total WT Tregs (Fig. 2) or to WT Foxp3hi and Foxp3hi thymocytes, suggesting that some factors present as a result of the concomitant autoimmunity associated with IL-2R deficiency may somewhat compensate for impaired IL-2R signaling by increasing the numbers of immature Tregs. We cannot rule out that the selective reduced impaired host Treg compartment of “cured” 2R−/− thymus might actually reflect competition for resources or niche due to the presence of donor WT Tregs used to prevent autoimmunity that also populate the thymus. However, we also noted that Foxp3hi and Foxp3hi Y3 thymic Tregs expressed lower Kif67, which is consistent with a role for IL-2 in supporting proliferation of developing Tregs. Although Bcl-2 is an important antiapoptotic gene, and its expression is regulated by IL-2 (28), we found normal Bcl-2 levels in developing Tregs from untreated 2R−/−, “cured” 2R−/−, and Y3 mice, indicating that IL-2 is not required for the expression of Bcl-2 by thymic Tregs and suggesting that IL-2R signaling may not significantly affect the survival of developing Tregs.

An important feature of developing Tregs is that these cells are exquisitely sensitive to IL-2. This was shown in two ways. First, WT thymic Tregs showed increased p-STAT5 activation compared with peripheral Tregs. Second, in competitive bone marrow chimeras, thymic Treg development was supported to a much greater level than was peripheral Treg homeostasis by Tregs expressing the Y3 mutant IL-2R. Thus, this increased IL-2R sensitivity to p-STAT5 activation is very effective in driving Treg development in the thymus. It will be of interest to determine the molecular basis of this phenomenon.

Past work indicates that CD4+ CD25+ Foxp3+ thymocytes contain precursors for the Treg lineage (18, 19). Our findings provide some information concerning the developmental origin of CD4+ Foxp3hi Tregs present in the thymus of WT and 2R−/− mice. It is unlikely that CD4+ Foxp3hi CD25− cells represent a mandatorily developmental intermediate between CD4+ CD25+ Foxp3+ thymocytes and mature CD4+ Foxp3hi CD25+ Tregs. First, ontogeny studies indicate that the appearance of CD4+ Foxp3hi CD25+ cells does not precede, but rather is concurrent with, the appearance of CD4+ Foxp3hi CD25+ Tregs. Second, the pattern of expression of CD24 by CD4+ Foxp3hi CD25− cells from the thymus of WT and 2R−/− mice was identical to that by CD4+ Foxp3hi CD25hi Tregs from WT mice, in which mature CD24− cells predominated. This latter result suggests that the CD4+ Foxp3hi CD25− cells undergo similar levels of some Treg maturation processes but fail to optimally express Treg functional molecules. Given the strong relationship between CD4+ Foxp3hi CD25− cells from WT and 2R−/− mice, the failure of these cells to mature fully is primarily accounted for by a lack of IL-2R signaling. The production of CD4+ Foxp3hi CD25− T cells likely depends on IL-7R signaling in the context of TCR and costimulatory signals, because no Foxp3+ cells are found when both IL-2 and IL-7 are absent (12). Therefore, we favor an alternative scenario wherein these CD4+ Foxp3hi CD25− Tregs represent abortive products of the Treg-developmental scheme. In this model, IL-2 directly drives maturation of CD4+ Foxp3+ CD25+ thymocytes into mature CD4+ Foxp3hi CD25+ Tregs; Foxp3 and CD25 are direct targets of IL-2R-dependent STAT5 activation (39). However, through the selection process, some thymocytes are also destined to become Tregs; however, without an IL-2 signal their full development is prevented, and they are arrested at the CD4+ Foxp3hi CD25+ stage. Because of their high Bcl-2 levels, these cells survive and seed the periphery, but they cannot suppress peripheral autoreactive T cells because of the impaired expression of Treg functional molecules. In WT mice, the detection of these cells in the thymus may simply represent developing Tregs that have never received an IL-2R signal. However, we cannot rule out that some of these CD4+ Foxp3hi CD25− T cells are derived from the dedifferentiation of mature CD4+ Foxp3hi CD25+ Tregs. We do not think that this is a major source of CD4+ Foxp3hi CD25− thymocytes because these cells are readily found in 2R−/− mice that contain very few mature CD4+ Foxp3hi CD25+ Tregs and because recent studies indicate that thymic-derived Foxp3+ Tregs are a stable lineage (40).

Disclosures

The authors have no financial conflicts of interest.

References


