Thymic regulatory T cell niche size is dictated by limiting IL-2 from antigen-bearing dendritic cells and feedback competition

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The thymic production of regulatory T cells (Treg cells) requires interleukin 2 (IL-2) and agonist T cell antigen receptor (TCR) ligands and is controlled by competition for a limited developmental niche, but the thymic sources of IL-2 and the factors that limit access to the niche are poorly understood. Here we found that IL-2 produced by antigen-bearing dendritic cells (DCs) had a key role in Treg cell development and that existing Treg cells limited new development of Treg cells by competing for IL-2. Our data suggest that antigen-presenting cells (APCs) that can provide both IL-2 and a TCR ligand constitute the thymic niche and that competition by existing Treg cells for a limited supply of IL-2 provides negative feedback for new production of Treg cells.

Tolerance to self requires an intact regulatory T cell (Treg cell) pool that acts to limit autoimmunity and maintain homeostasis within the immune system. Treg cells develop within the thymus from CD4+ single-positive (CD4SP) thymocytes, as well as extrathympically from conventional CD4+ T cells. Neonatal thymectomy leads to autoimmunity, which illustrates the importance of maintaining proper thymic Treg cell output1,2. Although thymus-derived Treg cells and extrathympically derived Treg cells overlap in their functional capacity, thymus-derived Treg cells are more stable under inflammatory conditions3. Therefore, understanding the factors that govern Treg cell development in the thymus is important for the design of strategies to generate large, stable Treg cell populations for immunotherapy4,5.

Several reports have delineated a two-step process that results in the thymic generation of Treg cells6–7. First, CD4SP thymocytes must receive relatively strong signals through the T cell antigen receptor (TCR), a process that allows transcriptional changes and increases in cell-surface expression of the high-affinity α-chain of the interleukin 2 (IL-2) receptor (CD25). Signaling by IL-2 via the signal transducer STAT3 is needed for complete development, which leads to induction of the Treg cell–defining transcription factor Foxp3. Although many studies have documented the requirements for strong TCR signals and IL-2 in Treg cell development6–9, less is known about how these requirements are integrated. In particular, it is not known whether TCR ligands and IL-2 signals must be spatially linked to efficiently promote Treg cell development.

Studies of mice expressing rearranged, Treg cell–biased Tcra and Tcrb transgenes have revealed that Treg cell development is most efficient when only a small fraction of thymocytes express a particular TCR10,11. In addition, limiting intraclonal competition leads to increased TCR signaling, which suggests that binding of the TCR to self peptide and major histocompatibility complex (MHC) can be a limiting factor when the frequency of thymocytes expressing a defined TCR is high8. Whether competition for IL-2 is also involved in establishing the size of the thymic Treg cell niche remains unknown.

Understanding the nature of the Treg cell niche is complicated by the fact that the thymic source of IL-2 remains unknown. In the periphery, T cells are the most abundant producers of IL-2, which has led to the suggestion that thymocytes may provide IL-2 to developing Treg cells. However, there are also reports that dendritic cells (DCs) can produce limited quantities of IL-2 in certain settings12,13. Given indications that IL-2 concentrations are limiting for the thymic development of Treg cells14–16, identifying the sources of IL-2 in the thymus, as well as the factors that govern its availability to developing Treg cells, is key to defining the thymic Treg cell niche.

We have developed an experimental system in which thymocytes expressing a transgene encoding a defined MHC class II–specific TCR are introduced into a thymic tissue slice in the presence of their cognate antigen, which leads to a synchronized wave of Treg cell development. Using this system, we found that antigen-bearing DCs provided a local source of IL-2 to promote Treg cell development. We also found that existing Treg cells within the thymic environment inhibited new Treg cell development by limiting the supply of available IL-2. Our data suggest a model in which localized antigen presentation and IL-2 supply, along with competition for IL-2 from existing Treg cells, establish a tightly controlled but flexible negative feedback loop to maintain balanced production of Treg cells.

RESULTS

Treg cell development in thymic tissue slices

Published reports have suggested that the thymic development of Treg cells is limited by the frequency of thymocytes specific for a particular

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self antigen and competition for the relevant self antigen\textsuperscript{8–11,17}. To further investigate the limited niche for T\textsubscript{reg} cell development, we used a thymic slice model in which a small number of thymocytes bearing a defined MHC class II–restricted TCR (OT-II) develop in the presence of their cognate antigen (ovalbumin (OVA)). We used OT-II thymocytes from a recombination activating gene 2–deficient (Rag\textsuperscript{2–/–}) background (called simply ‘OT-II thymocytes’ here) as a starting population with no detectable T\textsubscript{reg} cells; this provided a sensitive and synchronized system with which to track the development of new T\textsubscript{reg} cells in response to antigen encounter within the thymic slice. We gave wild-type mice an intravenous injection of 2 mg of soluble OVA protein, an approach that has been shown to induce antigen-specific thymic T\textsubscript{reg} cells in vivo\textsuperscript{18,19}. One hour after injection, we killed the mice, prepared thymic slices and introduced OT-II thymocytes into the slices. Flow cytometry revealed upregulation of CD25 expression on OT-II CD4SP thymocytes after 1 d of culture and the appearance of Foxp3\textsuperscript{+}CD25\textsuperscript{+} cells after 3 d (Supplementary Fig. 1), consistent with the timing of T\textsubscript{reg} cell development inferred from intrathymic transfer studies\textsuperscript{7}.

T\textsubscript{reg} cells can develop from immature T cells in the thymus or can be derived from mature peripheral CD4\textsuperscript{+} T cells by TCR stimulation in the presence of the immunosuppressive cytokine transforming growth factor–β (TGF-β)\textsuperscript{20}. To confirm that the thymic slice system recapitulated normal thymic T\textsubscript{reg} cell development, we compared the ability of OT-II thymocytes versus that of mature OT-II T cells to give rise to T\textsubscript{reg} cells upon introduction into thymic slices (Supplementary Fig. 2a). Mature OT-II T cells showed some upregulation of CD25 expression, but no Foxp3 expression, upon culture in thymic slices prepared from mice that had previously been given an injection of OVA protein (Supplementary Fig. 2a). The addition of TGF-β led to a small population of Foxp3\textsuperscript{+} cells; however, these cells had low expression of the thymus–derived T\textsubscript{reg} cell marker neuropilin-1 (refs. 21,22) (Supplementary Fig. 2a). In contrast, OT-II thymocytes cultured in thymic slices gave rise to Foxp3\textsuperscript{+} cells without the addition of TGF-β, and they also expressed neuropilin-1 (Supplementary Fig. 2a). Neither OT-II thymocytes nor mature T cells gave rise to T\textsubscript{reg} cells when stimulated with OVA-loaded DCs in vitro without thymic slices and without the addition of TGF-β (Supplementary Fig. 2b). Moreover, depletion of mature CD4SP cells from OT-II thymocyte samples before their addition to thymic slices delayed, but did not prevent, the development of T\textsubscript{reg} cells (Supplementary Fig. 2c,d and data not shown), which indicated that Foxp3\textsuperscript{+} cells arose from a relatively immature thymocyte population. These data indicated that the development of Foxp3\textsuperscript{+} cells from OT-II thymocytes on thymic slices was distinct from the in vitro development of T\textsubscript{reg} cells from mature conventional CD4\textsuperscript{+} T cells and suggested that the combination of the thymic environment and the developmental stage of the precursor cells was important for the development of thymus–derived T\textsubscript{reg} cells.

To probe the role of antigen availability in T\textsubscript{reg} cell development, we varied the antigen dose and delivery method. Injection of a high dose of soluble OVA led to the development of OT-II T\textsubscript{reg} cells after 3 d on thymic slices, whereas injection of one tenth the dose of OVA led to reduced but detectable development of T\textsubscript{reg} cells (Fig. 1a).

**Figure 1** Limited T\textsubscript{reg} cell development in thymic slices regardless of the route of antigen delivery. (a–c) Flow cytometry of OT-II thymocytes cultured for 3 d on thymic slices in the presence or absence of the cognate antigen OVA, obtained from wild-type mice given no injection (0) or intravenous injection of 0.2 or 2 mg of OVA protein 1 h before slice preparation (a), from RIP–mOVA transgenic or control nontransgenic mice (b), and from wild-type mice, with the addition of bone marrow–derived DCs loaded with various concentrations (above plots (left) or horizontal axis (right)) of OVA protein (c), presented as flow cytometry of gated OT-II donor CD4SP thymocytes (left) and quantification of the total OT-II T\textsubscript{reg} cells (CD25\textsuperscript{+}Foxp3\textsuperscript{+}) recovered per slice (right). Numbers in quadrants (left) indicate percent cells in each throughout. ND, not detectable. (d) Efficiency of T\textsubscript{reg} cell development in cultures of OT-II thymocytes on thymic slices as in a (OVA i.v.), b (RIP–mOVA) and c (DC + OVA), presented as the ratio of OT-II Foxp3\textsuperscript{+} CD4SP thymocytes recovered per slice to resident CD4SP thymocytes in the slice (OT-II T\textsubscript{reg}/CD4\textsuperscript{+} thymocyte), or as the ratio of T\textsubscript{reg} cells resident in the slice (Resident T\textsubscript{reg}) (pooled from a–c to resident CD4SP thymocytes (dotted line indicates mean resident T\textsubscript{reg} cell ratio). Each symbol represents an individual slice; small horizontal lines indicate the mean (± s.e.m.). *P < 0.01 and **P < 0.001 (one-way analysis of variance (ANOVA) with Tukey’s post-test analysis (a,c) or unpaired Student’s t-test (b)). Data are pooled from three experiments (a; mean and s.e.m. of n = 12 slices (0 mg), 10 slices (0.2 mg) or 13 slices (2 mg)) or are representative of three independent experiments (b; mean and s.e.m. of n = 3 slices (WT) or 13 slices (RIP–mOVA)), five independent experiments (c; mean and s.e.m. of n = 5 slices (0 mg/ml) or 6 (0.01 mg/ml, 0.1 mg/ml and 1 mg/ml)) or one experiment (d; DC + OVA (0 mg)), two experiments (d; OVA i.v. (0.2 mg or 2 mg) and DC + OVA (0.01, 0.1 or 1 mg/ml)), three experiments (d; OVA i.v. (0 mg) and (RIP–mOVA)) or four experiments (d; resident T\textsubscript{reg}).
We observed no expression of CD25 or Foxp3 when we cultured OT-II thymocytes in slices from control mice given an injection of PBS (Fig. 1a). As an alternative mode of antigen delivery, we used mice in which a membrane-associated form of OVA is transgenically expressed in medullary thymic epithelial cells and the pancreas (RIP-mOVA mice)29. Again, we observed a substantial population of CD25+Foxp3+ OT-II thymocytes after 3 d of culture on thymic slices from RIP-mOVA mice (Fig. 1b). As a third approach to introducing antigen, we incubated bone marrow–derived DCs with OVA protein and then introduced them into thymic slices containing OT-II thymocytes by overlaying them on top of the slice and allowing them to migrate into the tissue. Again we observed new Treg cell development, which increased with antigen dose (Fig. 1c). In contrast to published reports10,11, we did not observe any correlation between the frequency of OT-II thymocytes within thymic slices and the efficiency of Treg cell development (Supplementary Fig. 3), which indicated that the frequency of precursor cells was not a major limiting factor under our experimental conditions.

To compare the efficiency of Treg cell development across various routes of antigen delivery, we calculated the number of Foxp3+ OT-II Treg cells as a ratio of the total CD4SP thymocytes in the thymic slice. The efficiency of Treg cell development varied with antigen abundance (Fig. 1d), consistent with published studies8,18. However, the proportion of OT-II thymocytes that developed into Treg cells was generally less than 20% (Fig. 1 and Supplementary Fig. 3) and never exceeded the proportion of endogenous Treg cells within a slice (corresponding to 3–10% of total slice CD4SP thymocytes) (Fig. 1d). We observed this upper limit on Treg cell development even under conditions in which the majority of thymocytes responded to antigen, as indicated by the induction of CD25 expression (Fig. 1c, d). Together these results suggested there was a limited thymic niche for Treg cell development even when antigen encounter and the frequency of precursor cells were not limiting factors.

Existing thymic Treg cells reduce the niche size

The finding that the overall number of thymic Treg cells was limited to less than 10% of the total CD4SP population suggested that Treg cells themselves might inhibit new Treg cell development. To investigate whether existing Treg cells influence new Treg cell development, we prepared thymic slices from mice that lack thymic Treg cells (mice with transgenic expression of a TCR specific for MHC class II) together with mutation of Rag2 (AND × Rag2−/− mice)24. For these experiments, we introduced antigen by adding 1 μM OVA peptide (amino acids 323–339) to thymic slices containing OT-II thymocytes. We consistently observed a greater number of CD25+Foxp3+ OT-II thymocytes in Treg cell–deficient (AND × Rag2−/−) thymic slices than in Treg cell–sufficient (wild-type) thymic slices (Fig. 2a, b). We also observed enhancement of new Treg cell development in Treg cell–deficient slices when we used precursor cells with a different MHC class II–specific TCR (2D2 × Rag2−/−) (Supplementary Fig. 4a, b) and when we used thymic slices from a different Treg cell–deficient strain (mice with transgenic expression of a TCR specific for MHC class I together with mutation of Rag2 (F5 × Rag2−/−)) (Supplementary Fig. 4c, d). Finally, Treg cell development in thymic slices from mice with transgenic TCR expression (AND × Rag2−/−) was inhibited by the addition of sorted thymic Treg cells to thymic slices (Fig. 2c, d), which indicated that the lack of Treg cells, rather than other abnormal features of the thymic environment with transgenic TCR expression, was responsible for the enhanced Treg cell development. Combined these results provided evidence that existing thymic Treg cells limited the development of new Treg cells.

Treg cells do not interfere with peptide recognition

Treg cells can reduce the stimulatory capacity of antigen-presenting cells (APCs) in the periphery, which suggests that thymic Treg cells might inhibit new Treg cell development by interfering with the ability of thymic APCs to present agonist self peptides25. To test this idea we assessed expression of the transcription factor Nur77, which provides a quantitative ‘readout’ of accumulated TCR signal strength8. The addition of OVA peptide to Treg cell–sufficient or Treg cell–deficient thymic slices resulted in similar expression of CD25 and Nur77 by OT-II thymocytes at 6 h (Fig. 3a–c), which suggested that existing Treg cells did not influence the initial TCR signal received by OT-II thymocytes. As a measure of accumulated TCR signal, we also assessed Nur77 expression within the OT-II Treg cell population that developed after 3 d in the presence or absence of existing Treg cells (Fig. 3d). Consistent with published reports8, endogenous Treg cells from the thymic slice had slightly higher expression of Nur77 than did conventional CD4SP thymocytes (Fig. 3e), and newly developed OT-II Treg cells had slightly higher expression of Nur77 than did endogenous Treg cells in the slice (Fig. 3e, teal and magenta lines). Although the overall proportion of OT-II Treg cell development was enhanced in the absence of existing Treg cells, Nur77 expression within the OT-II...
T\textsubscript{reg} cell population was similar for OT-II T\textsubscript{reg} cells that developed on T\textsubscript{reg} cell–sufficient (wild-type) slices and those that developed on T\textsubscript{reg} cell–deficient (F5 \times Rag\textsuperscript{2/-/-}) thymic slices (Fig. 3e). Together these results provided evidence that existing T\textsubscript{reg} cells were able to limit new T\textsubscript{reg} cell development without altering TCR signaling.

**IL-2 availability influences the size of the T\textsubscript{reg} cell niche**

In addition to requiring agonist self peptide, developing T\textsubscript{reg} cells need IL-2 to fully mature and express Foxp3 (refs. 6, 7). To confirm the requirement for IL-2 in the thymic slice model, we used OVA protein–injected IL-2–/- mice as a source of thymic slices for T\textsubscript{reg} cell development. Half the number of OT-II thymocytes developed into T\textsubscript{reg} cells after 3 d on IL-2–/- slices relative to the number that developed on wild-type slices (Fig. 4a,b). This reduction in OT-II T\textsubscript{reg} cell development was mirrored by the reduction in endogenous T\textsubscript{reg} cells from IL-2–/- mice, which confirmed the requirement for IL-2 for efficient thymic T\textsubscript{reg} cell development and maturation\textsuperscript{14-16}. Notably, these data indicated that any IL-2 produced by added OT-II thymocytes was not able to fully compensate for the lack of IL-2 from the thymic environment, which highlighted the importance of IL-2 sources within the thymic slice itself.

To determine whether IL-2 is a limiting factor for T\textsubscript{reg} cell development, we assessed the effect of the addition of IL-2. We used complexes of IL-2 and antibody to IL-2 (anti-IL-2), which display an extended half-life compared with that of IL-2 alone\textsuperscript{26,27}. The addition of IL-2–anti-IL-2 complexes to thymic slices doubled the number of OT-II T\textsubscript{reg} cells that developed per slice in both the wild-type environment and the IL2–/- environment (Fig. 4a,b), which suggested that the availability of IL-2 was a limiting factor for expansion of the T\textsubscript{reg} cell niche. The addition of IL-2–anti-IL-2 complexes boosted the number of endogenous T\textsubscript{reg} cells within the slice (Fig. 4c), which indicated that IL-2 was also limiting for T\textsubscript{reg} cells in the steady-state thymus. It is noteworthy that the addition of IL-2–anti-IL-2 complexes did not fully restore the number of T\textsubscript{reg} cells in IL2–/- slices (Fig. 4b,c), which suggested a requirement for a localized source of IL-2 for efficient T\textsubscript{reg} cell development or an altered thymic environment in IL2–/- mice. Together these data indicated that IL-2 was both necessary and limiting for thymic T\textsubscript{reg} cell development within thymic slices.

**Existing T\textsubscript{reg} cells limit the availability of IL-2 and/or IL-15**

On the basis of reports that peripheral T\textsubscript{reg} cells can limit T cell effector function via absorption of IL-2 from the environment\textsuperscript{28,29}, we considered that thymic T\textsubscript{reg} cells might inhibit new T\textsubscript{reg} cell development by limiting the available cytokine supplies. To detect available IL-2 and IL-15 (a related cytokine that also supports T\textsubscript{reg} cell development\textsuperscript{14}), we used the mouse cell line CTLL2, which is dependent on IL-2 or IL-15 (ref. 30). CTLL2 cells proliferated when added to wild-type thymic slices (Fig. 5a), consistent with the presence of available IL-2 and IL-15 within the tissue. Moreover, CTLL2 cells cultured on T\textsubscript{reg} cell–deficient (AND \times Rag\textsuperscript{2/-/-}) slices showed enhanced survival at day 2 and more robust proliferation on day 4 (Fig. 5a,b), which suggested greater abundance of available IL-2 and/or IL-15 in the T\textsubscript{reg} cell–deficient environment than in the T\textsubscript{reg} cell–sufficient environment.
To further probe the role of Treg cells in limiting IL-2 availability within the thymus, we added IL-2−anti-IL-2 complexes to thymic slices prepared from wild-type and Treg cell–deficient (AND x Rag2−/−) mice that had been given an injection of OVA protein. The addition of IL-2−anti-IL-2 complexes to Treg cell–sufficient wild-type slices increased the frequency and absolute numbers of OT-II Treg cells twofold (Fig. 5c,d). Moreover, the addition of IL-2−anti-IL-2 complexes to Treg cell–deficient slices enhanced Treg cell development fourfold. These experiments demonstrated that while existing Treg cells within the thymus limited IL-2 availability, IL-2 remained a limiting factor for new Treg cell development even in the absence of existing Treg cells. These data also indicated that existing Treg cells were able to inhibit new Treg cell development even in the presence of large quantities of IL-2, which suggested tight feedback control of IL-2 availability.

APCs provide a local source of IL-2 for Treg cell development

We next investigated the cellular source of IL-2 for thymic Treg cell development. While T cells are typically considered the main producers of IL-2, our observation that wild-type OT-II thymocytes were unable to restore Treg cell development in IL2−/− thymic slices suggested that thymocytes might not provide an important source of IL-2 for Treg cell development. Published reports have suggested that DCs are able to express small amounts of IL-2 in certain settings<sup>12,13,31</sup>. To investigate whether antigen-bearing DCs might provide a local source of IL-2, we compared Treg cell development promoted by wild-type antigen-loaded DCs versus that promoted by IL2−/− antigen-loaded DCs. DCs derived in vitro from the bone marrow of young, asymptomatic IL2−/− mice expressed cell surface markers similar to those expressed by wild-type bone marrow–derived DCs (data not shown). Moreover, when we incubated IL2−/− DCs with OVA protein and introduced the cells into thymic slices, they led to normal upregulation of the expression of TCR-activation markers on OT-II thymocytes (Supplementary Fig. 5a–d), which indicated that they had a normal capacity to process and present antigen. However, OT-II Treg cell development promoted by OVA-loaded IL2−/− DCs was only half that promoted by OVA-loaded wild-type DCs (Fig. 6a–c). As expected, there was no change in overall number of endogenous Treg cells in the slices, regardless of whether the added DCs were IL-2 deficient or IL-2 sufficient (Fig. 6d), which indicated that the addition of wild-type DCs did not globally increase the abundance of IL-2 in the environment. The addition of IL-2–sufficient OVA-loaded DCs to IL-2–deficient thymic slices increased Treg cell development over that observed when both DC and slices were deficient in IL-2 (Supplementary Fig. 6). Together these results indicated that the

Figure 5 Existing Treg cells limit available IL-2 and/or IL-15 within the thymus. (a) Proliferation of eFluor 450–labeled CTL2 cells after culture for 4 d on thymic slices from wild-type mice (WT) or AND mice (−Treg), as well as of eFluor 450–labeled wild-type thymocytes in the same slice (non-dividing reference population (Ref)). (b) Survival of CTL2 cells following 2 d of culture as in a, presented relative to that of the reference wild-type thymocyte population (CTLL2/Ref). (c) Development of OT-II Treg cells on thymic slices prepared from wild-type or Treg cell–deficient mice given intravenous injection of 2 mg of OVA protein; cultures were treated with IL-2−anti-IL-2 complexes daily (+IL-2) or not (Ctrl) and assessed by flow cytometry. (d) Quantification of OT-II Treg cells (CD25+Foxp3+) recovered per slice, as in c, *P < 0.05 and **P < 0.001 (Student’s t-test) or one-way ANOVA with Tukey’s post-test analysis (d). Data are representative of n (a,b) or pooled from (c,d) three independent experiments (error bars, s.e.m. of n = 6 slices per group (b), or n = 9 (WT Ctrl), 11 (WT + IL-2 and −Treg + IL-2) or 15 (−Treg Ctrl) slices per group (d)).

Figure 6 Antigen-bearing DCs provide a local source of IL-2 to developing thymic Treg cells. (a–c) Development of OT-II Treg cells cultured for 3 d with wild-type thymic slices, along with wild-type or IL2−/− bone marrow–derived DCs loaded with OVA protein (1 mg/ml), assessed by flow cytometry of gated OT-II CD4+45 cells (a) and quantification of OT-II Treg cells (CD25+Foxp3+) recovered per thymic slice as in a (b). (c) Ratio of OT-II Treg cells to slice CD45+ thymocytes as in a. (d) Quantification of endogenous slice Treg cells recovered per slice as in a. (e) Quantitative RT-PCR analysis of Il2 expression in wild-type thymus dissociated by digestion with collagenase and separated into an adherent fraction (Stroma) and single-cell suspensions (Whole thymus), and single-cell suspensions after depletion of CD11c+ cells by magnetic beads (CD11c Dep), and after further enrichment of by flow cytometry to yield fractions of >85% CD11c+ cells (Thymic DC) (left), or wild-type or IL2−/− bone marrow–derived DCs, as well as the ‘thymic DCs’ described above (right); results were normalized to those of the control gene Gapdh and are presented relative to background values from IL2−/− bone marrow–derived DCs (for which no Il2 signal was observed after 40 cycles of PCR; thus, values reported are upper estimates); dashed line, lower limit of detection of the assay. Each symbol represents an individual well (technical replicates); small horizontal lines indicate the mean. NS, not significant (P > 0.05); *P < 0.05 and **P < 0.001 (unpaired Student’s t-test) or one-way ANOVA with Tukey’s post-test analysis (e). Data were pooled from three independent experiments (a–d; error bars, s.e.m. of n = 20 slices (WT) or 21 slices (IL2−/−)) or four experiments (e, error bars, s.e.m. of n = 12 technical replicates from four biological samples for all except ‘CD11c dep’ (n = 9 technical replicates from three biological samples) or ‘Stroma’ (n = 3 technical replicates from one biological sample).
antigen-bearing DCs were able to provide a relevant local source of IL-2 to promote Treg cell development.

Our data, together with published evidence that thymic DCs are important as APCs for Treg cell induction, suggested that thymic DCs might express IL-2. Although we were unable to detect IL-2 protein in the thymus (data not shown), we were able to measure Il2 mRNA from thymus by a sensitive quantitative RT-PCR assay (Fig. 6e). We found that purified thymic DCs expressed approximately fivefold more Il2 mRNA than did total, dissociated cells from the thymus, whereas the adherent thymic stromal cell compartment was depleted of Il2 mRNA compared with Il2 mRNA in the total thymus (Fig. 6e, left). While thymic DCs expressed more IL-2 than did thymocytes as a whole, it is noteworthy that depletion of CD11c+ cells from dissociated thymus samples did not significantly reduce the overall amount of Il2 mRNA in the samples (Fig. 6e, left). These data indicated that DCs were not the sole source of IL-2 within the thymus. Notably, while Il2 mRNA was also detectable over background in bone marrow–derived DCs, Il2 expression was ~250-fold lower in these cells than in thymic DCs (Fig. 6e, right). This indicated that very low levels of IL-2 had a potent effect on Treg cell development when IL-2 was expressed by the same cell that also displayed the antigenic peptide. Together our data support a model in which limited APC-derived IL-2, together with competition by existing Treg cells, defines the size of the thymic niche for new Treg cell development (Supplementary Fig. 6c).

DISCUSSION

The limited capacity of the thymus to support Treg cell development might be related to the dual requirements for TCR ligands and IL-2, but how these signals work together to define the size of the Treg cell niche is not well understood. Here we demonstrated that existing Treg cells within the thymus exerted control over the size of the thymic Treg cell niche by limiting the supply of available IL-2. We also showed that IL-2 produced by antigen-bearing cells, including DCs, promoted Treg cell development. Combined, our data suggest that the thymic niche for Treg cell development includes thymic APCs that can supply both a TCR ligand and IL-2 and indicate that existing Treg cells exert tight control over this niche by limiting available IL-2.

T cells are known to be the main producers of IL-2; thus, the indications that DCs provided an important source of IL-2 for Treg cell development were rather unexpected. Moreover, the finding that DC-derived IL-2 promoted the development of antigen-specific Treg cells in an IL-2-sufficient environment, and without boosting global Treg cell production, indicated that IL-2 is most effective when supplied by the same cell that is presenting the agonist peptide-MHC ligand. The physical linkage between antigen presentation and IL-2 supply might facilitate Treg cell development by increasing the probability that thymocytes will receive signals from both the TCR and IL-2. Moreover, the recognition of peptide-MHC by the TCR might help to position thymocytes near the source of IL-2 and allow them to compete more effectively for limiting supplies of the cytokine. Finally, our data suggest that thymic APCs that are able to provide both antigen and IL-2 might be particularly adept at supporting Treg cell development, which potentially explains the ability of certain thymic APCs to ‘preferentially’ induce Treg cell development rather than negative selection.

The role of IL-2 in thymic Treg cell development has been revealed by genetic approaches, but thymic concentrations of IL-2 are low, and the relevant cellular sources of the cytokine have remained unknown. Our data revealed that small amounts of IL-2 had a potent effect on Treg cell development when the IL-2 was produced by the same cell that presented self antigen. Evidence that DCs have an important role as APCs for Treg cell development, together with our data showing that thymic DCs expressed IL-2, suggests that thymic DCs are an important source of IL-2. However, our data do not rule out the possibility that other cell types might provide IL-2 in some settings. Of particular interest are medullary epithelial cells (mTECs), which can also serve as APCs to drive Treg cell development. Notably, mTECs express very little mRNA encoding IL-2 but express substantially more mRNA encoding the related cytokine IL-15 and its presenting receptor IL-15Rα. This suggests that mTECs might present IL-15 along with antigen to promote Treg cell development. Alternatively, the close contact and cell-to-cell transfer between mTECs and DCs might allow these cell types to contribute both self antigen and IL-2 to the same niche for Treg cell development.

Our data also indicated the involvement of existing thymic Treg cells in a negative feedback loop that limited the size of the thymic Treg cell niche via competition for IL-2. Modeling studies indicate that competition by mature Treg cells for peripheral sources of IL-2 is a local phenomenon, with most paracrine absorption occurring within 10–200 μm of the IL-2 source. That, together with our evidence showing that access to IL-2 was localized to the peptide-bearing DCs, suggests that competition between Treg cells and their precursors for IL-2 might also occur locally, perhaps during interactions with the same APC. Notably, IL-2 signaling also directly induces expression of the high-affinity α-chain of the IL-2 receptor (CD25), which further increases the competition for IL-2 binding. Tight feedback control on the availability of IL-2 might help to explain why manipulations designed to increase IL-2 availability (such as adding IL-2 or eliminating existing Treg cells) led to only modest (twofold) increase in new Treg cell development. As precursors of Treg cells are exposed to free IL-2, they rapidly become more efficient competitors for IL-2 and exert additional negative feedback on the niche.

Published studies of mice expressing Treg cell–biased TCRs have suggested that clonal competition for binding self antigen limits Treg cell development when the frequency of thymocytes specific for a particular self antigen is high. In contrast, we found little effect of precursor frequency on the efficiency of Treg cell development, and Treg cell development remained relatively inefficient, even when antigen abundance was high and the majority of OT-II thymocytes upregulated CD25 expression in response to antigen. We propose that the factors that limit access to the Treg cell niche might vary depending on the abundance of self antigen and the frequency of thymocytes specific for the self antigen. Under conditions of low precursor frequency and abundant self antigen, IL-2 becomes the main limiting factor for Treg cell development. On the other hand, if the abundance of self antigen is very low and/or the frequency of thymocytes specific for self antigen is high, competition for antigen recognition may become a limiting factor. In addition, while our study focused on the competition among Treg cells for IL-2, existing Treg cells may also inhibit new Treg cell development by competing for additional factors, such as B7 (the ligand for the coreceptor CD80) and/ligands for the tumor-necrosis factor receptor superfamily.

Together our data support a model in which IL-2-producing APCs within the thymus, in conjunction with the number of existing Treg cells within the vicinity of the IL-2 source, determine the size of the Treg cell niche. As thymocytes interact with APCs, mature into Treg cells and provide negative feedback by absorbing local supplies of IL-2, the niche remains balanced. Eventually, in a system with thymocyte egress intact, mature Treg cells would cycle out of the niche, which would ease the competition for IL-2 and allow further rounds.
of T<sub>reg</sub> cell development. Efforts to increase T<sub>reg</sub> cell production for therapeutic purposes should take into account the complex layering of feedback that exists to maintain proper niche output. In particular, linking IL-2 production and self-antigen presentation, as well as continuous removal of newly developing IL-2 competitors, should provide effective strategies for maximizing thymic T<sub>reg</sub> cell development.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

B.M.W. designed, performed and analyzed experiments and wrote the manuscript; N.K., J.B. and S.W.C. performed experiments; and E.A.R. supervised the study and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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1. Asano, M., Toda, M., Sakaguchi, N. & Sakaguchi, S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* **184**, 387–396 (1996).

2. Itoh, M. et al. Thymus and autoimmunity: production of CD25<sup>+</sup>CD4<sup>+</sup> naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunological self-tolerance. *J. Immunol.* **162**, 5317–5326 (1999).

3. Sawant, D.V. & Vignali, D.A. A two-step process for thymic regulatory T cell development. *Nat. Rev. Immunol.* **12**, 173–191 (2014).

4. Riley, J.L., June, C.H. & Blazar, B.R. Human T regulatory cell therapy: take a billion or so and call me in the morning. *J. Immunol.* **184**, 923–930 (1996).

5. von Boehmer, H. & Daniel, C. Therapeutic opportunities for manipulating T cell immunity. *Nat. Rev. Drug Discov.* **8**, 1533–1543 (2009).

6. Burchill, M.A. CD4<sup>+</sup>CD25<sup>+</sup> Treg cell suppressor function of IL-2.

7. Lio, C.W. & Hsieh, C.S. A two-step process for thymic regulatory T cell development. *Immunology* **135**, 387–396 (1996).

8. Granucci, F. et al. Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. *Nat. Immunol.* **10**, 610–617 (2009).

9. Molina, A.E. & Hogquist, K.A. T-cell receptor affinity in thymic development. *Immunochemistry* **135**, 261–267 (2012).

10. Jordan, M.S. et al. Thymic selection of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* **2**, 301–306 (2001).

11. Bauld, O.L. et al. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nat. Immunol.* **10**, 562–569 (2009).

12. Fontenot, J.D., Rasmussen, J.P., Gavin, M.A. & Rudensky, A.Y. A function for interleukin-2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* **6**, 1142–1151 (2005).

13. Lee, N.M., Bautista, J.L., Scott-Browne, J., Mohan, J.F. & Hsieh, C.S. A broad range of self-reactivity drives thymic regulatory T cell selection to limit responses to self. *Immunity* **37**, 475–486 (2012).

14. Caturegli, J., Caturegli, E. & Caturegli, L. CD4<sup>+</sup>CD25<sup>+</sup> T cell depletion in normal human thymus: reappraisal of a classic observation. *J. Immunol.* **183**, 7909–7918 (2009).

15. Atibalentja, D.F., Byersdorfer, C.A. & Unanue, E.R. Thymus-blood protein interactions of Polycomb silencing, and distribution of self-antigen expression in thymic central CD4<sup>+</sup> T cell tolerance. *Proc. Natl. Acad. Sci. USA* **105**, 18969–18974 (2008).

16. Kim, H.P., Kelly, J. & Leonard, W.J. The basis for IL-2-induced IL-2 receptor chain gene regulation: importance of two widely separated IL-2 response elements. *Proc. Natl. Acad. Sci. USA* **91**, 3435–3439 (1994).

17. Kim, H.P., Kelly, J. & Leonard, W.J. The basis for IL-2-induced IL-2 receptor α chain gene regulation: importance of two widely separated IL-2 response elements. *Immunity* **15**, 159–172 (2001).

18. Bartlott, T. et al. CD25<sup>+</sup>CD4<sup>+</sup> T cells compete with naive CD4<sup>+</sup> T cells for IL-2 and exploit it for the induction of IL-10 production. *Int. Immunol.* **17**, 279–288 (2005).

19. Caturegli, J.L. et al. Thymic selection of natural regulatory T cells. *Nat. Immunol.* **10**, 562–569 (2009).

20. Mahmoud, S.A. et al. Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nat. Immunol.* **15**, 473–481 (2014).

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ONLINE METHODS

Mice. All mice were bred and maintained under pathogen-free conditions within facilities approved by the American Association of Laboratory Animal Care at the University of California, Berkeley. The internal review board and the Animal Use and Care Committee at the University of California, Berkeley, approved all procedures. C57BL/6, B6.5L-Ptpcra Peephc/Boy (CD45.1), 2D2 TCR transgenic, AND, B6.129P2-H2m1Hor/J(J2t–/–), C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA) and Foxp3–GFP mice (expressing green fluorescent protein [GFP] under the control of the Foxp3 promoter) were from Jackson Laboratories. 2D2 x Rag2–/– mice were generated by crossing of 2D2 mice with Rag2–/– mice from Taconic. OT-II x Rag2–/– mice were from Taconic. F5 x Rag2–/– mice have been described45. Endotoxin-free soluble OVA was from Invivogen, and injections were performed intravenously, followed by killing of the mice 1 h later.

Thymocytes, Treg cell isolation and CTL2L culture. Thymocytes were isolated from OT-II x Rag2–/– thymi or 2D2 x Rag2–/– thymi through mechanical disruption. Cells were filtered through nylon mesh and were resuspended in complete DMEM (containing 10% FBS, non-essential amino acids, sodium pyruvate, 1-glutamine and 2-mercaptoethanol) for overlay onto thymic slices. OT-II thymocytes were distinguished from slice-resident thymocytes through use of the congenic markers CD45.1 and CD45.2, used in conjunction with staining for the specific TCR chain corresponding to the transgene expressed by the mice. Thymic Treg cells were isolated from Foxp3–GFP mice. Thymic cell suspensions were depleted of CD8+ single-positive thymocytes and CD4+CD8+ double-positive thymocytes through the use of anti-CD8 microbeads (Miltenyi), then the remaining CD4SP thymocytes were sorted by flow cytometry on the basis of GFP expression to generate >95% pure thymic Treg cells. Sorted thymic Treg cells were resuspended in DMEM complete medium for use with thymic slices. As a bioassay for available IL-2 and IL-15, we used the CTL2L cell line, a transformed IL-2- and/or IL-15-dependent cell line derived from C57BL/6 mice. CTL2L cells (a gift from the N. Shastri laboratory; verified free of mycoplasma before use) were cultured in complete RPMI medium (containing 10% FBS, non-essential amino acids, sodium pyruvate, 1-glutamine and 2-mercaptoethanol) along with 50 U/ml IL-2 until they were washed and overlaid on thymic slices. 1 x 106 CTL2L cells per slice were added and were allowed to migrate into slices for 4 h before samples were washed.

Thymic slices. Thymic slice preparation has been described46,47. Whole thymic lobes isolated from mice were embedded in 4% agarose with a low pyruvate, l-glutamine and 2-mercaptoethanol) for overlay onto thymic slices. Thymi from wild-type mice were dissociated by digestion with 0.05% collagenase for 1 h at 37 °C with collagenase (Sigma–Aldrich). Some of each sample was reserved for RNA isolation (whole-thymocyte samples), and the remainder was used for enrichment of DCs with MACS CD1c microbeads according to manufacturer’s instructions (Miltenyi), followed by flow cytometry to achieve a final purity of a population of >85% CD11c+Fcγ80+ cells (thymic DC samples). The thymus sample after removal of CD11c+ cells was also used for RNA isolation (CD11c-depleted samples). Cell samples were lysed in TRIzol (Life Technologies), and total RNA was prepared with an RNAeasy kit according to the manufacturer’s instructions (Qiagen). Reverse transcription was performed with a QuantiTect RT Kit (Qiagen), and cDNA was used for quantitative PCR with Taqman probes along with TaqMan Real-Time PCR master mix (Life Technologies). The PrimeTime probes Mm.PT.58.11478202 (for IL-2) and Mm.PT.39.1 (for GAPDH), were synthesized by IDT. All reactions were run on an Applied Biosystems 7300 RT PCR machine. IL-2 expression data were normalized to those of the control gene encoding GAPDH (glyceraldehyde phosphate dehydrogenase) and are presented relative to background expression in IL2–/– bone marrow-derived DCs, calculated by the change-in-cycling threshold (ΔΔCt) method. For IL2–/– DCs, no IL2 signal was observed after 40 cycles of PCR; therefore, values reported are upper estimates (gray shading in figures) and were used to determine the lower limit of detection of the assay.

Flow cytometry. The following antibodies were used for cell-surface staining: Alexa Fluor 700–anti-mouse CD4 (GR1.5), aliphophycin–eFlour 780–conjugated anti-CD8 (53-6.7), eFlour 450– or peridinin chlorophyll protein–cytochrome c 5.5–conjugated anti-CD25 (PC61.5), peridinin chlorophyll protein–eFlour 710–conjugated anti-CD4 (R03-16), phycoerythrin-indocarbocyanine–conjugated anti-CD45.1 (A20) and fluorescein isothiocyanate–conjugated anti-Vα2 (Y3.2–3.23; RR3-16), phycoerythrin-indocarbocyanine–conjugated anti-CD4 (53-6.7) (both from Biolegend); and phycoerythrin-conjugated anti-neuropilin-1 (3E12; Biolegend). Staining with the cell-proliferation dye eFlour 450 was performed for 10 min at 37 °C with 2 μM eFlour 450 (eBioscience). For intracellular staining, aliphophycin– or phycoerythrin–conjugated anti-Nur77 (12.14) and phycoerythrin–conjugated anti-Foxp3 (FJK-16s) were used (both from eBioscience). Cells were first labeled with Ghost Dye viability dye (Tonbo), then were surface stained for 15 min on ice, followed by fixation and permeabilization according to manufacturer’s instructions with the Foxp3 Transcription Factor staining buffer set (eBioscience). Flow cytometry was performed with a BD Fortessa or Fortessa X20 analyzer (BD Bioscience), and data were analyzed with FlowJo software (TreeStar).

Antibody complexes and peptides. Complexes of IL-2 and monoclonal antibody to IL-2 have been described26,27. 1.5 μg recombinant IL-2 (eBioscience) was incubated for 30 min at 37 °C with 7.5 μg of functional-grade monoclonal antibody to IL-2 (JES6–1; eBioscience). Complexes were added directly to thymic slices (10 μl per slice) daily over the course of the experiment. Peptides of mouse myelin oligodendrocyte glycoprotein amino acids 35–55 and chicken OVA amino acids 323–339 were from AnaSpec. Peptide was overlaid on thymic slices for 10–30 min after migration of thymocytes into the slice.

Statistics. The unpaired Student’s t-test and one-way ANOVA with Tukey’s post-test were calculated with Prism software (GraphPad). A P value of <0.05
was considered statistically significant. No data points were excluded, and the number of mice used was consistent with previous experiments using similar experimental designs.

45. Mamalaki, C. et al. Thymic depletion and peripheral activation of class I major histocompatibility complex-restricted T cells by soluble peptide in T-cell receptor transgenic mice. Proc. Natl. Acad. Sci. USA 89, 11342–11346 (1992).

46. Dzhagalov, I.L., Melichar, H.J., Ross, J.O., Herzmark, P. & Robey, E.A. in Current Protocols in Cytometry (ed., Robinson, J.P.) Ch 12, 12.26.1–12.26.20 (John Wiley and Sons, 2012).

47. Melichar, H.J., Ross, J.O., Herzmark, P., Hogquist, K.A. & Robey, E.A. Distinct temporal patterns of T cell receptor signaling during positive versus negative selection in situ. Sci. Signal. 6, ra92 (2013).

48. Ehrlich, L.I., Oh, D.Y., Weissman, I.L. & Lewis, R.S. Differential contribution of chemotaxis and substrate restriction to segregation of immature and mature thymocytes. Immunity 31, 986–998 (2009).

49. Haikias, J. et al. Opposing chemokine gradients control human thymocyte migration in situ. J. Clin. Invest. 123, 2131–2142 (2013).