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

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Abstract

Thymic regulatory T (T_{reg}) cell production requires interleukin 2 (IL-2) and agonist 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 show that IL-2 produced by antigen-bearing dendritic cells plays a key role in T_{reg} cell development, and that existing T_{reg} cells limit new T_{reg} cell development by competing for IL-2. . Our data suggest that antigen-presenting cells that can provide both IL-2 and a TCR ligand comprise the thymic niche, and that competition by existing T_{reg} cells for a limited supply of IL-2 provides negative feedback for new T_{reg} cell production.

Introduction

Tolerance to self requires an intact regulatory T (T_{reg}) cell pool, which acts to limit autoimmunity and maintain homeostasis within the immune system. T_{reg} cells develop within the thymus from CD4 single-positive (SP) thymocytes, as well as extrathymically from conventional $CD4^+$ T cells. Ablation of thymic T_{reg} cell generation via neonatal thymectomy leads to autoimmunity, illustrating the importance of maintaining proper thymic T_{reg} cell output[1,2]. Although thymic and extrathymic derived T_{reg} cells overlap in their functional capacity, thymic-derived T_{reg} cells appear to be more stable under inflammatory conditions[3]. Therefore, understanding the factors that govern T_{reg} cell development in the thymus is important for designing strategies to generate large, stable T_{reg} cell populations for immunotherapy[4,5].

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AUTHOR CONTRIBUTIONS

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

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Several reports have delineated a two-step process that results in thymic T_{reg} cell generation[6,7]. First, CD4SP thymocytes must receive relatively strong signals through the T cell receptor, a process that allows for transcriptional changes and increases in cell surface expression of the high-affinity alpha chain of the interleukin 2 (IL-2) receptor, CD25. IL-2 signaling via STAT5 is required to complete development, leading to induction of the T_{reg}-defining transcription factor, Foxp3. Although many studies have documented the requirements for strong TCR signals and IL-2 in T_{reg} cell development[6–9], 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 and temporally linked in order to efficiently promote T_{reg} cell development.

Thymic-derived T_{reg} cells represent a small proportion of the CD4SP thymocytes, suggesting that a limiting niche exists to support T_{reg} cell development. Moreover, studies using mice expressing rearranged, T_{reg}-biased *Tcra and Tcrb* transgenes reveal that T_{reg} cell development is most efficient when only a small fraction of thymocytes expressed a T_{reg}-biased TCR, pointing to intraclonal competition for access to a limited developmental niche[10,11]. Limiting intraclonal competition leads to increased TCR signaling, suggesting that access to peptide-MHC ligands can be a limiting factor when T_{reg} precursor frequency is high[8]. Whether competition for IL-2 is also involved in establishing the size of the thymic T_{reg} niche remains unknown.

Understanding the nature of the T_{reg} 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, leading to the suggestion that thymocytes may provide IL-2 to developing T_{reg} cells. However, there are also reports that dendritic cells (DCs) can produce limited quantities of IL-2 in certain settings[12,13]. Given indications that IL-2 concentrations are limiting for thymic T_{reg} cell development[14–16], uncovering the sources of IL-2 in the thymus, as well as the factors that govern its availability to developing T_{reg} cells is key to defining the thymic T_{reg} niche.

To address these questions, we have developed an experimental system in which thymocytes expressing a defined MHC class II specific TCR transgene are introduced into a thymic tissue slice in the presence of their cognate antigen, leading to a synchronized wave of T_{reg} cell development. Using this system, we provide evidence that antigen-bearing DCs provide a local source of IL-2 to promote T_{reg} cell development. We also show that existing T_{reg} cells within the thymic environment inhibit new T_{reg} 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 T_{reg} cells, establish a tightly controlled but flexible negative feedback loop to maintain balanced T_{reg} cell production.

Results

T_{reg} cell development in thymic tissue slices

Previous reports have suggested that thymic T_{reg} cell development is limited by T_{reg} precursor frequency and competition for antigen, implying the existence of a limiting niche for T_{reg} cell development[8–11,17]. To further investigate this niche, we utilized 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). We used OT-II TCR transgenic thymocytes from a *Rag2*$^{-/-}$ background (referred to as OT-II thymocytes) as a starting population with no detectable T_{reg} cells, providing a sensitive and synchronized system to track the development of new T_{reg} cell in response to antigen encounter within the thymic slice. We injected wild-type mice intravenously with 2 mg of soluble ovalbumin (OVA protein), an approach that was previously shown to induce antigen-specific thymic T_{reg} cells *in vivo*[18,19]. One hour after injection, we sacrificed the mice, prepared thymic slices, and introduced OT-II thymocytes. Flow cytometric analysis revealed CD25 upregulation on OT-II CD4SP thymocytes after 1 day of culture, and the appearance of Foxp3$^+$CD25$^+$ cells after 3 days (Supplementary Fig. 1), consistent with the timing of T_{reg} cell development inferred from intrathymic transfer studies[7].

T_{reg} cells can develop from immature T cells in the thymus, or can be derived from mature peripheral CD4$^+$ T cells by TCR stimulation in the presence of the immunosuppressive cytokine transforming growth factor-β (TGF-β)[20]. To confirm that the thymic slice system recapitulated normal thymic T_{reg} cell development, we compared the ability of OT-II thymocytes versus mature OT-II T cells to give rise to T_{reg} cells upon introduction into thymic slices (Supplementary Fig. 2a). Mature OT-II T cells showed some CD25 upregulation, but no Foxp3 induction, upon culture in thymic slices from mice injected with OVA protein. Addition of TGF-β led to a small population of Foxp3$^+$ cells, however these cells expressed low amounts of the thymus-derived T_{reg} marker, neuropilin-1 (Supplementary Fig. 2a)[21,22]. In contrast, OT-II thymocytes cultured in thymic slices gave rise to Foxp3$^+$ cells without the addition of TGF-β, and expressed neuropilin-1. Neither OT-II thymocytes nor mature T cells gave rise to T_{reg} cells when stimulated with OVA-loaded DCs *in vitro* without thymic slices and without addition of TGF-β (Supplementary Fig. 2b). Moreover, depletion of mature CD4SP from OT-II thymocytes prior to addition to thymic slices delayed, but did not prevent, T_{reg} cell development (Supplementary Fig. 2c,d and data not shown) implying that Foxp3$^+$ cells arose from an immature thymocyte population, rather than mature CD4SP thymocytes. These data indicate that the development of Foxp3$^+$ cells from OT-II thymocytes on thymic slices is distinct from *in vitro* T_{reg} cell development from mature conventional CD4$^+$ T cells, and suggest that the combination of the thymic environment and the developmental stage of the T_{reg} precursor are important for thymus-derived T_{reg} cell development.

To probe the role of antigen availability for T_{reg} cell development, we varied the antigen dose and delivery method. Injection of a high dose of soluble OVA led to OT-II T_{reg} cell development after 3 d on thymic slices, whereas injection of one-tenth the dose of OVA led to reduced, but detectable T_{reg} cell development. No CD25 or Foxp3 expression was observed when OT-II thymocytes were cultured in slices from control mice injected with PBS (Fig. 1a). As an alternative mode of antigen delivery, we utilized transgenic mice in which a membrane-associated form of ovalbumin is expressed in medullary thymic epithelial cells and the pancreas under the control of the rat insulin promoter (RIPmOVA)[23]. Again, we observed a substantial population of CD25$^+$Foxp3$^+$ OT-II thymocytes after 3 d of culture on thymic slices from RIPmOVA transgenic mice (Fig. 1b). As a third approach to

introduce antigen, we incubated bone marrow-derived DCs with ovalbumin protein and then introduced them to thymic slices containing OT-II thymocytes. Again we observed new T_{reg} cell development, which increased with antigen dose (Fig. 1c). In contrast to previous reports[10,11] we did not observe any correlation between the frequency of OT-II thymocytes within thymic slices and the efficiency of T_{reg} cell development (Supplementary Fig. 3), implying that T_{reg} precursor frequency is not a major limiting factor under our experimental conditions.

To compare the efficiency of T_{reg} cell development across various routes of antigen delivery, we expressed the number of Foxp3$^+$ OT-II T_{reg} cells as a ratio of the total CD4SP thymocytes in the thymic slice. The efficiency of T_{reg} cell development varied with antigen abundance, consistent with previous studies[9,18]. However, the proportion of OT-II thymocytes that developed into T_{reg} cells was generally less than 20% (Fig. 1 and Supplementary Fig. 3), and never exceeded the endogenous proportion of T_{reg} cells within a slice (corresponding to 3–10% of total slice CD4SP thymocytes) (Fig. 1d). This upper limit on T_{reg} cell development was observed even under conditions in which the majority of thymocytes responded to antigen, as indicated by CD25 induction (Fig. 1c,d). Together these results suggest that there is a limited thymic niche for T_{reg} cell development even when antigen encounter and T_{reg} precursor frequency are not limiting factors.

Existing thymic T_{regs} cells reduce the niche size

The finding that the overall number of thymic T_{reg} cells seems to be limited to fewer than 10% of the total CD4SP population suggests that T_{reg} cells themselves may inhibit new T_{reg} cell development. To test whether existing T_{reg} cells influence future T_{reg} cell development, we prepared thymic slices from AND×*Rag2*$^{-/-}$ mice for use as a thymic environment that does not contain T_{reg} cells[24]. For these experiments, we introduced antigen by adding 1 μM OVA peptide to thymic slices containing OT-II thymocytes. Interestingly, we observed a consistent increase in the number of CD25$^+$ Foxp3$^+$ OT-II thymocytes in T_{reg}-deficient (AND×*Rag2*$^{-/-}$) compared to T_{reg}-sufficient (wild-type) thymic slices (Fig. 2a,b). Enhancement of new T_{reg} cell development in T_{reg}-deficient slices was also observed using a different MHC class II specific TCR (2D2 ×*Rag2*$^{-/-}$) as the T_{reg} precursor population (Supplementary Fig. 4a,b), and using a different Rag2-deficient TCR transgenic model (F5×*Rag2*$^{-/-}$) as a source for T_{reg}-deficient thymic slices (Supplementary Fig. 4c,d). Finally, the enhanced T_{reg} cell development in thymic slices from TCR transgenic mice could be reversed by addition of sorted thymic T_{reg} cells to thymic slices (Fig. 2c,d), indicating that the lack of T_{reg} cells, rather than other abnormal features of TCR transgenic thymic environment, was responsible for enhanced T_{reg} cell development. Combined these results provide evidence that existing thymic T_{reg} cells limit the development of new T_{reg} cells.

T_{reg} cells do not interfere with peptide recognition

T_{reg} cells can reduce the stimulatory capacity of antigen-presenting cells (APCs) in the periphery, suggesting that thymic T_{reg} cells may inhibit new T_{reg} cell development by interfering with the ability of thymic APCs to present agonist self-peptides[25]. To test this idea we examined the expression of the transcription factor Nur77, which provides a quantitative readout of accumulated TCR signal strength[8]. Addition of OVA peptide to T_{reg}-

sufficient or T_{reg}-deficient slices resulted in similar expression of CD25 and Nur77 induction by OT-II thymocytes at 6 h (Fig. 3a–c), suggesting that existing T_{reg} cells did not influence the initial TCR signal received by T_{reg} progenitors. As a measure of accumulated TCR signal, we also examined Nur77 expression within the OT-II T_{reg} cell population that developed after 3 d in the presence or absence of existing T_{reg} cells (Fig. 3d). Consistent with previous reports[8], endogenous T_{reg} cells from the thymic slice expressed slightly more Nur77 than conventional CD4SP thymocytes (Fig. 3e), and newly developed OT-II T_{reg} cells expressed slightly more Nur77 than endogenous slice T_{reg} cells (Fig. 3e, teal and magenta lines). Although the overall proportion of OT-II T_{reg} cell development was enhanced in the absence of existing T_{reg} cells, Nur77 expression within the OT-II T_{reg} cell population was similar for OT-II T_{reg} cells that developed on wild-type or T_{reg}-deficient slices (Fig. 3e). Together these results provide evidence that existing T_{reg} cells can limit new T_{reg} cell development without altering TCR signaling in T_{reg} precursors.

IL-2 availability influences the size of the T_{reg} niche

In addition to agonist self-peptide, developing T_{reg} cells require IL-2 to fully mature and express Foxp3[6,7]. To confirm the requirement for IL-2 in the thymic slice model, we used *Il2*$^{-/-}$ mice injected with OVA protein as a source of thymic slices for T_{reg} cell development. Half the number of OT-II thymocytes developed into T_{reg} cells after 3 d on *Il2*$^{-/-}$ slices as compared to wild-type slices (Fig. 4a,b). This reduction in OT-II T_{reg} cell development is mirrored by a reduction in endogenous T_{reg} cells from *Il2*$^{-/-}$ slices, and confirms the requirement for IL-2 for efficient thymic T_{reg} cell development and maturation[14–16]. Importantly, these data indicate that any IL-2 produced by added OT-II thymocytes cannot fully compensate for the lack of IL-2 from the thymic environment, highlighting the importance of IL-2 sources within the thymic slice itself.

To determine whether IL-2 is a limiting factor for T_{reg} cell development, we tested the impact of IL-2 addition. We used IL-2–anti-IL-2 antibody complexes, which display an extended half-life compared to IL-2 alone[26,27]. Addition of IL-2 complexes to thymic slices doubled the number of OT-II T_{reg} cells that developed per slice in both wild-type and *Il2*$^{-/-}$ environments, suggesting that availability of IL-2 was a limiting factor for expansion of the T_{reg} niche (Fig. 4a, b). IL-2 addition also boosted the number of endogenous T_{reg} cells within the slice, implying that IL-2 is also limiting for T_{reg} precursors in the steady-state thymus (Fig. 4c). It is noteworthy that IL-2 addition did not fully restore T_{reg} cell numbers in *Il2*$^{-/-}$ slices. This observation could reflect the requirement for a localized source of IL-2 for efficient T_{reg} cell development, or may be the result of the altered thymic environment in *Il2*$^{-/-}$ mice. Together these data indicate that IL-2 is both necessary and limiting for thymic T_{reg} cell development within thymic slices.

Existing T_{reg} cells limit IL-2/IL-15 availability

Based on reports that peripheral T_{reg} cells can limit effector function via absorption of IL-2 from the environment[28,29], we considered that thymic T_{reg} cells may inhibit new T_{reg} cell development by limiting available supplies of IL-2 and IL-15, a related cytokine which also supports T_{reg} cell development[14]. To detect available cytokines in the thymus, we used the murine IL-2 (or IL-15)-dependent cell line, CTLL2[30]. CTLL2 cells proliferated when added

to wild-type thymic slices, consistent with the presence of available IL-2 and IL-15 within the tissue (Fig. 5a). Moreover, culture of CTLL2 cells on T_{reg}- deficient (AND×*Rag2*$^{-/-}$) slices lead to enhanced survival at d 2, and more robust proliferation on d 4 (Fig. 5a,b), suggesting increased abundance of available IL-2 and/or IL-15 in the T_{reg}-deficient, compared to the T_{reg}-sufficient, environment.

To further probe the role of T_{reg} 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 T_{reg} -deficient mice that had been injected with OVA protein. Addition of IL-2–anti-IL-2 complexes to T_{reg}-sufficient wild-type slices increased the frequency and absolute numbers of OT-II T_{reg} cells 2 fold relative to that observed in T_{reg}-deficient slices without added IL-2 (Fig. 5c,d). Moreover addition of IL-2 complexes to T_{reg}-deficient slices further enhanced T_{reg} cell development 4 fold. These experiments demonstrate that while existing T_{reg} cells within the thymus limit IL-2 availability, IL-2 remains a limiting factor for new T_{reg} cell development even in the absence of existing T_{reg} cells. These data also indicate that existing T_{reg} cells can inhibit new T_{reg} cell development even in the presence of large quantities of IL-2, suggesting tight feedback control of IL-2 availability.

APCs provide a local source of IL-2 to T_{reg} precursors

We next investigated the cellular source of IL-2 for thymic T_{reg} cell development. While T cells are typically considered the major producers of IL-2, our observation that wild-type OT-II thymocytes could not rescue T_{reg} cell development in *Il2*$^{-/-}$ thymic slices suggested that thymocytes might not provide an important source of IL-2 for T_{reg} cell development. Previous reports have suggested that DCs are capable of expressing low amounts of IL-2 in certain settings[12,13,31]. To investigate whether antigen-bearing DCs might provide a local source of IL-2, we compared T_{reg} cell development promoted by wild-type versus *Il2*$^{-/-}$ antigen-loaded DCs. Dendritic cells derived *in vitro* from the bone marrow of young, asymptomatic *Il2*$^{-/-}$ mice expressed similar cell surface markers compared to wild-type bone marrow derived DCs (data not shown). Moreover, when *Il2*$^{-/-}$ DCs were incubated with OVA protein and introduced into thymic slices, they led to comparable upregulation of TCR activation markers on OT-II thymocytes compared to wild-type DCs (Supplementary Fig. 5a–d), indicating that they have normal capacity to process and present antigen. However, OT-II T_{reg} cell development was reduced by nearly half using OVA-loaded *Il2*$^{-/-}$ DCs as compared to OVA-loaded wild-type DCs (Fig. 6 a–c). As expected, there was no change in overall numbers of endogenous slice T_{reg} cells, regardless of whether added DCs were IL-2 deficient or sufficient, indicating that addition of wild-type DCs did not globally increase IL-2 abundance in the environment (Fig. 6d). Addition of IL-2-sufficient OVA-loaded DCs to IL-2-deficient thymic slices increased T_{reg} cell development over that observed when both DC and slices were deficient for IL-2 (Supplementary Fig. 6). Combined, these results indicate that antigen-bearing DCs can provide a relevant local source of IL-2 to promote T_{reg} cell development.

Our data, together with evidence that thymic DCs are important as APCs for T_{reg} cell induction[18,19,32,33], suggest that thymic DCs may 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 using a sensitive qRT-PCR assay (Fig. 6e). We found that purified thymic DCs expressed approximately 5 fold more *Il2* mRNA compared to total, dissociated cells from the thymus, whereas the adherent thymic stromal cell compartment is depleted for *Il2* mRNA compared to total thymus (Fig. 6e left hand plot). While thymic DCs express more IL-2 compared to 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. These data indicate that DCs are a substantial but not the sole, source of IL-2 within the thymus. Interestingly, while *Il2* mRNA was also detectable over background in bone marrow derived DC, the level of expression was ~250 fold lower than the levels found in thymic DCs (Fig. 6e, right hand plot). This indicates that very low levels of IL-2 can have a potent impact on T_{reg} cell development when expressed by the same cell that also displays the antigenic peptide. Altogether our data support a model in which limited APC-derived IL-2, together with competition by existing T_{reg} cells, define the size of the thymic niche for new T_{reg} cell development (Supplemental Fig. 6c).

Discussion

The limited capacity of the thymus to support T_{reg} cell development may be related to the dual requirements for TCR ligands and IL-2, but how these signals work together to define the size of the T_{reg} niche is not well understood. Here, we demonstrate that existing T_{reg} cells within the thymus exert control over the size of the thymic T_{reg} niche by limiting the supply of available IL-2. We also show that IL-2 produced by antigen-bearing cells, including dendritic cells, can provide a potent local source of IL-2 for T_{reg} cell development. Combined, our data suggest that the thymic niche for T_{reg} cell development consists of thymic APCs that can supply both a TCR ligand and IL-2, and indicate that existing T_{reg} cells exert tight control over the availability of this niche by limiting available IL-2.

T cells are known to be the major producers of IL-2, and thus indications that DCs provide an important source of IL-2 for T_{reg} cell development was rather surprising. Moreover, the fact that DC-derived IL-2 promoted the development of antigen-specific T_{reg} cells in an IL-2-sufficient environment, and without boosting global T_{reg} cell production, implies 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 may facilitate Treg development by increasing the probability that T_{reg} precursors will receive both TCR and IL-2 signals. Moreover, TCR recognition of peptide-MHC may help to position T_{reg} precursors 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 may be particularly adept at supporting T_{reg} cell development, potentially explaining the ability of certain thymic APCs to preferentially induce T_{reg} cell development rather than negative selection[32].

The role of IL-2 in thymic T_{reg} cell development has been revealed by genetic approaches[16,31,34], but concentrations of thymic IL-2 are low, and the relevant cellular sources of the cytokine remained unknown. Our data reveal that low amounts of IL-2 can have a potent effect on T_{reg} cell development when produced by the same cell that presents self antigen. Evidence that DCs play an important role as APCs for T_{reg} cell

development[18,32,33,35], together with our data showing that thymic DCs express IL-2, strongly suggests that thymic DCs are an important source of IL-2. However, our data do not rule out the possibility that other cell types may provide IL-2, in some settings. Of particular interest are medullary thymic epithelial cells (mTECs), which can serve as APCs to drive T_{reg} cell development[36,37]. Interestingly, mTECs express very little *Il2* mRNA, but express substantially higher levels of the related cytokine *Il15,* and its presenting receptor, *Il15ra*[38], suggesting that mTECs may present IL-15 along with antigen to promote Treg cell development. Alternatively, the close contact and cell-to-cell transfer between mTECs and DCs[39] may allow these cell types to contribute both self antigen and IL-2 to the same niche for T_{reg} cell development.

Our data also implicate existing thymic T_{reg} cells in a negative feedback loop that limits the size of the thymic T_{reg} niche via competition for IL-2. Modeling studies indicate that mature T_{reg} cell competition for peripheral sources of IL-2 is a local phenomenon, with most paracrine absorption occurring within 10–200 microns of the IL-2 source[40]. This, together with our evidence that access to IL-2 is localized to the peptide-bearing DCs, suggests that competition between T_{reg} cells and T_{reg} precursors for IL-2 may also occur locally, perhaps during interactions with the same APC. Interestingly, IL-2 signaling also directly induces expression of the high-affinity IL-2Rα chain, CD25, which further increases the competition for IL-2 binding[40–42]. The tight feedback control of IL-2 availability may help to explain why manipulations designed to increase IL-2 availability (such as adding IL-2 or eliminating existing T_{reg} cells) led to only modest (2-fold) increase in new T_{reg} cell development. As T_{reg} precursors are exposed to free IL-2, they rapidly become more efficient competitors for IL-2, exerting additional negative feedback on the niche.

Previous studies using mice expressing T_{reg}-biased TCRs have suggested that clonal competition for binding self antigen limits T_{reg} cell development under conditions of high T_{reg} precursor frequency[8–10]. In contrast, we see little impact of precursor frequency on the efficiency of T_{reg} cell development, and T_{reg} cell development remains relatively inefficient, even when antigen abundance is high and the majority of OT-II thymocytes upregulate CD25 in response to antigen. We propose that the factors that limit access to the T_{reg} niche may 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 T_{reg} cell development. On the other hand, if self antigen abundance is very low and/or T_{reg} precursor frequency is high, competition for antigen recognition may become a limiting factor. In addition, while the current study focuses on T_{reg} cell competition for IL-2, existing T_{reg} cells may also inhibit new T_{reg} cell development by competing for additional factors, such as B7 and/or TNF family ligands [43,44].

Combined, our data support the notion that IL-2-producing APCs within the thymus, in conjunction with the number of existing T_{reg} cells within the vicinity of the IL-2 source, determines the size of the T_{reg} niche. As thymocytes interact with APCs, mature into T_{reg} 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 T_{reg} cells would cycle out of the niche, easing the competition for IL-2, and allowing for further rounds of

T_{reg} cell development. Efforts to increase T_{reg} 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_{reg} cell development.

Methods

Mice

All mice were bred and maintained under pathogen-free conditions within American Association of Laboratory Animal Care approved facilities at the University of California, Berkeley. The internal review board and the Animal Use and Care Committee at UC Berkeley approved all procedures. C57BL/6, B6.SJL-Ptprca Pepcb/BoyJ (CD45.1), 2D2 TCR transgenic, AND TCR transgenic, B6.129P2-Il2tm1Hor/J(IL2−/−), C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIPmOVA), and Foxp3-GFP mice were purchased from Jackson. 2D2×*Rag2*$^{-/-}$ mice were generated by crossing 2D2 mice with *Rag2*$^{-/-}$ mice purchased from Taconic. OT-II×*Rag2*$^{-/-}$ TCR transgenic mice were purchased from Taconic. F5×*Rag2*$^{-/-}$ mice have been described previously[45]. Endotoxin-free soluble ovalbumin was purchased from Invivogen, and injections were performed via i.v. injection, followed by sacrifice 1 h later.

Thymocytes, T_{reg} cell isolation and CTLL2 culture

Thymocytes were isolated from TCR transgenic (OT-II×*Rag2*$^{-/-}$, 2D2×*Rag2*$^{-/-}$) thymi via mechanical disruption. Cells were filtered through nylon mesh, and resuspended in DMEM complete media for overlay onto thymic slices. Thymocytes were distinguished from slice by use of congenic markers for CD45.1 and CD45.2, used in conjunction with staining for the specific TCRα chain corresponding to the transgenic mice. Thymic T_{reg} cells were isolated from Foxp3-GFP mice. Thymic cell suspensions were depleted of CD8SP and DP thymocytes using anti-CD8 microbeads (Miltenyi), then the remaining CD4 single positive thymocytes were sorted by flow cytometry based on GFP expression to obtain >95% pure thymic T_{reg} cells. Sorted thymic T_{reg} cells were resuspended in DMEM complete media for use with thymic slices. As a bioassay for available IL-2 and IL-15, we used a transformed IL-2/IL-15-dependent cell line derived from C57BL/6 mice called CTLL2. CTLL2 cells (a gift from N. Shastri lab, UC Berkeley, verified mycoplasma free prior to use) were cultured in RPMI complete media containing 10% FBS along with 50 U/ml IL-2 until being washed and overlaid on thymic slices. 1×10^6 CTLL2 cells were added per slice, and allowed to migrate into slices for 4 h prior to washing.

Thymic slices

Thymic slice preparation has been described previously[46,47]. Briefly, whole thymic lobes were isolated from mice, embedded in 4% low melt agarose and cut into 400 μm sections using a vibratome. Slices were cultured in 0.4 μm tissue culture inserts set in DMEM complete media. 1×10^6 thymocytes resuspended in 10 μl of DMEM were overlayed on the slice and allowed to migrate into thymic slices for 2–4 h, followed by gentle pipetting to remove thymocytes that did not migrate into the slice. Under these conditions, 3000–10,000

thymocyte enter the slice, and migrate to their normal locations following chemokine gradients and other guidance cues within the tissue slice, as we and other have previously reported[48,49]. Media was changed daily during the 3-day culture period. For sorted T_{reg} cell experiments, OT-II thymocytes were first overlaid for 4 h, followed by washing and addition of 1×10^5 Foxp3-GFP T_{reg} cells for 4 h. Thymic slices derived from each thymus were randomized by order of slicing after the slicing process, prior to use for experiments. To minimize the impact of variation in the size of thymic slice and the proportion of cortex and medulla, we used adjacent thymic slices for experimental comparisons. In some cases, we normalized T_{reg} cell numbers to the number of total CD4SP (including slice-resident CD4SP), allowing us to pool data from multiple experiments into a single graph. For mature OT-II T cell experiments, T cells were isolated from the spleens of OT-II×*Rag2*$^{-/-}$ mice and 1×10^6 cells were overlaid onto thymic slices. In some cases, 5 ng/ml of TGF-β (Peprotech) was added along with the mature T cells to the slice.

Bone marrow derived dendritic cell cultures

Bone marrow was flushed from femurs and tibias of mice, followed by RBC lysis using ACK buffer. Cells were resuspended in 10 ng/ml GM-CSF and IL-4 (Peprotech), plated at 0.5×10^6 /ml and cultured for 7 d. A half media change was performed on d2, and full media change with GM-CSF + IL-4 was performed on d5. Semi-adherent cells were isolated on d7, washed, and loaded with soluble OVA protein (1 mg/ml, Invivogen) for 30 min at 37 °C. Cells were washed 3x with HBSS, and overlaid onto thymic slices 4 h after addition of OT-II thymocytes. Cells were allowed to migrate into thymic slices for at least 4h prior to washing off excess. Bone marrow from *Il2*$^{-/-}$ mice was harvested when mice were < 4 weeks of age, before the onset of pathology.

Quantitative PCR

Thymi from wild-type mice were dissociated using collagenase (Sigma-Aldrich.) digestion for 1 h at 37°C. Some sample was reserved for RNA isolation (“whole thymocyte” samples), and the remainder was used for DC enrichment using MACS CD11c microbeads according to manufacturer’s instructions (Miltenyi), followed by flow cytometry to reach a final purity >85% CD11c$^+$ F4/80$^-$ population (“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 prepared using the RNAeasy Kit (Qiagen) as per manufacturer’s instructions. Reverse transcription was performed using the Quatitect RT Kit (Qiagen) and cDNA was utilized to run qPCR reactions using Taqman probes along with TaqMan Real-Time PCR master mix (Life Technologies). PrimeTime probes, (Mm.PT.58.11478202 (IL-2) and Mm.PT.39a.1 (GAPDH)), were synthesized by IDT. All reactions were run on an Applied Biosystems 7300 RT PCR machine. IL-2 expression data were normalized to GAPDH and expressed as fold increase over the background values from *Il2*$^{-/-}$ bone marrow-derived DCs using $\Delta\Delta C_t$ values. (For *Il2*$^{-/-}$ DCs, no IL2 signal was observed after 40 cycles of PCR, therefore values reported are upper estimates (indicated by grey shading), and were used to determine the lower limit of detection of the assay.

Flow cytometry

The following antibodies were used for cell surface staining: anti-mouse CD4-Alexa700 (clone GK1.5), CD8-APC-eFluor780 (clone 53–6.7), CD25-eFluor450 or PerCPCy5.5 (clone PC61.5), Vα2TCR-PerCP eFluor710 or APC (clone B20.1), Vα3.2TCR-FITC (clone RR3-16), CD45.1-PE-Cy7 (clone A20), CD45.2-FITC (clone 104) (eBioscience), or CD4-redfluor710, CD8-APC-Cy7 (Tonbo Bioscience), and Neuropilin-1-PE (clone 3E12) (Biolegend). Cell proliferation dye eFluor450 staining was performed at 2 μM for 10 min at 37 °C (eBioscience). For intracellular staining: Nur77-PE (clone 12.14), and Foxp3-PE (clone FJK-16s) or -APC were used (eBioscience). Cells were first surface stained for 15 min on ice, followed by fixation and permeabilization according to manufacturer's instructions using Foxp3 Transcription Factor staining buffer set (eBioscience). Flow cytometry was performed using a BD Fortessa or Fortessa X20 analyzer (BD Bioscience), and data were analyzed using FlowJo software (Treestar).

Antibody complexes and peptides

IL-2:anti-IL-2 mAb complexes were described previously[26,27]. Briefly, 1.5 μg IL-2 recombinant protein (eBioscience) was incubated with 7.5 μg of functional grade anti-IL-2 mAb (clone JES6-1, eBioscience) for 30 min at 37 °C. Complexes were added directly to thymic slices (10 μl/slice) daily over the course of the experiment. Mouse MOG 35–55 and chicken OVA 323–339 peptides were purchased from Anaspec. Peptide was overlaid on thymic slices for 10–30 min after thymocyte migration into the slice.

Statistics

Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) test with Tukey's post-test were calculated using Prism software (Graphpad). A P value <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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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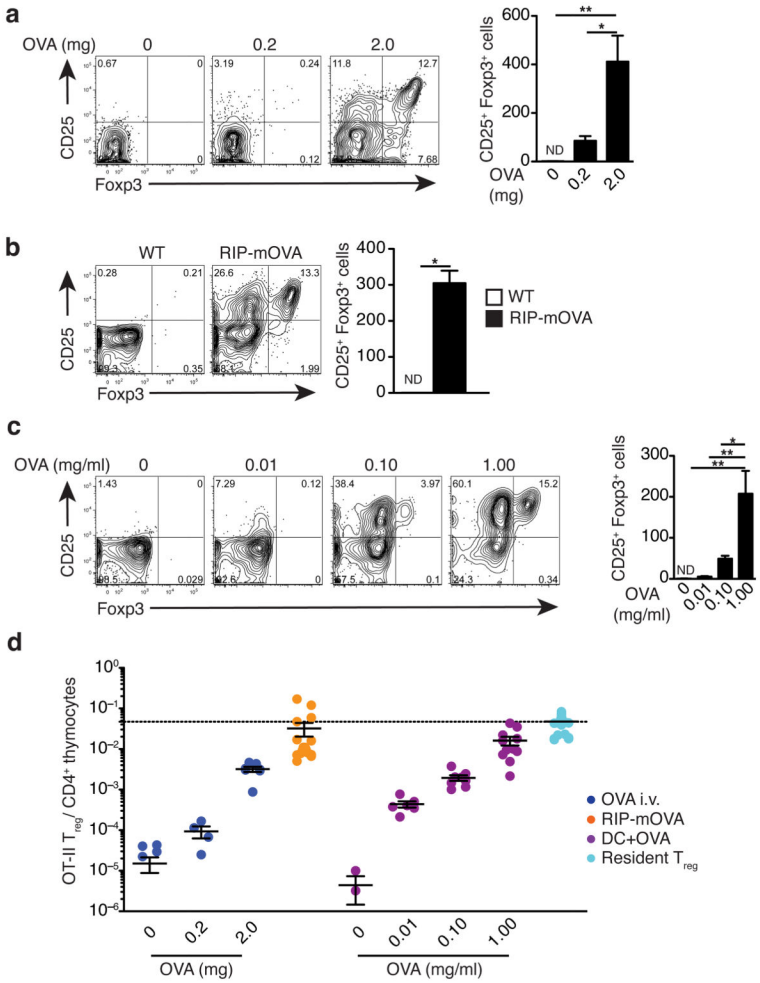

Figure 1.

Limited T_{reg} cell development in thymic slices regardless of the route of antigen delivery. Thymocytes from OT-II TCR transgenic *Rag2* −/− mice (OT-II) were cultured on thymic slices in the presence or absence of the cognate antigen ovalbumin for 3 days prior to flow cytometric analysis. (a-c) Left hand panels show representative flow cytometry plots gated on OT-II donor CD4SP thymocytes and right panels shows total number of OT-II T_{reg} cells (CD25+Foxp3+) recovered per slice. (a) Wild type mice were injected i.v. with 0.2 or 2 mg of ovalbumin protein 1h prior to sacrifice, preparation of thymic slices, and addition of OT-II thymocytes. n=12,10, and 13 slices pooled from 3 experiments. (b) Thymic slices were prepared from mice expressing a RIPmOVA transgene. n=3, 13 slices. (c) Bone marrow derived DCs loaded with the indicated concentrations of OVA protein were added to thymic slices after addition of OT-II thymocytes. n=5, 6, 6, and 6 slices respectively. (d) T_{reg} cell development efficiency shown as a ratio of number of OT-II FoxP3+ CD4 single positive thymocytes recovered per slice relative to the number of resident CD4 single positive thymocytes within the slice for various routes and concentration of antigen. Each dot represents a value from an individual slice compiled from multiple experiments: PBS (3 exp.); 0.2mg (2 exp.); 2mg (2 exp.); mOVA (3 exp.); none (1 exp.); 0.01mg/ml (2 exp.); 0.1mg/ml (2 exp.); 1mg/ml (2 exp.); resident T_{reg} cells (4 exp.). Error bars represent +/−

SEM. *p<0.01 and **p<0.001 by unpaired student's *t*-test (b), or one-way ANOVA using Tukey's post test analysis (a,c). Panels b-c show representative data from 3 (b) or 5 (c) independent experiments.

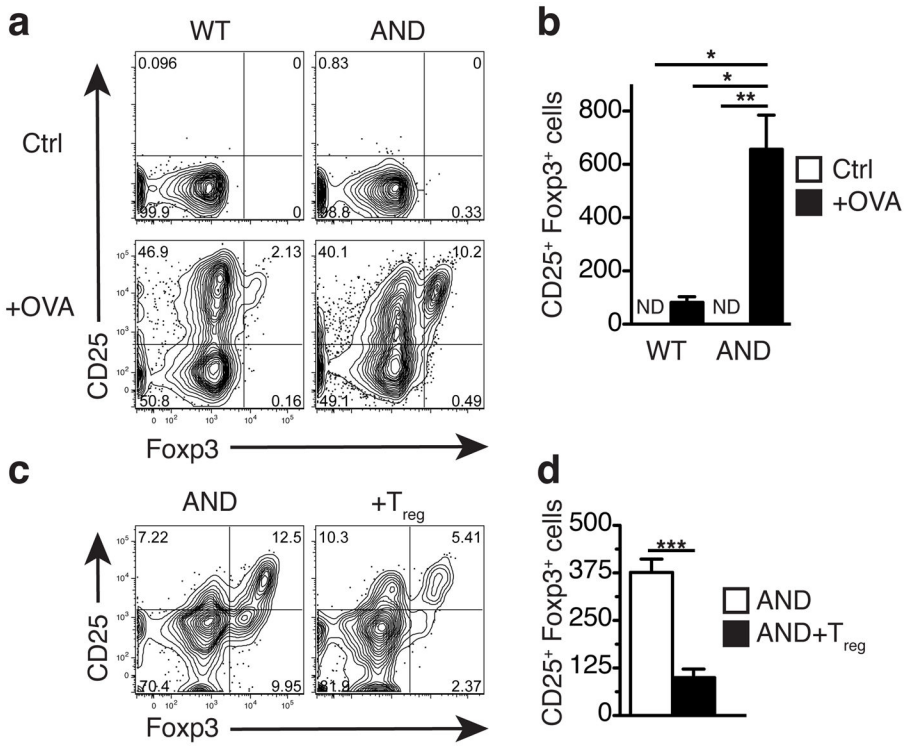

Figure 2.

Existing thymic T_{reg} cells reduce the capacity of the T_{reg} developmental niche. (a,b) OT-IIx*Rag2*−/− (OT-II) thymocytes overlaid onto thymic slices from WT or AND TCRx*Rag2*−/− (AND) mice in the presence of 1μM OVA peptide. T_{reg} cell development measured by flow cytometric analysis at d3. (a) Representative flow cytometry plots of CD25 and Foxp3 expression on gated OT-II donor CD4SP thymocytes. (b) Number of OT-II T_{reg} cells (CD25+Foxp3+) recovered per thymic slice, n=3,3,6,7 slices. (c-d) Thymic T_{reg} cells ($1x10^5$) sorted from Foxp3-GFP mice were added to thymic slices from WT or AND mice that were injected with 2mg OVA protein. OT-II T_{reg} cell development measured at d3. (c) Representative flow cytometry plots gated on OT-II donor CD4SP thymocytes. (d) Number of OT-II T_{reg} cells (CD25+Foxp3+) recovered per thymic slice, n=7 slices. Data representative of 3 (a, b) and 5 (c, d) independent experiments. Error bars represent +/− SEM. *p<0.01,**p<0.001, ***p< 0.0001 by one-way ANOVA using Tukey's post test analysis (b) or unpaired student's t-test (d).

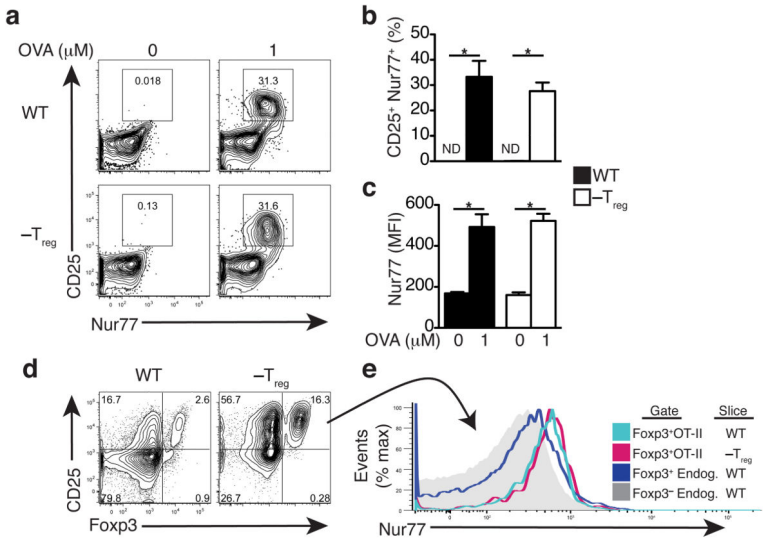

Figure 3.

Existing thymic T_{reg} cells do not prevent TCR signaling within T_{reg} precursors. (a-c) OT-II thymocytes cultured in the presence of 1μm OVA peptide for 6h within WT or AND TCR (T_{reg} deficient) thymic slices and analyzed by flow cytometry. (a) Representative flow cytometry plots showing gated OT-II CD4SP thymocytes. (b) Percent of CD25+ Nur77+ OT-II thymocytes. (c) Nur77 MFI of OT-II single positive thymocytes. (d) Analysis of OT-II T_{reg} cell development 3d after culture on WT or $–T_{reg}$ thymic slices in the presence of 1μm OVA peptide. (e) Comparison of Nur77 levels between the indicated CD4SP populations at d3. Error bars represent +/− SEM. All figures representative of 3 independent experiments. n=6 slices per condition. *p<0.001 by one-way ANOVA using Tukey's post test analysis (b,c).

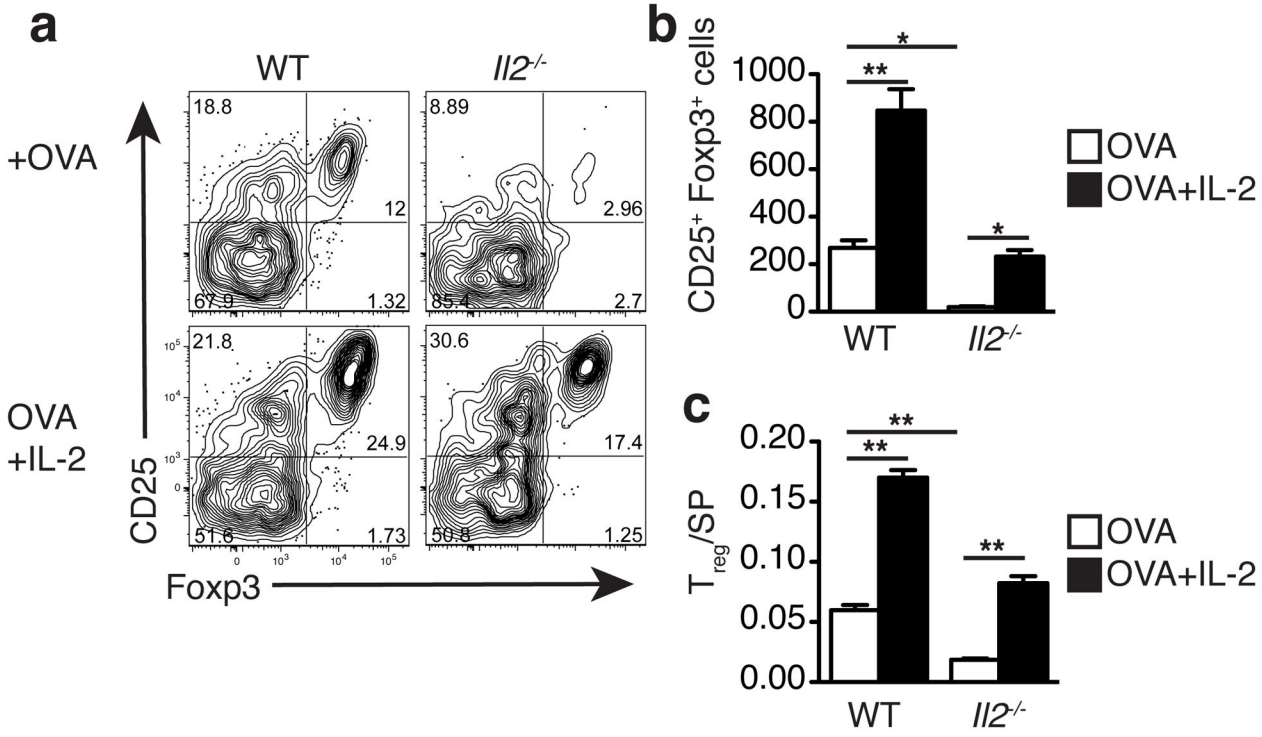

Figure 4.

IL2 availability from the thymic environment influences the T_{reg} niche. WT and *Il2*$^{-/-}$ mice were injected with 2mg of OVA protein 1h prior to sacrifice and preparation of thymic slices. OT-II thymocytes were added to thymic slices and were treated with IL2 complexes daily. OT-II T_{reg} cell development was assessed by flow cytometry at d3. (a) Representative flow cytometry profiles. (b) Number of OT-II T_{reg} cells (CD25+Foxp3+) recovered per thymic slice. (c) Endogenous T_{reg} cell population expressed as the ratio relative to slice resident CD4 single positive thymocytes. Error bars represent +/− SEM. Data representative of 5 independent experiments, n=6,7,6, and 7 slices respectively. *p<0.05 and **p<0.001 by one way ANOVA using Tukey's post test analysis.

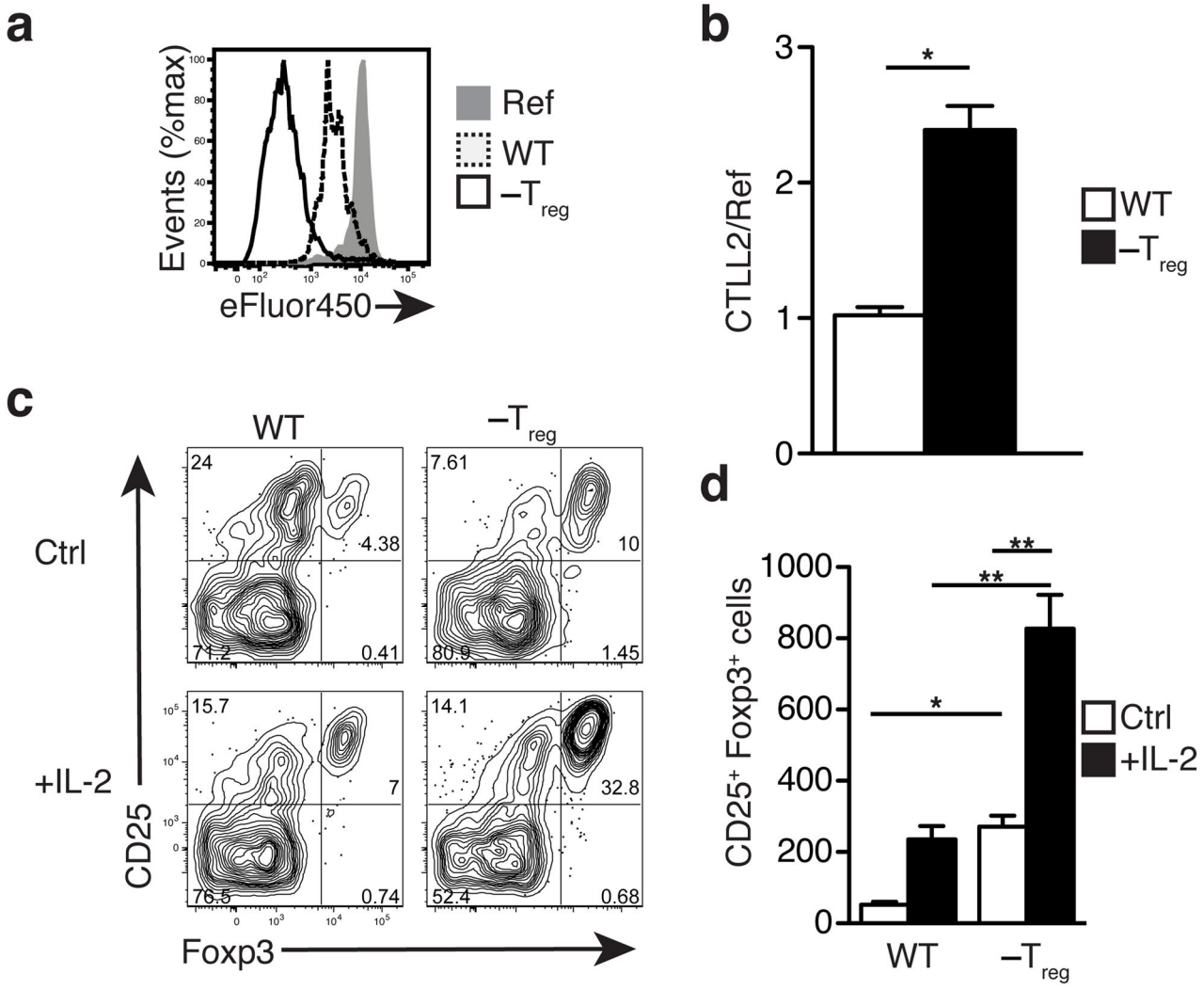

Figure 5.

Existing T_{reg} cells limit available IL-2/IL-15 within the thymus. (a,b) The IL-2/IL-15-dependent CTLL2 cell line was labeled with eFluor450 and cultured on either WT or T_{reg} deficient (AND TCR) thymic slices. (a) Proliferation assessed at d4. Wild type thymocytes labeled with eFluor450 were included as a reference population to provide an indication of the level of fluorescence in a non-dividing population. (b) Survival relative to reference wild type thymocyte population following 2d of culture, n=6 slices. (c,d) OT-II T_{reg} cell development on WT or T_{reg} deficient slices in the presence or absence of added IL-2 complexes. Antigen was introduced by i.v. injection of mice with 2mg of OVA protein. (c) Representative flow cytometry plots. (d) Number of OT-II T_{reg} cells (CD25+Foxp3+) recovered per slice, n=9, 11,15, and 11 slices respectively. Error bars represent +/− SEM. Data representative (a,b) or pooled (c,d) from 3 independent experiments, *p<0.05, and **p<0.001 by student's *t-test* (b) or one-way ANOVA using Tukey's post test analysis (d).

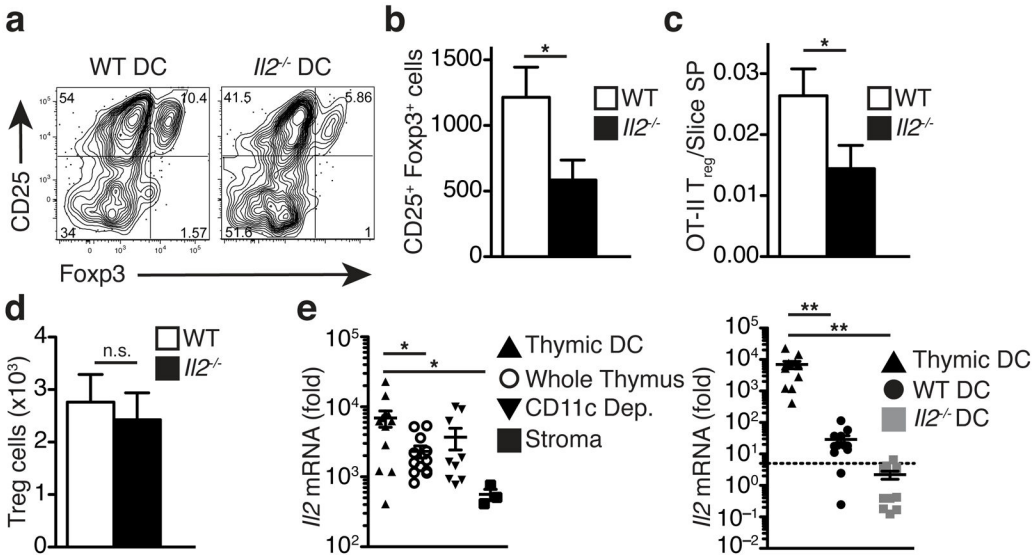

Figure 6.

Antigen-bearing dendritic cells provide a local source of IL-2 to developing thymic T_{reg} cells. (a-d) Impact of IL2 mutation on ability of DCs to support T_{reg} cell development. Bone marrow derived DCs from WT or *Il2*−/− mice were incubated with 1 mg/ml OVA protein and added to WT thymic slices along with OT-II thymocytes. OT-II T_{reg} cell development was assessed after 3 days of culture. (a) Representative flow cytometry analysis of gated OT-II CD4SP. (b) Number of OT-II T_{reg} cells (CD25+Foxp3+) recovered per thymic slice. (c) Ratio of OT-II T_{reg} cells to slice CD4 single positive thymocytes. (d) Quantification of endogenous slice T_{reg} cells recovered per slice. (a-d) n=20 and 21 slices respectively, data pooled from 3 independent experiments. (e) qRT-PCR analysis of IL-2 expression. RNA was prepared from the indicated samples and analyzed by qRT-PCR using TaqMan probes. Data are normalized to GAPDH and presented as fold increase over the background values from *Il2*$^{-/-}$ bone marrow derived DCs. Thymi from wild type mice were dissociated with collagenase-digestion and separated into single cell suspension “whole thymus” and an adherent fraction “stroma:. For some samples, single cell suspensions were depleted of CD11c+ cells using magnetic beads: “CD11c depleted”. CD11c enriched fractions were further purified by FACS to yield > 85% CD11c+ cells: “thymic DC”. Bone marrow derived DCs from wild type or *Il2*$^{-/-}$ mice were included for comparison. For *Il2*$^{-/-}$ DCs, no IL2 signal was observed after 40 cycles of PCR, therefore values reported are upper estimates (indicated by grey shading), and the dashed line indicates the lower limit of detection of the assay. Data from thymic DC samples are included on both plots for comparison. n=12 experimental replicates from 4 biological samples except “CD11c depleted” n=9 experimental replicates, 3 biological samples or “stroma” n=3 experimental replicates, 1 biological sample. Error bars represent +/− SEM. *p<0.05, **p<0.001, and n.s. = not significant by unpaired student’s *t*-test or one-way ANOVA using Tukey’s post test analysis.