Abrogation of CD40–CD154 Signaling Impedes the Homeostasis of Thymic Resident Regulatory T Cells by Altering the Levels of IL-2, but Does Not Affect Regulatory T Cell Development

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Abrogation of CD40–CD154 Signaling Impedes the Homeostasis of Thymic Resident Regulatory T Cells by Altering the Levels of IL-2, but Does Not Affect Regulatory T Cell Development

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Identification of costimulatory signals required for murine regulatory T (Treg) cell development relies on measuring the frequency of total thymic Treg cells. However, the thymus contains both resident and newly developed Treg cells; whether such signals target both populations is unknown. In this study, we show that CD40–CD154 blockade specifically targeted thymic resident Treg cells, but not, as was previously believed, newly developed Treg cells. Unlike CD28–CD80/CD86 signals, CD40–CD154 signals were not required for Treg cell precursor development. Instead we demonstrate that homeostatic proliferation of thymic resident Treg cells was dependent on CD40–CD154 signals maintaining IL-2 levels. Furthermore, in newborn mice, where all Treg cells are newly developed, blockade of CD40–CD154 signals had no effect on thymic Treg numbers or their proliferation. Our studies highlight the complexity in the study of thymic Treg cell development due to the heterogeneity of thymic Treg cells. The Journal of Immunology, 2012, 189: 1717–1725.
into account by previous studies. We show that the abrogation of the CD40–CD154 signal results in a large decrease in this resident Treg cell population, and link this reduction to a decrease in the cytokines required for homeostatic proliferation. In contrast to several previous reports, we find that the CD40–CD154 pathway has only a minor effect on actual Treg cell development. These data indicate that signals that affect the homeostasis of thymic resident Treg cells will have a dominant effect on thymic Treg cell levels.

Materials and Methods

Animals

All animals were bred and maintained under specific pathogen-free conditions. RAG2p-GFP mice were purchased from The Jackson Laboratory and have been described previously (16). C57BL/6 mice have been described previously (17). C57BL/6 and C57BL/6 CD45.1 mice were purchased from The Jackson Laboratory. All animal work was conducted under Home Office project license regulations after approval from Cambridge University Ethical Review Committee.

Flow cytometry

Single-cell suspensions were labeled with Abs against CD3 (145-2C11), CD4 (RM4-5 or GK1.5), CD8α (53-6.7), CD25 (PC61.5), CD45.1 (A20), CD45.2 (104), Foxp3 (FJK-16s), GITR (DTA-1), Ki67 (B5; BD Biosciences), or isotype Abs (all Abs from eBioscience unless specified). Intracellular labeling of Foxp3 and Ki67 was performed using eBioscience fixation/permeabilization buffers, as per manufacturer’s instructions. Labeling of Foxp3 in RAG2p-GFP thymocytes was performed after fixation and permeabilization in BD Bioscience buffers and was with anti–Foxp3-EF450 Ab. Flow cytometry was performed on a CyAn ADP (Dako) and analyzed using FlowJo software (Tree Star).

In vivo studies

For costimulatory blockade, 300 μg anti-CD154 (MR1) Ab (Bio X Cell), 100 μg anti-CD80 Ab (16-10A1) with 100 μg anti–IL-2 Ab (clone JES6-1A12; eBioscience), and 10 μg anti-IL-2 (Bio X Cell), or 300 μg hamster IgG (hIgG; Jackson ImmunoResearch Laboratories) was i.p. injected into recipient mice. Mice were injected five times every other day for 9 d. Mice were analyzed 24 h after final injection. For IL-2 treatment, mice were injected i.p. with a mixture of 1 μg mouse rIL-2 (eBioscience) and 10 μg anti–IL-2 Ab (clone JES6-1A12; eBioscience), prepared as described previously (18). Control mice were injected with 10 μg rat IgG (rIgG). Mice were given three injections, every other day for 5 d, and were analyzed 48 h after the final injection.

Real-time PCR

Thymi were digested in RPMI 1640 medium containing 0.5 mg/ml collagenase and 0.3 mg/ml dispase, and RNA was then extracted using a RNeasy kit (Qiagen), according to manufacturer’s instructions. cDNA was generated using oligo-dT primers and SuperScript II, as per manufacturer’s instructions. Gene expression was quantified with primer/probe mixes from Applied Biosystems. Samples were run on an ABI7300 real-time PCR machine. GAPDH was used as an endogenous control, and relative quantities of cDNA were calculated using the 7000 SDS system software. Data are normalized to wild type (WT).

Bone marrow chimera mice

Recipient mice were irradiated with two doses of 550 rad, separated by 3 h. Donor bone marrow was depleted of T cells by CD4 and CD8 microbeads (Miltenyi Biotec), as per manufacturer’s instructions, prior to injection into recipient mice. Mice were then analyzed 8–10 wk later.

Statistical analysis

Analysis of experimental and control groups was performed by a two-tailed nonparametric Mann-Whitney U test. Statistical significance was assigned if p value < 0.05. Statistics given in the text are mean ± SE.

Results

Impaired CD40–CD154 signaling decreases thymic resident Treg cell numbers

A recent study using RAG2p-GFP mice has revealed that two populations of Treg cells reside in the thymus, as follows: GFP−
resistant Treg cells and newly developed GFP+ Treg cells (13). Flow cytometric analysis of thymi from RAG2p-GFP mice has established that thymic resident Treg cells constitute the major fraction of thymic Treg cells in adult mice, as ~60% of thymic Treg cells are GFP+ (Fig. 1A) (13). Because resident Treg cells represent the major proportion of thymic Treg cells, they are likely to have a large effect on analysis of Treg cell development; this has not been previously taken into account. The CD40–CD154 costimulatory pathway has been shown to be important for thymic Treg cells, as abrogation of CD40–CD154 signals resulted in a significant reduction in total thymic Treg cell numbers (5–7). We asked whether this costimulatory pathway was important for numbers of resident Treg cells and/or newly developed Treg cells. To address this, we injected RAG2p-GFP mice with an anti-CD154 Ab that blocks the CD40–CD154 pathway, and measured the impact such costimulatory blockade had on the Treg cell populations.

Flow cytometric analysis showed that blockade of the CD40–CD154 pathway caused a significant decrease in both the frequency and the absolute number of thymic resident Treg cells in comparison with control Ab-treated mice (Fig. 1B, 1C, Supplemental Fig. 1). However, whereas blockade of this pathway significantly decreased the frequency of newly developed Treg cells when compared with the frequency of newly developed Treg cells in control Ab-treated mice, no effect was seen in absolute number (Fig. 1D, 1E). The reduction seen in newly developed Treg cells was minor in comparison with the large reduction in thymic resident Treg cells: after anti-CD154 treatment, resident Treg cells were decreased by 47% ± 3.5, whereas the reduction in newly developed Treg cells was merely 16% ± 3.6, in comparison with control Ab-treated mice. This indicates that the CD40–CD154 pathway has a minor effect on the development of Treg cells, and instead mainly affects levels of thymic resident Treg cells. This conclusion is supported by our data showing that newly developed Treg cells were significantly increased as a percentage of thymic Treg cells in the absence of the CD40–CD154 pathway (Fig. 1F).

The use of RAG2p-GFP mice to identify newly developed Treg cells in the thymus is novel, and so we wished to test whether a signal with a well-established role in Treg cell development would reduce the numbers of GFP+ newly developed Treg cells, thus validating our analysis. For this purpose, we blocked the CD28–CD80/CD86 pathway, as the role of this pathway has been demonstrated to be early in Treg cell development (9–11). Treatment of RAG2p-GFP mice with blocking anti-CD80 and anti-CD86 Abs resulted in a significant reduction of the frequency and absolute number of newly developed Treg cells, compared with control Ab-treated mice (Fig. 1D, 1E). We examined the effect of the CD28–CD80/CD86 pathway on the thymic resident Treg cells, and found that these cells were also significantly reduced in both frequency and absolute number (Fig. 1B, 1C).

Whereas we have identified that the CD40–CD154 pathway has little role in the development of Treg cells in the thymus, it is important to verify whether thymic output is affected. RD Treg cells can be detected in the periphery, as they retain some GFP (14). However, it should be noted that these cells may not represent total recent thymic emigrants, as some Treg cells may egress at the GFP+ stage. There was a significant decrease in the frequency and absolute number of both RD Treg cells and non-RD Treg cells (Fig. 2A–D). The decrease was smaller for RD Treg cells than for non-RD Treg cells (25% ± 4.8 versus 37% ± 1.7), and, as a percentage of total Treg cells, GFP+ cells were significantly increased (Fig. 2E). Therefore, these data imply that the greatest cause of the reduced splenic Treg cell population after CD40–CD154 blockade was decreased homeostasis of peripheral Treg cells.

**FIGURE 2.** CD40–CD154 blockade mainly causes a reduction in non-RD GFP+ peripheral Treg cells. (A and B) RAG2p-GFP mice were injected with anti-CD154, anti-CD80, and anti-CD86, or hlgG Abs every other day for 9 d, and the frequency (A) and absolute number (B) of RD GFP+ Foxp3+ CD4+ splenocytes were determined 24 h after the last injection. (C and D) RAG2p-GFP mice were injected with Abs, as described above, and the frequency (C) and absolute number (D) of non-RD GFP+ Foxp3+ CD4+ splenocytes were determined. (E) RAG2p-GFP mice were injected with Abs, as described above, and the frequency of newly developed GFP+ splenocytes within the Foxp3+ CD4+ subset was determined. The data presented in (A)–(E) are representative of three individual experiments giving similar results. *p < 0.05, **p < 0.01.
CD40–CD154 signals are not important for Treg cell precursor development

It is important to examine the effect of the CD40–CD154 pathway on Treg cell precursors, especially as Treg cell precursors contain preformed CD154 (data not shown). In other contexts, blocking signals that have been shown to affect the development of Treg cell precursors caused a reduction in their numbers (9, 11), whereas signals that affected the progression of Treg cell precursors to maturity caused an increase in their numbers (19). Thus, Treg cell precursor levels are a useful tool for analyzing Treg cell development. GITR<sup>high</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> CD4SP thymocytes (Treg cell precursors) had a higher GFP intensity than GFP<sup>+</sup> Foxp3<sup>+</sup> CD4SP thymocytes; the GFP mean fluorescence intensity was 87% ± 2.5 higher in the Treg cell precursors (Fig. 3A), implying that they are earlier in the Treg cell developmental pathway, as predicted.

We analyzed the frequency of Treg cell precursors in RAG2p-GFP mice receiving anti-CD154 Abs in comparison with control Ab-treated mice. Although the frequency of Treg cell precursors was slightly reduced by 11% ± 3.4 following blockade of CD40–CD154 signals in comparison with control mice, no effect was seen in absolute numbers (Fig. 3B, 3C). In addition, these Treg cell precursors were not reduced in frequency or absolute number in CD154<sup>−/−</sup> mice when compared with WT mice (Fig. 3D, 3E). These data demonstrate that the role of the CD40–CD154 pathway in Treg cell development is late in the process, and probably after the cells become Foxp3<sup>+</sup>. To confirm our strategy for measuring Treg cell precursor levels, we measured these cells after blockade of the CD28–CD80/CD86 pathway. This resulted in a reduction in the frequency and absolute number of these Treg cell precursors compared with control mice (Fig. 3B, 3C), as expected (9).

CD40–CD154 signals are required for efficient homeostatic proliferation of thymic resident Treg cells

We have previously demonstrated that thymic Treg cell proliferation is reduced in the absence of the CD40–CD154 pathway (6). However, it is not known whether thymic resident Treg cells are undergoing homeostatic proliferation or whether this is affected by the CD40–CD154 pathway. Using Ki67, a marker of cells in active stages of the cell cycle (20), we found that few newly developed Treg cells were Ki67<sup>+</sup> (Fig. 4A). Instead, 16% ± 1.1 of thymic resident Treg cells were Ki67<sup>+</sup> (Fig. 4A). When the CD40–CD154 pathway was blocked, there was a 50% ± 5.7 drop in actively cycling thymic resident Treg cells (Fig. 4B) compared with control Ab-treated mice, indicating that this pathway is needed for efficient proliferation of these cells. To our knowledge, this is the first demonstration of homeostatic proliferation of thymic resident Treg cells.

We confirmed this finding by comparing CD154<sup>−/−</sup> mice with WT; the reduction in the percentage of total Foxp3<sup>+</sup> CD4SP thymocytes that were Ki67<sup>+</sup> in the CD154<sup>−/−</sup> mice was 40% ± 1.2 (Fig. 4C).

As we have shown that the reduction in RD peripheral Treg cells after CD40–CD154 blockade was smaller than the reduction in non-RD Treg cells (Fig. 2E), it implies that mechanisms apart from thymic output must be affecting peripheral Treg cells. The literature is not in agreement as to whether the CD40–CD154 pathway affects the turnover of Treg cells in the periphery (5, 6). After anti-CD154 Ab treatment, we found a 44% ± 1.6 reduction in the frequency of splenic Treg cells that were Ki67<sup>+</sup>, compared with control Ab-treated mice (Fig. 4D). A reduction was also seen when comparing CD154<sup>−/−</sup> mice with WT (Fig. 4E).

Levels of intrathymic IL-2 and IL-15 are reduced in CD154<sup>−/−</sup> mice

These data have indicated that, in the absence of the CD40–CD154 pathway, the niche for thymic resident Treg cells was

**FIGURE 3.** CD40–CD154 signals are not important for Treg cell precursor development. (A) Thymocytes were isolated from RAG2p-GFP mice, and the GFP intensity of double-positive, Treg cell precursors (GITR<sup>high</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> CD4SP), mature Treg (Foxp3<sup>+</sup> CD4SP), and non-Treg (CD25<sup>+</sup> Foxp3<sup>+</sup> CD4SP) thymic subsets was determined. The data are representative of three independent experiments performed, a total of 14 mice examined. (B and C) RAG2p-GFP mice were injected with anti-CD154, anti-CD80, and anti-CD86, or hlgG Abs every other day for 9 d, and the frequency (B) and absolute number (C) of GITR<sup>high</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> CD4SP Treg cell precursors were determined 24 h after the last injection. (D and E) Thymocytes were isolated from CD154<sup>−/−</sup> mice or WT mice, and the frequency (D) or absolute number (E) of GITR<sup>high</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> CD4SP Treg cell precursors was determined. The data are representative of three independent experiments (B, C, and E) or eight independent experiments (D). *p < 0.05.
FIGURE 4. In the absence of the CD40–CD154 pathway, the steady-state proliferation of thymic resident Treg cells is reduced. (A) Thymocytes were isolated from RAG2p-GFP mice, and the percentage of Ki67+ thymocytes within the Foxp3+ CD4SP subset was compared between resident (GFP+) and newly developed (GFP−) cells. (B) RAG2p-GFP mice were injected with anti-CD154 or hlgG Abs every other day for 9 d, and the frequency of Ki67+ thymocytes within the GFP+ Foxp3+ CD4SP subset was determined 24 h after the last injection. (A) and (B) are representative of two independent experiments. (C) Thymocytes were isolated from CD154−/− mice or WT mice, and the frequency of Ki67+ cells within the Foxp3+ CD4SP subset was determined. Data are representative of five independent experiments. (D) RAG2p-GFP mice were injected with Abs, as described in (B), and the frequency of Ki67+ splenocytes within the Foxp3+ CD4− SP subset was determined. Data are representative of three independent experiments. (E) Splenocytes were isolated from CD154−/− mice or WT mice, and the frequency of Ki67+ cells within the Foxp3+ CD4− SP subset was determined. Data are representative of six independent experiments. *p < 0.05, **p < 0.01.

reduced. Others have used an in vitro system to link similar reductions in the periphery to lower IL-2 production (5), as Treg cell homeostasis is very closely linked to IL-2 levels (21). We used real-time PCR analysis on thymic levels of IL-2 and IL-15, two cytokines closely linked to Treg cell development (3, 22), to show that these cytokines were reduced in the steady-state thymus of CD154−/− mice in comparison with WT (Fig. 5A, 5B). IL-2 was reduced by 65% ± 5.9, and IL-15 was reduced by 52% ± 6.1, compared with WT.

To determine whether decreased IL-2 levels were responsible for the effects on thymic resident Treg cells upon CD40–CD154 pathway blockade, we treated mice with IL-2 coupled to anti–IL-2 Ab. A complex of IL-2 with this anti–IL-2 Ab is believed to enhance the in vivo activity of IL-2 by increasing its 1/2 (23). After IL-2:anti–IL-2 Ab treatment of anti-CD154 Ab-treated mice, thymic resident Treg cells were significantly increased over mice treated with hlgG and anti-CD154 Ab, to near the levels of control mice treated with hlgG and rlgG (Fig. 5C). As expected, changes in the frequencies of newly developed Treg cells were much smaller between all three groups (Fig. 5C). Furthermore, treatment of CD154−/− mice with IL-2:anti–IL-2 Ab also resulted in an increase in thymic Treg cells over untreated CD154−/− mice, to near levels in WT mice (data not shown). To determine whether replenishment of IL-2 levels increased the cycling activity of thymic resident Treg cells, we measured the frequency of Ki67+ resident thymic Treg cells. As shown in Fig. 5D, there was an increase in the frequency of actively cycling thymic resident Treg cells after IL-2:anti–IL-2 Ab treatment of anti-CD154 Ab-treated mice, compared with mice treated with hlgG and anti-CD154 Ab.

Because thymic dendritic cells (DCs) and Foxp3+ CD4SP thymocytes can produce IL-2, we looked to see whether abrogation of CD40–CD154 signals impacted on the frequency of either population. In CD154−/− mice, thymic DCs are only slightly reduced compared with WT mice, whereas conventional CD4SP thymocytes are increased (Fig. 5E, 5F). So the effect of the CD40–CD154 pathway on IL-2 production is not simply by affecting the levels of IL-2-producing cells. The increase in the conventional CD4SP thymocytes in CD154−/− mice has been noted before (7, 17), and may be due to decreased negative selection (24).

CD154 has a cell-extrinsic role in thymic resident Treg cell homeostasis

Although classically CD154 signals through cross-linking of CD40 on target cells, some reports have suggested that intrinsic back signals through CD154 can modulate T cells (25, 26), affecting processes such as thymic Treg cell development (6). To determine whether CD154 affects thymic Treg cell numbers via a cell-intrinsic or cell-extrinsic mechanism, we generated bone marrow chimera with CD45.1 WT cells that had been reconstituted with a 50:50 mixture of CD45.1 WT and CD45.2 CD154−/− bone marrow. To control for differences between the CD45.1 and CD45.2 strains that could be independent of CD154, we also generated CD45.1 WT versus CD45.2 WT chimeras. All data shown are CD45.2+ thymocytes, normalized to the CD45.1+ thymocyte data. No significant difference in Foxp3+ Treg cell frequency was seen between the CD45.2 CD154−/− and the CD45.2 WT thymocytes (Fig. 6A). This indicates that the role the CD40–CD154 pathway plays in Treg cell development is extrinsic and not specific to those thymocytes expressing CD154 (6).

To ascertain whether the difference in proliferation seen between Treg cells from CD154−/− and WT mice is also cell extrinsic, we examined the percentage of Ki67+ Foxp3+ CD4SP thymocytes. No significant difference was seen in the percentage of CD154−/− or WT Treg cells that were Ki67+ when in chimera with CD45.1 WT cells (Fig. 6B), again suggesting that CD40–CD154 signals act extrinsically.

Finally, we checked the chimeric mice for a difference in Treg cell precursor frequencies. There was no reduction in CD154−/− Treg cell precursors when compared with the CD45.2 WT (Fig. 6C), as expected.

The CD40–CD154 pathway has little effect on thymic Treg cell levels in neonates

Our model of Treg cell development states that the effect of the CD40–CD154 pathway is on longer-lived thymic Treg cells; as
such, it would be expected that, in neonatal mice, where all Treg cells are newly developed (27), Treg cell levels should be unaffected by the presence or absence of the CD40–CD154 pathway. Our data demonstrate that, at least until day 4, Treg cells were near WT levels in CD154−/− mice (Fig. 7A). A clear reduction in the frequency of CD154−/− Treg cells compared with WT was observed.

FIGURE 5. Thymic resident, but not developing, Treg cell homeostasis is sensitive to changes in intrathymic IL-2 levels. (A) The relative quantity of IL-2 mRNA from whole thymus was compared between WT and CD154−/− mice. The data have been normalized to WT samples and pooled from two independent experiments. (B) The relative quantity of IL-15 mRNA from whole thymus was compared between WT and CD154−/− mice. The data have been normalized to WT samples and pooled from two independent experiments. (C) RAG2p-GFP mice were injected with anti-CD154 Ab or control hlgG every other day for 9 d; starting on the fifth day of treatment, mice were also injected with 1 μg IL-2:10 μg anti–IL-2 complex or control rlgG every other day for 5 d. The frequency of Foxp3+ CD4SP thymocytes was determined, and data are separated into GFP− resident Treg cells (left panel) or GFP+ newly developed Treg cells (right panel). (D) RAG2p-GFP mice were treated as described above, and the percentage of Ki67+ thymocytes within the GFP+ Foxp3+ CD4SP resident Treg cell subset was determined. (E) Total thymic suspensions were isolated from WT or CD154−/− mice, and the frequency of CD11c+ cells was determined. Data are representative of five independent experiments. (F) Thymocytes were isolated from WT or CD154−/− mice, and the frequency of CD25+ Foxp3+ CD4SP cells was determined. Data are representative of 12 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 6. The effects of CD154 on thymic Treg cells are cell extrinsic. (A) Bone marrow chimeric mice were generated by reconstitution of irradiated CD45.1 mice with a 50:50 mixture of CD45.1 WT and CD45.2 CD154−/− bone marrow (left two groups), or CD45.1 WT and CD45.2 WT bone marrow (right two groups). Mice were analyzed after 2 mo, and the percentage of Foxp3+ CD4SP thymocytes within each CD45 subtype was determined. The frequencies of CD45.2 WT or CD45.2 CD154−/− thymocytes were normalized to CD45.1 WT thymocytes in the respective chimeras. (B) Chimeric mice were generated, as in (A), and the percentage of Ki67+ thymocytes within the Foxp3+ CD4SP subset was determined. The data were normalized as in (A) above. (C) Chimeric mice were generated, as in (A), and the frequency of GITRhigh CD25+ Foxp3+ CD4SP Treg precursors within CD45 subtype was determined. The data were normalized as before. The CD45.1 WT versus CD45.2 CD154−/− chimera experiment was performed twice, with at least four mice each time.
detected at ∼1 wk after birth. This reduction of CD154−/− Treg cells when compared with WT Treg cells continued to increase as older mice were examined.

We examined proliferation of Treg cells during ontogeny. At the earliest stages, there was no difference in the percentage of Ki67 Treg cells between WT and CD154−/− mice (Fig. 7B). As the mice aged, the percentage of Ki67 Treg cells rapidly decreased, and by approximately day 10 a clear difference between WT and CD154−/− mice was seen. This difference remained small, but increased as the mice age.

To confirm that in neonatal mice all Treg cells are RD, and that resident Treg cells can be detected by the time that CD154 affects thymic Treg cell levels, we examined RAG2p-GFP mice at 4 and 8 d old (Fig. 7C). At day 4, there was no peak of GFP Foxp3 thymocytes, indicating that all Treg cells were newly formed. By day 8, a clear peak of GFP Foxp3 thymocytes was seen, although these cells were a much smaller proportion of the thymic Treg cells than in adult mice. Therefore, our data suggest that the role of CD40–CD154 signals in Treg cell development gradually increases as the mice age because the resident Treg cells are not present at the earliest time points, but then gradually increase as a percentage of total thymic Treg cells. This is in keeping with our overall model for the role of CD40–CD154 signals in Treg cell development (Fig. 8).

Discussion

Several studies have shown that Treg cell development is reduced in the absence of the CD40–CD154 pathway (5–7). However, these studies, and in fact most of the wider Treg cell development field, do not take into account the recently discovered thymic resident Treg cells. We examined Treg cell development in the context of these cells and showed that, in the absence of the CD40–CD154 pathway, there was only a small drop in Treg cells at all stages of development. However, in both the thymus and the periphery, there was a much larger drop in the populations of already existing Treg cells. We link this reduction in thymic resident Treg cells to reduced intrathymic cytokine, in particular IL-2, which in turn reduced the homeostatic proliferation of resident Treg cells (summarized in Fig. 8). To our knowledge, no one has directly examined the homeostatic proliferation of the resident Treg cell subset, and our data indicate that changes in the rate of homeostasis can have the dominant effect on thymic Treg cell levels.

Our data have important implications, as they demonstrate that levels of thymic Treg cells are not indicative of the rate of development. Whereas we have used the CD40–CD154 pathway as
an example of a signal whose role has been overestimated, the reverse is potentially possible. In other contexts, it has been demonstrated that Treg cell niches are filled rapidly (28); thus, it is likely that even if Treg cell development were reduced to very low levels, this would manifest as only a small drop in thymic Treg cell levels, as the majority of thymic Treg cells are resident. An example of this may be the work of Liu et al. (29); the system they studied is an interesting reversal of ours. They find that, in the absence of TGF-β signaling, a difference can be seen in neonatal Treg cells, but not adult, and they link this to increased IL-2. In their model, we hypothesize that the increased IL-2 would most likely affect the resident Treg cells. This highlights the attractiveness of using the RAG2p-GFP system to allow clarification of the role of TGF-β and many other signals in adult Treg cell development.

In our model, we also found that Treg cells in neonatal mice were affected differently by signal blockade, demonstrating that CD40–CD154 signals were not required in newborn mice. We believe that the independence from such CD40–CD154 signals in newborns is because all the Treg cells are newly developed at these early stages, and resident Treg cells are not yet a large population in the thymus. Thus, the CD40–CD154 pathway has little effect on thymic Treg cells just after birth.

Two cytokines closely linked to Treg development are IL-2 and IL-15 (3, 22), and we see a decrease in both when CD40–CD154 signals are lacking, suggesting that reduction in these two cytokines may be indicative of a general reduction in thymic cytokine production. However, despite these cytokine reductions, we did not see a large reduction in the number of newly formed thymic Treg cells. This may be because very low levels of cytokine signaling are required for Treg cell development (30), whereas higher cytokine levels are essential for maintaining resident Treg cell numbers. IL-2 in particular has been demonstrated to be important for the homeostasis of Treg cells (21). We expand on this observation to show that it is the homeostasis of the resident Treg population that is most sensitive to reduction in IL-2 levels by abolition of CD40–CD154 signals, and their frequency and cycling ability are restored following IL-2 administration.

The significant reduction in IL-2 mRNA due to blockade of CD40–CD154 signals led us to ask whether such signals were responsible for maintaining normal numbers of IL-2-producing cells in the thymus. Although we saw a decrease in numbers of thymic DCs in the absence of CD40–CD154 signals, the slight reduction in the frequency of thymic DCs is unlikely to directly translate to a 65% reduction in IL-2 mRNA. In contrast to thymic DCs, conventional CD4SP thymocyte numbers increased in the absence of CD40–CD154 signals, a finding that has previously been noted (7, 17). Therefore, it seems likely that CD40–CD154 signals are involved in maintaining IL-2 RNA levels by mechanisms other than simple maintenance of adequate numbers of IL-2-producing cells.

It is not known how thymic resident Treg cells accumulate in the thymus. It is possible that they either migrate back into the thymus or that they are retained after development. The retention of Treg cells in the thymus may explain why our results differ from the work of Zhan et al. (7). Their study used mice that lack peripheral CD4+ T cells, due to expression of a transgene encoding anti-CD4 Ab. This allowed study of Treg cell development without contamination by migrating Treg cells. Their results indicate that CD154−/− mice still have a large reduction in thymic Treg cells even without migration. We believe that preventing migration will not prevent the formation of the thymic resident subset, as retained cells will fill the niche. Therefore, in the model lacking peripheral CD4+ T cells, the CD40–CD154 pathway will be required for efficient expansion of the thymic resident Treg cell population.

We demonstrate that without the CD40–CD154 pathway, peripheral Treg cells are reduced independently of thymic output. As these Treg cells may be able to migrate back into the thymus, it is possible that the reduction in the thymic Treg cell population is due to fewer peripheral Treg cells being available for migration into the thymus. Although this may play a role, this is unlikely to be the sole reason for the reduction in thymic resident Treg cells, as the drop in Treg cells is larger in the thymus than in the periphery. Furthermore, we have shown that thymic resident Treg cell numbers are maintained in their own right and the homeostatic proliferation of thymic Treg cells is more sensitive to the CD40–CD154 pathway than peripheral Treg cells.

One caveat is that the reduction in resident Treg cells following CD40–CD154 ablation could be due to GFP dilution caused by proliferation (13). As Treg cell proliferation is lower after anti-CD154 Ab treatment, this could potentially cause the reduction in GFP+ Treg cells, as fewer newly formed Treg cells would proliferate until they had undetectable GFP levels. However, the newly formed Treg cells appear to have a low level of proliferation, and their GFP intensity was not increased after anti-CD154 Ab; thus, it appears unlikely that decreased GFP dilution due to proliferation could be responsible for the large reduction seen in GFP+ Treg cells.

Lastly, it is important to clarify how the role of the CD40–CD154 pathway in Treg cell development compares with the role of the CD28–CD80/CD86 pathway, because CD40 signaling is known to increase CD28 ligands on APCs (8). The CD28–CD80/CD86 pathway has a well-established role in Treg cell development (9–11), and the CD40–CD154 pathway could affect thymic Treg cells by decreasing CD28–CD80/CD86 signals. In addition, CD154 has been reported to directly back signal (25, 26, 31–33), and some have linked this to association with a CD28 splice variant (12). However, our data show that the role of CD154 in Treg development is unlike that of CD28; where CD28–CD80/CD86 signals are essential at the Treg cell precursor stage, CD40–CD154 signals are less so. Furthermore, CD40–CD154 signals act in a cell-extrinsic manner to maintain thymic resident Treg cells. Therefore, explanations via the CD28 do not explain the effects of CD154 on Treg cell development, and it also appears unlikely that a CD154 back signal has a major effect in the context.

In summary, we have demonstrated that effects on the homeostatic proliferation of thymic resident Treg cells can have a dominant effect on thymic Treg cell levels. These novel findings add a significant layer of complexity to Treg cell development analysis.

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Disclosures
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