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Regulatory T Cells Dynamically Control the Primary Immune Response to Foreign Antigen

Dipica Haribhai,* Wen Lin,† Lance M. Relland,* Nga Truong,† Calvin B. Williams,*2* and Talal A. Chatila2†

The population dynamics that enable a small number of regulatory T (T_R) cells to control the immune responses to foreign Ags by the much larger conventional T cell subset were investigated. During the primary immune response, the expansion and contraction of conventional and T_R cells occurred in synchrony. Importantly, the relative accumulation of T_R cells at peak response significantly exceeded that of conventional T cells, reflecting extensive cell division within the T_R cell pool. Transfer of a polyclonal T_R cell population before immunization antagonized both polyclonal and TCR transgenic responses, whereas blocking T_R cell function enhanced those responses. These results define an inverse quantitative relationship between T_R and conventional T cells that controls the magnitude of the primary immune response. The high frequency of dividing T_R cells suggests degenerate TCR specificity enabling activation by a broad spectrum of Ags. The Journal of Immunology, 2007, 178: 2961–2972.

Among the several subpopulations of regulatory T (T_R) cells identified to date, the naturally arising CD4⁺CD25⁺ T_R cells have emerged as particularly critical for the maintenance of immunological tolerance (1). CD4⁺CD25⁺ T_R cells arise in the thymus, represent 5–10% of CD4⁺ T cells in the periphery, and are distinguished by their specific and universal expression of the forkhead type transcription factor Foxp3, which serves as a master switch factor for their development and function (2–4). In vitro, T_R cells are anergic, do not produce IL-2, and fail to proliferate in response to mitogenic stimulation (5, 6). They constitutively express the IL-2γc-chain (CD25) and a number of other surface markers that are not specific for T_R cells (1, 7). Lack of specific markers has complicated the in vivo identification and analysis of T_R cells.

The mechanisms by which the smaller subset of T_R cells regulate the responses of the much larger subset of conventional T cells are of considerable interest. The essential function of T_R cells in the maintenance of tolerance to self-Ags is established, with dominant, contact-dependent suppression of autoimmune responses emerging as a central feature of T_R cell activity (8). Indeed, the absence of T_R cells due to Foxp3 deficiency results in a fatal lymphoproliferative disease marked by autoimmunity and inflammation in multiple organs (4, 9–15).

More problematic is the role of T_R cells in the control of immune responses to foreign Ags. This dilemma is due to the minimal overlap of their TCR repertoire with that of conventional T cells and their decided bias toward self-reactivity (16–18). Consistent with such regulation, T_R cell depletion and/or blocking of T_R cell activity by means of an anti-CD25 mAb enhances the immune response to infectious agents and to immunization (19–22). This process is postulated to involve increased APC function, an enhanced expansion phase or a prolonged contraction phase of the primary immune response (23, 24). Although control of immune responses is beneficial in most settings, T_R cell inhibition of effector T cell responses to infections may allow for persistence of pathogens such as parasites, viruses, and indolent bacterial species (25). Therefore, a detailed understanding of mechanisms involved in T_R cell control of immune responses to foreign Ags is essential to the rational manipulations of these responses.

Given the fact that in vitro T_R cells are generally viewed as anergic, the stimulatory effects of anti-CD25 mAb treatment on the immune response to foreign Ags could be interpreted as the result of overcoming a tonic, basal inhibitory effect by T_R cells. However, adoptive transfer studies with TCR transgenic T_R cells demonstrate that T_R cells can proliferate in vivo in response to primary immunization with cognate Ag and that they suppress the expansion of TCR transgenic conventional T cells (26, 27). These data suggest that Ag-driven proliferation of T_R cells occurs in vivo. However, two caveats limit a broad interpretation of these results. The first is the forced expression of the same transgenic TCR on both conventional and T_R cells. This condition is not likely to occur in normal mice, because these two populations have largely distinct TCR repertoires. The second issue is the inflated frequencies of Ag-specific conventional and T_R cells used in these experiments. This approach does not replicate the stoichiometry of the interaction between conventional and T_R cells seen in a normal polyclonal response.

Attempts to study the more physiological in vivo polyclonal T_R cell response to foreign Ags have yielded conflicting results and are subject to the same difficulties in interpretation noted above. Although some adoptive transfer studies demonstrate suppression by polyclonal T_R cells of primary and memory immune responses

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of conventional T cells. The extent of cell division within the TR reveal that TR cells proliferate vigorously in vivo after immunization. The kinetics of TR cell expansion and contraction parallel that of effector T cells.

In this study, we investigated the mechanisms by which polyclonal TR cells regulate the immune responses to foreign Ags. To that of effector T cells).

Genera and Methods

Generation of Foxp3EGFP mice

Foxp3 genomic DNA was isolated from a bacterial artificial chromosome clone (Genome Systems) and subcloned into the plasmid vector pKo (Lexicon). A DNA cassette composed of an internal ribosomal entry sequence (IRES) linked to downstream EGFP and SV40 poly-A sequences (BD Clontech) was inserted by blunt-end ligation at the SSPI restriction site immediately downstream of the Foxp3 translational stop codon and upstream of the endogenous polyadenylation signal. A PKG-neo cassette was also inserted at the EcoRI site in intron 9 of Foxp3 in the same orientation as Foxp3 and was flanked (floxed) by two LoxP sites to allow excision by Cre-mediated recombination. The targeting construct also included a diphtheria toxin gene (DT) for negative selection against randomly inserted targeting constructs.

Targeting plasmids were introduced by electroporation into SC10 embryonic stem cells and subjected to G418 selection. Resistant clones were screened by Southern blotting. Successfully targeted clones were injected into C57BL/6 blastocysts, and chimeric males were mated with wild-type females to generate fostering chimeras, and the resulting progeny were backcrossed into the C57BL/6J background to obtain homozygous Foxp3EGFP mice.

PCR screening of the mutant allele was also achieved by PCR amplification using genomic DNA and the following primers: forward sense 5'-GGTGAAGATGGATGCTAGTT-3' and reverse 5'-GGTGCTGGCTGTCTGTCC-3'. PCR-based discrimination between WT and mutant alleles was conducted by analyzing the presence of the residual LoxP-sequence retained in the mutant allele following Cre-mediated excision of the PGK-neo cassette. The following primers were used: sense 5'-GGTTAAGCGAGGATGAGG-3' and antisense 5'-GCATGGTCAAGGGTGAT-3'.

Quantitative real-time PCR analysis

Real-time PCR for Foxp3 expression was performed as described previously (15). Samples were analyzed in triplicate, and the relative expression was determined by normalizing expression of each target to the endogenous reference, hypoxanthine phosphoribosyltransferase (Hprt) transcript. Primers and internal fluorescent probes were as follows: Foxp3 exon 7, sense 5'-CCCAAGGAAGAGCACAACCTT-3' and antisense 5'-TTCTTCAACACGAGCCCTTGG-3', and probe 5'-FAM-ATCTTACCCACCGTGGCAGGTGACCT-3' and probe 5'-FAM-ATCTTACCCACCGTGGCAGGTGACCT-3'.

Antibodies

PE anti-mouse Thy1.2, allophycocyanin anti-mouse Thy1.1, allophycocyanin anti-mouse CD44, allophycocyanin B220/ Flow Kit, and PE anti-mouse CD4 were obtained from BD Biosciences. Pacific Blue anti-mouse CD4, Pacific Orange anti-mouse CD8, biotin anti-DO11.10 TCR, PE-Texas Red anti-mouse CD62L, allophycocyanin Cy5.5 anti-mouse CD19, and streptavidin-PE-Cy5.5 were obtained from Invitrogen Life Technologies. Pacific Blue anti-mouse Foxp3 was purchased from eBioscience and used following the manufacturer's instructions. The clone Pori was purchased from the American Tissue Culture Collection, and the Abs were generated and purified in the laboratory.

Flow cytometry

For cell surface markers, single-cell suspensions of lymphocytes were stained as previously described (30). Intracellular Foxp3 staining was conducted using an anti-murine Foxp3-Pacific Blue Ab (15). Samples were analyzed on an LSRII using a live cell gate, and a minimum of 1 × 10⁴ live cell events were collected per sample.

Suppression assays

Plates were coated with anti-CD3 mAb at 2.5 μg/ml in PBS for 6 h at 37°C. CD4+ cells were enriched from whole splenocytes using mouse CD4 column (R&D Systems), and CD4+ EGFP+ and CD4+ EGFP- cells were further purified by cell sorting. Purified cell populations were suspended in culture medium containing RPMI 1640, 10% FBS, 5 mM glutamine, and 10 μg/ml anti-CD28 mAb. Cells were added to coated wells, and the number of responder cells (R) was kept constant at 5 × 10⁵ cells per well. The number of suppressor cells (S) was titrated to achieve the R:S ratio as indicated (see Fig. 2D). Triplicate wells were set at each R:S ratio. Cultures were incubated for 48 h at 37°C with 5% CO2, pulsed with 0.4 μM/well [3H]TdR for an additional 18 h, harvested onto fiber filters using a Micro96 harvester (Skatron), and counted.

Immunization

Foxp3EGFP mice were immunized s.c. with CFA emulsified 1:1 with PBS (100 μl per mouse). At various times after immunization, the draining lymph nodes were removed and the total number of lymphocytes and those of the respective subsets were determined by counting with a hemocytometer and by flow cytometric analysis, respectively.

Immunocytochemistry

Tissues were fixed in 4% formaldehyde in PBS for 1 h at 4°C, transferred to 20% sucrose in PBS, and incubated 1 h at 4°C, embedded in the OCT reagent, and frozen in liquid nitrogen. Sections were cut at a thickness of 7 μm, fixed with acetone for 10 min at 4°C, and blocked with 5% BSA in PBS for 30 min in a humidified chamber. Abs and secondary reagents were diluted in the blocking buffer to a final concentration of 2 μg/ml. Primary reagents were applied to the sections, and incubated for 12 h in a humidified chamber at 4°C. In the case of biotinylated primary Abs (anti-I-A, blocking reagents (Vector Laboratories) were each applied for 15 min at room temperature before applying the primary Ab. Following incubation with primary Abs, the slides were washed and secondary reagents were applied as appropriate for 1 h at 4°C. The slides were then mounted in ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Molecular Probes). Sections were visualized by laser scanning confocal microscopy with a Leica TCS SP II microscope equipped with 488, 568, and 633 nm lasers. Images were collected using Leica acquisition software at ×10, ×20, ×40, and a ×63 objective with a ×4 optical zoom for close-up.
CD4 T cells from lymph nodes and spleens were purified by negative selection using mouse CD4 columns following the manufacturer’s recommendations (R&D Systems). Purified CD4+ T cells were stained with anti-CD4-PE mAb and sorted into CD4+ and CD4− populations using a FACSAria or a FACSvantage SE cell sorter (BD Biosciences).

**Isolation of EGFP+ and EGFP− T cells**

CD4+ T cells from lymph nodes and spleens were purified by negative selection using mouse CD4 columns following the manufacturer’s recommendations (R&D Systems). Purified CD4+ T cells were stained with anti-CD4-PE mAb and sorted into CD4+ EGFP+ and CD4+ EGFP− populations using a FACSAria or a FACSvantage SE cell sorter (BD Biosciences).

**In vivo suppression**

A total of 2 × 106 CD4+ EGFP+ T cells was adoptively transferred into BALB/c mice. Mice were immunized the following day with CFA. At 10 days after immunization, the draining lymph nodes were analyzed by cell count and FACS.

For suppression of Ag-specific T cells, 2.5 × 105 CD4+ Rag−/− DO11.10 T cells were adoptively transferred i.v. into BALB/c mice. Two to 3 h later, 5 × 104 CD4+ EGFP+ T cells were also adoptively transferred i.v. into the same mice. Mice were immunized s.c. with 2 nmol of OVA (323–339) peptide emulsified in CFA. At the peak of the immune response, draining lymph nodes were analyzed by cell count and by FACS.

**Antagonizing Tr cell function with a blocking anti-CD25 mAb**

Mice were given 250 μg of PC61 mAb i.p. at day −4 and day −1. Mice were immunized with CFA s.c. on day 0. Lymphocytes from draining lymph nodes were analyzed by cell count and by FACS.

**BrdU treatment and detection**

Mice were given a single i.p. injection of 1 mg BrdU, followed by continuous administration of BrdU (0.8 mg/ml) in the drinking water. On the same day as the i.p. injection, mice were immunized s.c. with CFA. The water containing BrdU was changed on a daily basis. At various times after immunization, the draining lymph nodes were analyzed by cell count and by FACS. Unimmunized mice were used as controls. Single-cell suspensions from the draining lymph nodes were subject to cell surface staining for CD4, CD8, CD62L, and CD44 followed by intranuclear staining for BrdU incorporation using a BrdU Flow Kit (BD Pharmingen) following the manufacturer’s protocol. Analysis was based on sequential gating using live cells followed by a CD4+ gate.

**Conversion of polyclonal and monoclonal T cells**

A total of 5 × 106 CD4+ EGFP− T cells were adoptively transferred into BALB/c Thy1.1 mice. Transfer recipients were immunized s.c. the following day with CFA. Ten days after immunization, the draining lymph nodes were analyzed by cell count and flow cytometry. In similar experiments, 5 × 105 CD4+ DO11.10 T cells from DO11.10 RAG−/− mice were adoptively transferred into BALB/c recipients. In some experiments, cells were labeled with CFSE before adoptive transfer as described previously (31).

**Results**

**Derivation and characterization of Foxp3EGFP mice**

A cassette encoding an IRES followed the EGFP, and the polyadenylation signal from SV40 (SV40 poly-A) was inserted into the 3′ untranslated region of Foxp3 to generate a bicistronic locus encoding both Foxp3 and EGFP under the control of the Foxp3 promoter (Foxp3EGFP) (Fig. 1). Both male and female mice harboring the Foxp3EGFP allele were phenotypically indistinguishable from...
Foxp3 expression in CD4 T cells in the peripheral blood of hemizygous males and heterozygous females. Results are means ± SEM of percentage of EGFP+ CD4+ T cells in circulation (males, 5.30 ± 0.50; females, 2.82 ± 0.46; n = 5 mice each; p = 0.0064). B, Foxp3 mRNA expression was found restricted to the T cell population in the thymus, inguinal lymph nodes, and spleen is shown in Table I. Finally, CD4+EGFP+ TR cells in the immune responses to foreign Ags, Foxp3EGFP+/− female mice were immunized with CFA. The draining lymph nodes were examined at the times indicated by cell count and analytical flow cytometry. The results were compared with those of unimmunized control mice.

Expansion of the total lymphocyte compartment as well as individual CD4+ and CD8+ conventional T cell populations peaked at day 10, as expected, before contracting again back to baseline levels by day 20 (Fig. 3, A–C). Individual CD4+ EGFP+ and CD8+ EGFP+ TR cell populations followed a similar time course of expansion and contraction. In the early stages of the primary response, the EGFP+ and EGFP− T cell populations accumulated in the draining lymph nodes at a similar rate. For example, on day 5, a 1.2-fold expansion is seen in both the CD4+ EGFP+ (conventional) and CD4+ EGFP− (regulatory) T cell populations. However, by day 10, the conventional CD4+ T cell population expanded by an average of 2.3-fold, whereas the CD4+ EGFP+ TR cells reached an average of 3.3-fold (Fig. 3D). Thus, the ratio of CD4+ EGFP+ T cells:CD4+ EGFP− T cells increased by 43%, demonstrating a significant and preferential accumulation of TR cells at the peak of the primary immune response (p = 0.005, two-tailed paired t test) (Fig. 3D). Similarly, by day 10, the comparatively much smaller CD8+ EGFP+ TR cell population had expanded ~3.5-fold compared with a 2-fold increase in the CD8+ EGFP− T cell population (p = 0.006, two-tailed paired t test) (Fig. 3D). The differences in fold expansion between the CD4+ EGFP+ and CD8+ EGFP+ TR cell populations and their respective conventional counterparts narrowed by day 15 postimmunization, and both populations approached their baseline status by day 20.

Table I. The percentage and total number of EGFP+ CD4+ and CD8+ T cells*

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Total Lymphocyte Count (×10^6)</th>
<th>%CD4+EGFP+</th>
<th>No. of CD4+EGFP+ (×10^6)</th>
<th>%CD8+EGFP+</th>
<th>No. of CD8+EGFP+ (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus (SP)</td>
<td>9</td>
<td>114 ± 15</td>
<td>3.4 ± 0.35</td>
<td>3.0 ± 0.25</td>
<td>0.7 ± 0.11</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Spleen</td>
<td>16</td>
<td>63 ± 3.3</td>
<td>8.3 ± 0.38</td>
<td>12 ± 0.9</td>
<td>0.1 ± 0.01</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td>Lymph node</td>
<td>39</td>
<td>10 ± 0.7</td>
<td>7.0 ± 0.40</td>
<td>0.8 ± 0.1</td>
<td>0.1 ± 0.01</td>
<td>0.4 ± 0.05</td>
</tr>
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</table>

*Enumeration of the percentages and cell counts of CD4+ EGFP+ and CD8+ EGFP+ cell population in the thymus, spleen, and lymph nodes of naive (unimmunized) Foxp3EGFP+/− hemizygote males, consistent with random X-inactivation in females (Fig. 2A). Real-time PCR analysis of Foxp3 mRNA expression from EGFP+ and EGFP− CD4+ T cells that were purified by cell sorting revealed that Foxp3 mRNA localized (>70-fold enrichment) to EGFP+ CD4+ T cells (Fig. 2B). Analysis of Foxp3 expression by intracellular staining with anti-murine Foxp3 revealed that ≥97.5% of Foxp3+ T cells detected were EGFP+. The percentage and total number of EGFP+ CD4+ and CD8+ T cells in the thymus, inguinal lymph nodes, and spleen is shown in Table I. Finally, CD4+EGFP+ T cells was also detected (Fig. 2C). The percentage and total number of EGFP+ CD4+ and CD8+ T cells in the thymus, inguinal lymph nodes, and spleen is shown in Table I. Finally, CD4+EGFP+ T cells suppressed the proliferation CD4+ EGFP+ effector T cells in response to stimulation with anti-CD3 and anti-CD28 mAb (Fig. 2D). These results confirmed that EGFP accurately and specifically identifies Foxp3+ TR cells, consistent with other mice expressing fluorescent proteins under the control of the Foxp3 promoter (32, 33).

Foxp3+ T cells accumulate in the draining lymph nodes during a primary immune response

To study the role of Foxp3+ TR cells in the immune responses to foreign Ags, Foxp3EGFP+/− female mice were immunized with CFA. The draining lymph nodes were examined at the times indicated by cell count and analytical flow cytometry. The results were compared with those of unimmunized control mice.
postimmunization. These data suggested that the ratio of T<sub>R</sub>conventional T cells in the course of a primary immune response is tightly controlled, implicating T<sub>R</sub> cells in the control of these responses.

**T and B cell zone-specific changes in the density of CD<sup>4</sup><sup>+</sup> EGFP<sup>+</sup> T cells in draining lymph nodes**

T<sub>R</sub> cells are hypothesized to regulate both T and B cell responses, implying that they will be found within both T and B cell zones. Therefore, we determined the location and density of EGFP<sup>+</sup> cells within the draining lymph nodes during the primary response by immunocytochemistry. Analysis using hemizygous male mice revealed large clusters of EGFP<sup>+</sup> cells in their draining lymph nodes that made it difficult to accurately count the number of cells in a given area. Heterozygous female mice were therefore selected for this analysis, because only half of their T<sub>R</sub> cell population expresses EGFP due to random X chromosome inactivation (Fig. 2 and data not shown). Restricting analysis to heterozygous females minimized the T<sub>R</sub> cell clustering effect.

One draining lymph node from each heterozygous female mouse used in Fig. 3 was sectioned and stained with conjugated Abs specific for CD4, CD8, B220, CD11c, and EGFP. Lymphocytes were visualized by scanning confocal laser microscopy, and the images were aligned to create a composite of each lymph node. Anti-EGFP Ab was used to enhance image quality and to accurately detect these cells. Representative data from each time point at ×20 and ×40 magnification are shown in Fig. 4A. At early time points (day 2 and day 5), most EGFP<sup>+</sup> cells are localized in T cell areas. By day 10, EGFP<sup>+</sup> cells are found within B cell zones, but relatively few cells are seen within the germinal centers from day 15 and day 20 lymph nodes.

Visual inspection of the images also suggested that the relative density of EGFP<sup>+</sup> cells in the T cell zones increased by day 10. To further examine this possibility, the density of EGFP<sup>+</sup> cells was determined by counting these cells in multiple areas from multiple lymph nodes at each time point. The T and B cell zones and the B/T borders were delineated as described in Materials and Methods. These quantitative measurements demonstrated that, in female Foxp3<sup>EGFP</sup> mice, the density of EGFP<sup>+</sup> cells within the B cell zones increased by day 10 (Fig. 4B, top left panel), from a mean density of 46 × 10<sup>3</sup> cells/mm<sup>3</sup> in immunized controls to 59 × 10<sup>3</sup> cells/mm<sup>3</sup> in immunized mice (p = 0.046). This result was followed by a decrease into the normal range for EGFP<sup>+</sup> cells found in the B cell zones on day 15, and a secondary increase in the density of these cells on day 20 (66 × 10<sup>3</sup> cells/mm<sup>3</sup>; p = 0.034). A similar biphasic response was also observed for T<sub>R</sub> cells at the B/T borders. The decrease in the T<sub>R</sub> cell density on day 15 likely reflects a relative expansion of the B cell zones. The density of conventional CD<sup>4</sup><sup>+</sup> T cells found in the B cell zones is increased on day 10 and day 20, but plateaus on day 15, consistent with the T<sub>R</sub> cell data (Fig. 4B).

At baseline, the density of EGFP<sup>+</sup> cells in the T cell zones was 254 × 10<sup>3</sup> cells/mm<sup>3</sup>. The T<sub>R</sub> cell density increased on the 10th day after immunization to 354 × 10<sup>3</sup> cells/mm<sup>3</sup> (p = 0.038), and remained near this level at day 15 and day 20 (Fig. 4B). Examination of EGFP<sup>+</sup> cells found at the B/T borders under high-power magnification revealed dire contact between EGFP<sup>+</sup> cells and CD<sup>4</sup><sup>+</sup> T cells, CD<sup>8</sup><sup>+</sup> T cells, B cells, and CD11c<sup>+</sup> APCs (Fig. 4C). Furthermore, clusters of EGFP<sup>+</sup> cells are seen, suggesting the possibility of T<sub>R</sub> cell-cell interaction. Together, the data in Figs. 3 and 4 demonstrate that both the number and relative concentration of T<sub>R</sub> cells increase in the draining lymph nodes following immunization, consistent with a role in regulating both B and T cell primary responses.

**T<sub>R</sub> cells control the magnitude of the primary response**

To investigate the influence of T<sub>R</sub> cells on primary responses, we antagonized T<sub>R</sub> cell function by pretreating Foxp3<sup>EGFP</sup><sup>+</sup> female mice with an anti-CD25 mAb. This approach, which takes advantage of the high level of expression of CD25 on T<sub>R</sub> cells and the critical function of IL-2 in T<sub>R</sub> cell function, blocks T<sub>R</sub> cell activity while sparing the expansion of conventional T and B cells (34). Accordingly, mice were treated with anti-CD25 mAb (clone PC61) on days −4 and −1 before their immunization with CFA. Ten days after immunization, draining lymph nodes were harvested and analyzed for lymphocyte cell numbers and phenotype (Fig. 5A). The total number of lymphocytes per draining lymph node was increased after treatment with anti-CD25 mAb (13.54 vs 8.45 × 10<sup>6</sup>; n = 14 and 15 respectively; p = 0.001), primarily due to the expansion of CD<sup>4</sup><sup>+</sup> T cell and CD19<sup>+</sup> B cell populations (Fig. 5B). Analysis of EGFP expression in the CD<sup>4</sup><sup>+</sup> T cell...
compartment showed a decrease in the percentage of CD4+ EGFP+ T cells in anti-CD25 mAb-treated mice as compared with sham-treated mice (4.4 vs 6.8%; \( p < 0.007 \)). Simultaneous staining for CD25 also showed a similar decrease in the CD25+ EGFP+ population (Fig. 5C). This reflected the expansion of CD4+ EGFP+ T cells in the anti-CD25 mAb treated mice, because the total number of CD4+ EGFP+ cells was increased after treatment with anti-CD25 mAb as compared with the sham treatment (3.77 vs 2.72 \( \times \) 10^6; \( p = 0.02 \)). The total number of CD4+ EGFP+ T cells was not significantly changed due to the 55% increase in lymph node size after PC61 treatment (Fig. 5D).

Next, we determined whether tipping the balance in favor of T_R cells would suppress the magnitude of the T and B cell expansion following immunization with CFA. CD4+ T cells were isolated from Foxp3^EGFP^ mice by negative selection, the EGFP+ and EGFP^- populations were purified by cell sorting, and 2 \( \times \) 10^5 EGFP+ T_R cells or 5 \( \times \) 10^6 EGFP^- (control) T cells were adoptively transferred into BALB/c recipients. The following day, recipient mice and untransferred mice were immunized with CFA. The draining lymph nodes were analyzed for cell number and phenotype 10 days after immunization. The adoptive transfer of T_R cells resulted in a 22% decrease in lymph node cellularity, and a 39% decrease in the number of CD4+ T cells relative to the controls (Fig. 6A; \( p = 0.047 \) and \( p = 0.002 \), respectively). B cell numbers were unchanged. The adoptive transfer of EGFP+ T_R cells increased the proportion of T_R cells in the draining lymph nodes from 11% to 14% of the total CD4+ population (\( p = 0.008 \); data not shown). This change in the proportion of T_R to conventional CD4+ T cells is due to the decreased numbers of the latter population, because only a small number of transferred T_R cells (~500 cells/lymph node) were identified in the lymph nodes of transfer recipients at the peak of the response.

The effects of adoptively transferred T_R cells on lymphocyte expansion are likely to reflect inhibition of conventional T cell Ag-specific responses. However, the precursor frequency of the responding cells and the magnitude of their expansion could not be directly determined from the previous experiments. In addition, the Ag specificity of the T_R cell response is an important and unresolved question. We addressed these issues by adoptively transferring...
2.5 × 10⁵ TCR transgenic DO11.10⁺, Rag⁻⁻⁻⁻Thy1.2⁺ conventional T cells, and 5.0 × 10⁵ polyclonal EGFP⁺Thy1.2⁺ T<sub>R</sub> cells into Thy1.1⁺ BALB/c recipients. Mice were immunized with OVA (323–339) peptide in CFA, and the number of D011.10 T cells in the draining lymph nodes was quantified by FACS at the peak of the immune response. Control mice received only the DO11.10 T cells and were immunized following the same protocol. Results showed that increasing the number of T<sub>R</sub> cells with a normally distributed polyclonal TCR repertoire before immunization resulted in 46% reduction in the frequency and a 41% reduction in the total number of the OVA-specific DO11.10 T cells (Fig. 6B; p < 0.008 and p < 0.005, respectively). As in the prior experiments, a small number of transferred T<sub>R</sub> cells were recovered from the draining lymph nodes at the peak of the response (1,900 T<sub>R</sub> cells/lymph node). These data demonstrated that increasing the number of T<sub>R</sub> cells before immunization reduces the magnitude of the primary response. The frequency of OVA-specific T<sub>R</sub> cells in the adoptively transferred polyclonal T<sub>R</sub> cell population is likely to be immeasurably low compared with the transgenic DO11.10 T cells. Therefore, these results indicated that T<sub>R</sub> cell TCR specificity need not be directed against the foreign Ags that mediate the conventional T cell responses.

The increase in T<sub>R</sub> cells after immunization is associated with heightened cell division

Several mechanisms were possible for the relative expansion of EGFP⁺ T<sub>R</sub> cells in the draining lymph nodes following immunization. First, the increase in T<sub>R</sub> cells might be attributed to a non-dividing T<sub>R</sub> cell population that accumulated due to increased trafficking and/or preferential retention. Second, the observed increase might largely involve the local expansion of T<sub>R</sub> cells by means of cell division. Finally, T<sub>R</sub> cells could be generated by in situ conversion of conventional T cells.

To determine the proliferative activity of T<sub>R</sub> cells in the draining lymph nodes, the DNA precursor BrdU was administered to immunized mice and unimmunized controls. BrdU incorporation into DNA and the cell surface phenotype were determined by FACS after 5 and 10 days of continuous BrdU labeling. In these experiments, CD4⁺ T cells were divided into four main groups (Fig. 7A): T<sub>R</sub> cells that have divided (EGFP⁺ BrdU⁺); T<sub>R</sub> cells without
cells were CD44highCD62Lhigh/low, consistent with the homeostatic time frame of the experiment (Fig. 7). Dot plot analysis showing BrdU incorporation in CD4+ T cells with and without immunization (day 5, n = 5; day 10, n = 10). B, The percentage of ThR cells and conventional T cells that incorporate BrdU with and without immunization. C, The total number of dividing cells in the EGFP+ and EGFP− populations. D, Phenotypic analysis of dividing and nondividing ThR and conventional T cells based on CD44 and CD62L expression. *, p < 0.05 and **, p < 0.005.

Recent cell division (EGFP+BrdU−); conventional T cells that have divided (EGFP−BrdU+); and conventional T cells without recent cell division (EGFP−BrdU−). As expected, a small percentage (1.9%) of the resident lymph node conventional T cells in unimmunized mice have undergone cell division within the time frame of the experiment (Fig. 7B). A majority of these cells were CD44highCD62Lhigh/low, consistent with the homeostatic proliferation of memory T cells (35) (Fig. 7D). In contrast, 6.6% of the ThR cell population divided, representing a 3-fold increase in the baseline cell division (n = 10; p = 0.0054, two-tailed paired t test). Nearly all of the dividing ThR cells also had an activated phenotype (CD44highCD62Lhigh/low), in agreement with previous reports (Fig. 7D) (36). After immunization, the percentage of ThR cells that divided was increased 3-fold compared with ThR cells in unimmunized mice, suggesting extensive cell division (20.1 vs 6.5%; n = 10 per group; p = 0.007) (Fig. 7B, left panel). These values were used to calculate the total number of ThR cells per lymph node that had divided, and the results were compared with immunized controls (0.46 × 105 vs 0.068 × 105 BrdU+ cells per lymph node on day 10; p = 0.0001) (Fig. 7C, left panel), confirming heightened cell division within the ThR cell pool. After immunization, virtually all ThR cells that had divided exhibited an activated phenotype (CD44highCD62Lhigh/low) (Fig. 7D).

The percentage of conventional T cells that had divided also increased ~3-fold 5 and 10 days after immunization, compared with immunized controls (day 5, p = 0.013 and day 10, p = 0.012) (Fig. 7B, right panel). Consistent with this observation, the number of dividing conventional T cells was also significantly increased at these time points (day 5, p = 0.022 and day 10, p < 0.0001) (Fig. 7C, right panel). Similar to their ThR cell counterparts, dividing conventional T cells had an activated phenotype (CD44highCD62Lhigh/low) (Fig. 7D). Importantly, whereas the number of dividing conventional T cells was greater than the number of dividing ThR cells, the percentage of dividing ThR cells was nearly 3-fold greater than that seen within the conventional CD4+ EGFP+ population (20.1 vs 7.3%; p = 0.0006). These data demonstrated that at the peak of the primary immune response, ~20% of the ThR cell pool in the draining lymph nodes is dividing, a much higher frequency of cell division than in the conventional T cell population. However, they also show the same 3-fold expansion in both the conventional and ThR cell populations, implying that the rates of cell division in the two types of cells are equivalent. Thus, the preferential accumulation of ThR cells described in the preceding section involves both cell division and other mechanisms. Possibilities include selective retention/survival of ThR cells, efflux of conventional CD4+ effector T cells, or both.

In situ ThR cell conversion contributes marginally to ThR cell expansion

Conventional CD4+Foxp3− T cells can be converted to express Foxp3 after activation in the presence of TGF-β (37). The extent of conversion in vivo and the role of converted cells in regulating immune responses is controversial. Repertoire studies comparing TCR CDR3 regions have identified limited overlap between the conventional and ThR cell pools, suggesting that in vivo conversion has a minimal role in generating long-lived ThR cells. To determine the contribution of conventional T cell conversion to the observed expansion of ThR cells after immunization, a polyclonal population of Thy1.2+CD4+EGFP− T cells was adoptively transferred into Thy1.1+BALB/c recipients. The Thy1.2+CD4+EGFP− population was isolated by cell sorting to >99% purity, hence EGFP expression marked only those transferred cells that up-regulated Foxp3. Recipient mice were immunized the following day with CFA. The composition of the draining lymph nodes was analyzed at the peak of the T cell response on day 10. Live cells were sequentially gated on CD4 and Thy1.2 to identify the transferred
population, and CD4⁺Thy1.2⁺ cells were analyzed for EGFP fluorescence (Fig. 8A). Thy1.2⁺ cells represented 0.9% of the CD4⁺ compartment found in the draining lymph nodes. Within this transferred population, 1.5% of the CD4⁺ T cells were also EGFP⁺. When corrected for lymph node size, these percentages result in ~270 CD4⁺Thy1.2⁺EGFP⁺ and 20,270 CD4⁺Thy1.2⁺EGFP⁻ cells per draining lymph node. These data suggest that in vivo conversion is a very limited process that cannot explain the large number of proliferating Tr cells shown in Fig. 7.

In theory, only those T cells that are activated through their TCR are available for conversion. Thus, the maximum rate of conversion might be underestimated using the polyclonal population of T cells described above, where the frequency of cells specific for the Ags in CFA is unknown. To maximize the potential conversion process and directly examine Foxp3 expression, we transferred Ag-specific T cells isolated from DO11.10 × Rag⁻/⁻ mice into BALB/c recipients. DO11.10 × Rag⁻/⁻ mice do not normally develop Foxp3⁺Tr cells. Foxp3⁺Tr cells can be generated from DO11.10 × Rag⁻/⁻ T cells by ex vivo conversion with TCR cross-linking in the presence of TGF-β (data not shown). Recipient mice received 0.5 × 10⁶ CD4⁺DO11.10 T cells and were immunized with OVA (323–339) peptide emulsified in CFA. Control mice received the adoptively transferred T cells but were not immunized. After immunization, 5% of the total CD4⁺DO11.10 T cells express Foxp3 (Fig. 8B). DO11.10 Foxp3⁺ T cells were not seen in unimmunized mice.

Although all adoptively transferred DO11.10 T cells were potentially available to participate in the immune response, it was possible that a large number of these cells were not activated. This situation would result in underestimating the contribution of conversion to Tr cell expansion. To determine the extent and the rate of cell division for both conventional and converted DO11.10 T cells, the cells were labeled with CFSE before adoptive transfer. At the peak of DO11.10 expansion, lymphocytes from the draining lymph nodes were stained for CD4, the DO11.10 TCR, and Foxp3. Results confirm that 99% of DO11.10 T cells have undergone cell division, and that the number of converted Tr cells remains at ~2% (Fig. 8C). A small but measurable population of DO11.10 T cells expresses Foxp3 before cell division. These results demonstrated that only a small percentage of conventional T cells undergoing Ag-driven proliferation converted into Tr cells and, consequently, that conversion did not contribute substantially to the expanding Tr cell pool.

**Discussion**

By engineering mice with a bicistronic Foxp3 allele that links the expression of Foxp3 with that of a fluorescent reporter protein (EGFP), the expansion and function of Foxp3⁺Tr cells during the course of a primary immune response could be accurately analyzed in vivo. Previous studies have described an increase in Tr cells in vivo in response to antigenic stimulation (26, 27). In this study, we establish that the kinetics of Tr cell expansion parallels that of conventional T cells, and that the vast majority of Tr cells are found within T cell zones. More notably, our data demonstrate a relative accumulation of Tr cells within the draining lymph nodes in excess of that seen for conventional T cells. Consistent with this observation, we also find a significant increase in the density of Tr cells within T and B cell areas as the primary immune response progresses. Continuous BrdU labeling identified extensive Tr cell proliferation as one mechanism driving the buildup of Tr cells. This result suggests that Tr cell activation and expansion is integral to the regulation of primary immunity. The biological relevance of this finding was confirmed in adoptive transfer experiments that demonstrated a modest increase in the ratio of Tr: conventional T cells before immunization diminished the primary immune response. These results indicate that there is an inverse quantitative relationship between the number of responding Tr and conventional cells that regulates the magnitude of the primary immune response to foreign Ags.

Adoptive transfer experiments may not faithfully reproduce the normal kinetics of Ag-specific responses (38, 39). Nevertheless, they illustrate that the dynamic relationship between Tr cells and conventional CD4⁺ T is particularly sensitive to perturbations early in the response. Experiments using intravital microscopy to
track transferred T<sub>R</sub> cells have demonstrated reduced CD4-dendritic cell interaction times and a reduction in the half-life of affected dendritic cells (40). Thus, one way that T<sub>R</sub> cells may work is by decreasing dendritic cell maturation and function. This mechanism certainly does not preclude T<sub>R</sub> cell regulation at later time points in the course of the immune response. For example, stimulated T<sub>R</sub> cells can kill activated targets, including T cells, B cells, and dendritic cells (41–43). Importantly, the adoptive transfer experiments also show that matched Ag specificity between conventional T cells and T<sub>R</sub> cells is not a prerequisite for effective regulation of the primary response.

Previous studies tracking T<sub>R</sub> cells in vivo have frequently identified T<sub>R</sub> cells using CD25, a strategy that fails to recognize up to 30% of T<sub>R</sub> cells and does not distinguish between T<sub>R</sub> cells and activated conventional T cells (33). In contrast, our data demonstrate that >20% of the T<sub>R</sub> cell population is dividing in the draining lymph nodes at the peak of the primary response, clearly indicating activation of T<sub>R</sub> cells after immunization. Activation of T<sub>R</sub> cells requires TCR engagement by Ag/MHC complexes and is essential for T<sub>R</sub> cell function (5, 6). Once activated, T<sub>R</sub> suppression of conventional T and B cell responses is thought to be independent of T<sub>R</sub> cell Ag specificity (41–44). Our data is consistent with the interpretation that T<sub>R</sub> cell regulation of primary immunity depends upon activation of a sizable portion of the T<sub>R</sub> cell pool. Several mechanisms could contribute to this extensive activation, including the propensity of T<sub>R</sub> cells for self-reactivity, increased T<sub>R</sub> cell repertoire diversity, and reduced T<sub>R</sub> cell TCR specificity.

The BrdU data also provides comparative information on T<sub>R</sub> cells and conventional T cells at steady state in the absence of stimulation by foreign Ag. We found that ~7% of T<sub>R</sub> cells had divided during the 10 days of continuous BrdU labeling, while only 2% of conventional T cells had incorporated BrdU during the same time period. Cell division of conventional CD4<sup>+</sup> T cells at steady state depends upon lower affinity interactions between the TCR and self-peptide MHC complexes than those required for T cell activation (45, 46). In the absence of foreign Ag, broadened TCR specificity and a T<sub>R</sub> cell bias toward self-recognition may also account for the relatively higher rates of T<sub>R</sub> cell division. Blocking steady-state T<sub>R</sub> cell proliferation with an anti-IL-2 Ab results in accelerated and multimer autoimmune disease in NOD mice, consistent with the activation of T<sub>R</sub> cells on self-peptide/MHC complexes (47). Previous BrdU studies show that most conventional CD4<sup>+</sup> T cells that divide at steady state are CD4<sup>4hi</sup>, and homeostasis in lymphocyte-replete animals has therefore been postulated to largely involve the memory pool (35). A rapidly dividing CD4<sup>4hi</sup> T<sub>R</sub> cell pool was observed in another study, and our data demonstrated a similar population (36). Importantly, we find that ~25% of the cells that divided in the draining lymph nodes were T<sub>R</sub> cells. These cells were exclusively CD4<sup>4hi</sup>, suggesting an activated/memory T<sub>R</sub> cell phenotype. No cells in the dividing T<sub>R</sub> cell pool have a naive phenotype (CD62L<sup>hi</sup>/CD4<sup>4lo</sup>), although such cells can be seen in the conventional EGFP<sup>+</sup> BrdU<sup>+</sup> population. The extensive contribution of T<sub>R</sub> cells to steady-state cell division within the CD<sup>+</sup> T cell compartment implicates continual T<sub>R</sub> cell activation as an important mechanism in controlling spontaneous autoimmune responses generated by conventional T cells (47).

In the thymus, agonist-mediated selection of T<sub>R</sub> cells has been observed in a TCR transgenic model (48). Although this point has been debated, most experiments examining T<sub>R</sub> cell development demonstrate that T<sub>R</sub> cells are relatively resistant to negative selection (48–50). In the absence of efficient negative selection, positive selection of T<sub>R</sub> cells might result in a TCR repertoire that is highly peptide degenerate and biased toward MHC recognition, analogous to the model that has been developed for the selection of conventional T cells (51–53). Our data demonstrating a significant increase in T<sub>R</sub> cell division at steady state and after immunization are arguably most consistent with a T<sub>R</sub> cell pool characterized by degenerate TCR recognition and activation largely on self-Ags. We propose a model whereby T<sub>R</sub> cells are progressively enlisted to regulate immune responses based on the level of conventional T cell activation and the local amount of IL-2 produced. Constitutive expression of the high-affinity IL-2R (CD25) and the dependence on exogenous IL-2 enforces T<sub>R</sub> cell participation commensurate with the conventional T cell response. The postulated degenerative nature of T<sub>R</sub> cell TCR recognition then allows for recruitment of a sufficient number of T<sub>R</sub> cells to control the magnitude of the response.

In contrast, in situ generation of T<sub>R</sub> cells did not appear to play a role in the accumulation of T<sub>R</sub> cells during the course of the primary immune response to CFA immunization. Previous studies have demonstrated that T<sub>R</sub> cells may arise from activation of conventional T cells, either by stimulation with low doses of Ag in the absence of professional APC stimulation (54), or by TCR cross-linking in the presence of TGFB (37). This conversion results in the expression of Foxp3 and the acquisition of a regulatory phenotype (32, 37, 55, 56). Converted T<sub>R</sub> cells exhibit suppressor function and are capable of down-regulating Ag-specific immune responses in a manner similar to that of thymus-derived or “natural” T<sub>R</sub> cells. Recent studies have documented the production of converted T<sub>R</sub> cells in the context of an adjuvant (aluminum hydroxide)-driven immune response (57). We also find that conversion can occur in vivo. However, our adoptive transfer experiments revealed that in situ T<sub>R</sub> conversion contributed little to the increase in T<sub>R</sub> cells in draining lymph nodes of CFA-immunized mice, making a measurable regulatory role for these cells less likely. These data do not rule out more extensive conversion at later time points, as has been seen in other systems (58).

Whereas the T<sub>R</sub> cell expansion in the course of a primary immune response may take advantage of degenerate TCR recognition and a more diverse TCR repertoire, chronic antigenic stimulation, such as that seen with parasitic infections, can be associated with a more focused, Ag-specific T<sub>R</sub> cell response. This is demonstrated in the recent reports examining mouse models of Leishmania infection where Leishmania Ag-responsive T<sub>R</sub> cells were prominently represented at the infection site and suppressed the in situ action of effector T cells, allowing for parasite persistence (59). In both acute and chronic responses to foreign Ags, T<sub>R</sub> cells regulate the development of conventional T cell effector function. However, the differential dependence on foreign Ag specificity displayed by T<sub>R</sub> cells in the course of chronic vs acute responses may be the consequence of the manipulation of the local environment by pathogens leading to Ag persistence. Such divergence in T<sub>R</sub> cell response may explain some of the detrimental effects associated with fixing T<sub>R</sub> cell Ag specificity during chronic antigenic stimulation, which may allow the persistence of pathogens such as parasites in the face of an ongoing immune response (25).

In addition to CD4<sup>+</sup> EGFP<sup>+</sup> T<sub>R</sub> cells, we also observed a small population of CD8<sup>+</sup> EGFP<sup>+</sup> cells that follow the same kinetics as the CD4<sup>+</sup> EGFP<sup>+</sup> population. Expansion and contraction of this Foxp3<sup>+</sup> population during the primary response suggests a regulatory role, although this is not confirmed by our studies. The small number of these cells makes their potential effects more difficult to dissect. We have used the neonatal transfer of purified CD4<sup>+</sup> T cells to rescue Foxp3<sup>−/−</sup> mice, suggesting that CD8<sup>+</sup> Foxp3<sup>−/−</sup> cells are not essential for preventing autoimmunity (D. Haribhai, unpublished results). However, CD8<sup>+</sup>EGFP<sup>+</sup> cells arise in the thymus and form a distinct lineage, making it likely that they play...
some role in regulating certain cell populations or immune responses. Assuming that CD8+ Foxp3+ Treg cells require activation via their MHC class I-restricted TCR for their function, targets might include cells of the innate immune system, APCs, and CD4+ T effector cells.

In summary, this study provides new data about Treg cell proliferation, localization, and Ag independence during the primary immune response. This study also confirms related observations in the literature using suboptimal markers. Collectively, the data raise many questions about the specific factors that drive a large fraction of the Treg cell pool to divide in naive and immunized animals. Why is the baseline frequency of Treg cell division so high? Are there specific APC populations or cytokines that modulate Treg cell division? What is the role of TCR signal strength and of TLR signaling? How degenerate is Treg cell TCR specificity, and might this impact Treg cell function? The Foxp3+ Treg mice provide a useful tool designed to answer these and other aspects of the biology of Treg cells.

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Disclosures

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