Deficiency of the Src Homology Region 2 Domain-Containing Phosphatase 1 (SHP-1) Causes Enrichment of CD4⁺CD25⁺ Regulatory T Cells

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Immune tolerance and the prevention of autoimmune diseases are thought to occur at various levels in the immune system. During thymic T cell development, the majority of thymocytes expressing TCRs with high affinity for self-peptides undergo negative selection, resulting in their apoptosis. This process is referred to as central tolerance (reviewed in Ref. 1). Despite this mode of eliminating autoreactive T cells, every healthy adult still carries potentially harmful self-reactive T cells (2). In the peripheral mode of eliminating autoreactive T cells, such autoreactive T cells are rendered inert by mechanisms termed peripheral tolerance (3). In addition, a subpopulation of T cells, named regulatory T cells (Treg cells), has been shown to play a key role in tolerance and the prevention of autoimmunity. It is not known how changes in TCR signal strength during thymic T cell development affect the generation of a Treg population. In this study, we took two different strategies to modulate the TCR signal strength: an intrinsic approach, where signaling was enhanced by the loss of a negative regulator, and an extrinsic approach, where signaling strength was altered through variations in the concentrations of the selecting peptide. The tyrosine phosphatase Src homology region 2 domain-containing phosphatase 1 (SHP-1) is a known negative regulator of TCR-mediated signaling. motheaten mice, lacking expression of SHP-1, showed a 2- to 3-fold increase in the percentage of CD4^{+}CD25^{+} T_{reg} cells within the CD4^{+} T cells. Similarly, the percentage of T_{reg} cells was heightened in fetal thymic organ cultures (FTOCs) derived from DO11.10 TCR transgenic mice, we demonstrated that exposure to increasing concentrations of the cognate OVA peptide favored the appearance of T_{reg} cells. Our data suggest that the development of CD4^{+}CD25^{+} T_{reg} cells is intrinsically different from non-T_{reg} cells and that T_{reg} cells are selectively enriched under conditions of enhanced negative selection. We also revealed a key role for the SHP-1-mediated regulation of TCR signal strength in influencing the ratio of T_{reg} vs non-T_{reg} cells. The Journal of Immunology, 2005, 174: 6627–6638.

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A subpopulation of T cells, named regulatory T cells (T_{reg} cells), has been shown to play a key role in tolerance and the prevention of autoimmunity. It is not known how changes in TCR signal strength during thymic T cell development affect the generation of a T_{reg} population. In this study, we took two different strategies to modulate the TCR signal strength: an intrinsic approach, where signaling was enhanced by the loss of a negative regulator, and an extrinsic approach, where signaling strength was altered through variations in the concentrations of the selecting peptide. The tyrosine phosphatase Src homology region 2 domain-containing phosphatase 1 (SHP-1) is a known negative regulator of TCR-mediated signaling. motheaten mice, lacking expression of SHP-1, showed a 2- to 3-fold increase in the percentage of CD4^{+}CD25^{+} T_{reg} cells within the CD4^{+} T cells. Similarly, the percentage of T_{reg} cells was heightened in fetal thymic organ cultures (FTOCs) derived from DO11.10 TCR transgenic mice, we demonstrated that exposure to increasing concentrations of the cognate OVA peptide favored the appearance of T_{reg} cells. Our data suggest that the development of CD4^{+}CD25^{+} T_{reg} cells is intrinsically different from non-T_{reg} cells and that T_{reg} cells are selectively enriched under conditions of enhanced negative selection. We also revealed a key role for the SHP-1-mediated regulation of TCR signal strength in influencing the ratio of T_{reg} vs non-T_{reg} cells. The Journal of Immunology, 2005, 174: 6627–6638.
via cross-linking of the TCR/CD3 complex or costimulation of CD28, renders them refractory to suppression (16). Although energy is a hallmark of Treg cells in vitro, they are capable of considerable expansion in vivo without losing their suppressive ability (12, 17). Treg cells must be stimulated to exert their suppressive effects; however, once stimulated, Treg cells act in an Ag-nonspecific manner (8, 9, 18, 19). In vitro, the suppressive abilities of the Treg cells are connected to the anergic state, because addition of IL-2 not only breaks the anergy of the Treg cells but also abrogates their ability to suppress proliferation (20).

Src homology region 2 domain-containing phosphatase 1 (SHP-1) is a non-transmembrane protein tyrosine phosphatase that is expressed predominantly in hemopoietic cells of all lineages and all stages of maturation as well as at lower levels in epithelial cells (21–25). The existence of a murine genetic model for SHP-1 deficiency has significantly aided our understanding of the biological function of SHP-1 (26, 27). A splicing mutation in the H11032 locus causes the motheaten (me/me) phenotype. This mutation leads to a frameshift near the 5′-end of the SHP-1 coding sequence, resulting in no detectable SHP-1 protein. me/me mice are therefore effectively SHP-1 nulls. A number of studies have suggested that SHP-1 is a negative regulator of TCR-mediated signaling (28, 29). We had previously reported that SHP-1 plays a role during thymic T cell development by influencing the TCR signal strength (30). Using a transgenic TCR system, bred into the motheaten background, we observed that SHP-1 is involved in setting the thresholds for positive and negative selection (31). Other reports have also noted similar effects of SHP-1 on thymic selection (32–34). Taken together, these studies are consistent with a regulatory role for SHP-1 during T cell development.

In this study, we determined whether SHP-1 plays a role in the development of Treg cells. me/me mice show an increase in the percentage of functional CD4+CD25+ Treg cells compared with their +/+ littermates in the thymus and spleen. These SHP-1-deficient Treg cells are thymus derived and functionally indistinguishable from wild-type Treg cells based on their suppressive efficiency and expression of a number of surface markers. Moreover, in fetal thymic organ cultures (FTOCs) derived from TCR-Tg mice, addition of the cognate at concentrations that induce negative selection results in an enrichment of CD4+CD25+ T cells within the CD4+ single-positive (SP) population. These data suggest that the development of CD4+CD25+ Treg cells is intrinsically different from Treg cells. Our data also provide evidence that the tyrosine phosphatase SHP-1, through its regulation of signaling strength downstream of the TCR and its influence on negative selection, favors the development of Treg cells over Treg cells.

Materials and Methods

Mice

(BALB/c/mel+), (BALB/c:++/+; DO11.10 TCR-Tg+), and (BALB/c/mel+;DO11.10 TCR-Tg+) mice (31) were bred in our colony to generate the various genotypes. Genotyping for all mice was performed as described (31). Unless otherwise noted, 15- to 19-day-old mice were used throughout the study. All mice were bred and maintained in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC) at the University of Virginia. All experiments involving mice were conducted with the approval of IACUC.

Electronic cell purification

Splenocytes were dispersed from the spleen, and RBCs were lysed using PharM Lyse (BD Pharmingen). Cell suspensions were precleared of macrophages and B cells by adherence to tissue culture plates (Corning) at 1 x 106 cells/plate for 1 h at 37°C followed by incubation onto a nylon wool column (Polysciences) (4 x 106 cells/g wool) for 1 h at 37°C. Cells were subsequently stained with anti-CD4-PE and anti-CD25-allophycocyanin, (used at 2 μg/ml each) (BD Pharmingen). CD4+CD25+ and CD4+CD25− populations were electronically purified using a FACSVantage SE cell sorter (BD Biosciences).

Flow cytometry

Total splenocytes or thymocytes were isolated and stained with Abs to the indicated surface markers in PBS supplemented with 1% BSA and 0.1% sodium azide. CD4-PE, CD25-allophycocyanin, CD25-PE, CD25-biotin, CD44-allophycocyanin, streptavidin-PerCP, CD69-PE (used at 1 μg/ml), CD8-FITC, CD8-allophycocyanin, Vβ8-FITC, Vβ8-biotin (clone MR5-2), Vα2-FITC, CD45RB-PE, CD62L-PE, CD38-PE, TLR4-PE, CTLA-4-PE (used at 2.5 μg/ml), and streptavidin-allophycocyanin (used at 0.25 1 μg/ml) were purchased from BD Pharmingen, KJ1-26-PE and KJ1-26-FITC (used at 1 μg/ml) were purchased from Caltag, and GITR-biotin (used at 0.075 μg/ml) was purchased from R&D Systems. Stained cells were collected on a FACSCalibur instrument calibrated with CaliBRITE beads using CellQuest software for collection and subsequent analyses (BD Biosciences). Analyses were conducted on live cells (>95%) as defined by forward- and side-angle scatter. Gates were set using isotype-matched control Abs.

Proliferation and suppression assays

These assays were performed as described previously (20, 35). Briefly, to assess proliferation, 2.5 x 104 CD4+CD25+ or CD4+CD25+ T cells (electrostatically sorted to a purity of >95% as described above) were plated in triplicate in 200 μl of RPMI 1640 medium (supplemented with 10% FCS, 5 x 10−5 M 2-ME, 2 mM l-glutamine, and antibiotics) in 96-well plates. For assessment of suppressor activity, 2.5 x 104 CD4+CD25+ (effector) T cells plus 2.5 x 104 CD4+CD25+ (regulatory) T cells were plated in triplicate in 96-well plates. Irradiated (2000 rad) total splenocytes (from a DO11.10 TCR-Tg BALB/c mouse) were added at 5 x 104 cells per well along with anti-CD3 Ab (145-2C11, Southern Biotechnology) at 12 μg/ml. Cells were incubated at 37°C for 72 h before they were pulsed with 1 μCi of [³H]thymidine for 18 h. [³H]Thymidine incorporation was measured using a cell harvester and Betaplate counter (Wallac).

Expression of Foxp3

Total RNA was isolated from electronically purified CD4+CD25+ or CD4+CD25+ T cells using TRIzol reagent (Invitrogen Life Technologies). Purified RNA from 4 x 10⁶ cells/sample served as template for first-strand cDNA synthesis using the Superscript System (Invitrogen Life Technologies). Foxp3 levels were assessed using real-time PCR as described previously (36). For conventional PCR, products generated from 30-cycle reactions were analyzed. The primers for Foxp3 were the same as used for real-time PCR. The following primers were used for GAPDH assessment: 5′-GGC TTC ACC ACC ATG GAG-3′ and 5′-AAG TTG TCA TGG ATT GAC CTT GG-3′.

Immunoprecipitation and immunoblotting

These assays were performed as described previously (31). Briefly, SHP-1 was immunoprecipitated from lysates of the indicated numbers of purified CD4+CD25+ or CD4+CD25+ T cells using 2 μg of rabbit anti-SHP-1 Abs (Santa Cruz Biotechnology) followed by immunoblotting for SHP-1 with monoclonal anti-SHP-1 (clone 1SH01; NeoMarkers) at 1.35 μg/ml.

FTOCs

FTOCs were set up as previously described (37, 38). Briefly, females were checked for fertilization by the presence of a vaginal plug (day 0 gestation) and sacrificed on day 15 gestation when pups were harvested to remove fetal thymus. Thymi were cultured in six-well plates on a Transwell insert resting upon 1.5 ml of Iscove’s medium supplemented with 10% FCS (HyClone), 5 x 10−5 M 2-ME, 2 mM l-glutamine, and antibiotics. Days of subsequent culture are indicated for each experiment. Thymocytes were dispersed, counted, and stained for flow cytometric analysis as described above.

Peptide pulsing of FTOCs

FTOCs were established from day 15 gestation pregnant mice as described above. On day 2 of culture, FTOCs were incubated with OVA peptide (IQKVAHAAHEINAGR; synthesized at the Biomolecular Research Facility, University of Virginia) at concentrations indicated in the individual experiment. After 5 days of culture, organ cultures were transferred to OVA-free medium and cultured for an additional 48 h without OVA. Thymocytes were then dispersed, counted, and stained for flow cytometric analysis as described above. We observed that +/+ FTOCs derived from crosses of mel+/+;DO11.10 mice (as shown in Fig. 7) failed to show a
significant increase in the percentage of CD4+CD25+ cells until exposed to 2000 ng of OVA peptide, whereas the FTOCs derived from +/-: DO11.10 mice (as shown in Fig. 6) start to respond at ≈1000 ng of peptide.

Results
Thymi and spleens of me/me mice are enriched for CD4+ CD25+ T cells
Because high-avidity peptide/TCR interactions can lead to an increase in the number of Treg cells (11–13), it is thought that the strength of TCR signaling also regulates Treg cell development. We therefore examined whether SHP-1, through regulation of TCR signal strength, is involved in the generation of Treg cells. In the descriptions below, we refer to and discuss the absolute and relative cell numbers separately, because the motheaten mice are smaller in size, which in turn affects their thymic cellularity. Moreover, differences in relative and absolute cell numbers have also been useful in distinguishing between possible models of Treg cell generation. Thymi from SHP-1-deficient me/me mice showed consistently higher percentages of CD4+CD25+ T cells compared with their +/- littermates (4.2 vs 1.8%; p < 0.0013) (Fig. 1a and Table I). The absolute number of CD4+CD25+ T cells in the me/me mice is approximately the same as in their +/- littermates, despite the smaller size of the me/me mice and decreased total thymic cellularity. Importantly, there are only very few (<1%) CD25+ cells within the double-positive (DP) and the CD8 SP subpopulations in both me/me and +/- thymi, indicating that there is no overall increase in CD25+ surface expression in the me/me mice (Fig. 1a). Thymocytes that have undergone positive selection up-regulate the activation marker CD69 (39). In both the me/me and the +/- populations, the majority of CD4+CD25+ and CD4+CD25− are CD69high, indicating that they recently underwent positive selection (Fig. 1a), whereas the CD69low population represents the more mature CD4 SP thymocytes.

Spleens of me/me mice also have ~2-fold more CD4+CD25+ T cells than their +/- littermates, both in absolute number and as a percentage of the CD4+ cells (20 vs 10%, p < 0.001) (Fig. 1b and Table I). Because CD25 is also a marker for activated T cells, we examined whether there was a general activation of T cells in the me/me mice, but there was no overall increase in CD25+ populations in both me/me and +/- mice (Table I). The absolute number of CD4+CD25+ T cells was not found to be increased by IL-2 treatment (data not shown). The absolute number of CD4+CD25− T cells in the me/me mice is significantly decreased (2% of the splenic CD8 SP population, the majority of CD4+CD25− T cells are CD69low, indicating that they recently underwent positive selection (Fig. 1a), whereas the CD69low population represents the more mature CD4 SP thymocytes.

FIGURE 1. me/me mice have higher percentages of CD4+ CD25+ Treg cells than +/- mice. a and b, Thymocytes (a) and splenocytes (b) from +/- and me/me BALB/c mice were isolated and stained for flow cytometry with the indicated Abs. A total of 10⁶ live cells was analyzed per point. Gated subpopulations (based on CD4 and CD8 expression) were further analyzed for CD25 expression and the percentages of CD25+ cells within each subpopulation are shown. Overlay histograms of CD69 surface expression of +/- (open line) and me/me (gray shaded) on CD4+CD25+ as well as CD4+CD25− T cells are depicted below. c, CD4+CD25+ splenocytes isolated from +/- and me/me mice are anergic and suppress the proliferation of Treg cells. CD4+CD25+ (Treg) and CD4+CD25− (Teff) cells were isolated from splenocytes. A total of 10⁴ cells of each indicated cell type was assayed for proliferation/suppressor activity. Proliferation of +/- CD4+CD25− is set as 100%. Where indicated, IL-2 (30 U/ml) was added. APCs represent background proliferation from the irradiated splenocytes. Error bars denote ± SEM. Results are representative of five independent experiments.
CD4+ cells in the thymus and spleen cellularity is not due to increased deletion but rather reflects the smaller body size of the animals. The actual increase in thymic CD4+ cells isolated from both DO11:110 me/me mice expressed slightly lower CD4 levels on T cells than DO11:110 me/me mice is not due to a general increase in the percentage of CD25+ cells from either genotype, but it was also observed that the CD25+ population is enriched in the Treg me/me population, because the CD25+ populations of CD4+ T cells in the me/me and control background have a relatively low usage of the Vα2 chain (~4%) (Fig. 2c). Although there is an enrichment of Vα2 usage in the splenic CD4+ CD25+ cell population, there is no increase in Vα2 expression (up to 9%) in the CD25+ population due to a general increase in the percentage of T cells expressing an endogenous TCR instead of the transgenic αβTCR (Fig. 2d). Both thymic and splenic CD4+ CD25+ T cell populations still express the transgenic TCR on their surface, detected using the clonotypic Ab KJ1-26 (data not shown). The similarities of surface marker expression profiles between the +/- and me/me CD4+ CD25+ populations and the consistencies with previous reports for CD4+ CD25+ Treg cells further indicate that the CD4+ CD25+ T cells found in the me/me mice are Treg cells.

Although there are no unique Treg specific surface markers available at present, we analyzed the CD4+ CD25+ and the CD4+ CD25− populations from both +/- and me/me mice for the expression of a number of surface markers that have been associated with the Treg population, such as GITRpos, CD45RBlow, CTLA-4pos, CD62Lpos, CD38hi, and TLR4pos (41–45) (data not shown). The majority of cells within the CD4+ CD25+ population in me/me and control mice displayed comparable flow cytometric profiles similar to what has been reported for Treg cells. Moreover, the thymic and splenic CD4+ CD25+ populations from both +/- and me/me mice expressed slightly lower CD4 levels on their surface as it has been described for natural Treg cells (46) (Fig. 2e). These data further indicated that the me/me and the +/-

### Table I. Relative and absolute cell numbers in wild-type and SHP-1-deficient mice^a^

<table>
<thead>
<tr>
<th></th>
<th>% of CD4+ CD25+ T Cells within CD4+ Population</th>
<th>No. CD4+ CD25+ T Cells (%CP)</th>
<th>No. CD4+ CD25+ T Cells (%CP)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thymus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/-</td>
<td>1.8 ± 1.4</td>
<td>9.9 ± 3.9</td>
<td>1.8 ± 1.3</td>
</tr>
<tr>
<td>me/me</td>
<td>4.2 ± 1.6</td>
<td>4.4 ± 4.0</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>DO11: +/-</td>
<td>2.8 ± 1.0</td>
<td>11.6 ± 4.4</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>DO11:me/me</td>
<td>7.1 ± 2.3</td>
<td>8.7 ± 2.3</td>
<td>6.2 ± 1.9</td>
</tr>
</tbody>
</table>

| **Spleen** |                                              |                             |                             |
| +/-      | 10.4 ± 2.3                                    | 3.2 ± 0.8                   | 3.3 ± 0.8                  |
| me/me    | 19.9 ± 5.5                                    | 3.2 ± 1.3                   | 6.4 ± 1.7                 |
| DO11: +/- | 6.5 ± 2.7                                     | 5.1 ± 1.6                   | 3.5 ± 1.4                 |
| DO11:me/me | 20.2 ± 6.2                                    | 4.1 ± 1.3                   | 8.3 ± 2.5                 |

^a^ Thymi and spleens of mice carrying the indicated genotypes were analyzed for CD4, CD8, and CD25 surface expression. The relative (%) and absolute (no.) of cells displaying the indicated phenotypes are shown. Errors represent the SEM. n indicates the number of mice analyzed to determine percentage of CD4+ CD25+ within the CD4+ population.

^b^ p values were calculated for the changes in the absolute numbers of CD4+ CD25+ between me/me and +/- mice. ^c^ p value of 0.0013, ^d^ p value of <0.001, and ^e^ p value of <0.005 at a 95% confidence interval.

Although there is a significant increase in the relative number of CD4+ CD25+ thymocytes in the me/me mouse, the absolute numbers are comparable. However, the cell numbers are not normalized for the size difference between a me/me and a +/- thymus, although a me/me thymus is approximately one-half to two-thirds the size of a +/- thymus (as also reflected in the number of CD4 SP thymocytes). Based on thymic composition and surface marker profile of the me/me thymus (Ref. 28 and Fig. 1), the decreased thymic cellularity is not due to increased deletion but rather reflects the smaller body size of the animals. The actual increase in thymic CD4+CD25+ T cells in me/me mice is therefore likely underestimated.
CD4−CD25+ T cells are comparable, albeit selectively increased in the motheaten mouse. 

Recently, expression of Foxp3, a transcription factor, has been shown to be restricted to Treg cells. Moreover, expression of Foxp3 in T cells seems to be necessary and sufficient to induce the regulatory phenotype (47– 49), thereby making it a valuable marker in T cells. Recently, expression of Foxp3, a transcription factor, has been shown to be restricted to Treg cells. Moreover, expression of Foxp3 in T cells seems to be necessary and sufficient to induce the regulatory phenotype (47– 49), thereby making it a valuable marker for naturally occurring Treg cells. Sorted splenic CD4−CD25+ T cells from both wild-type and me/me mice showed comparable Foxp3 mRNA expression as assessed by quantitative real-time PCR (Fig. 3a) as well as conventional PCR (b). Under the same conditions, the CD4−CD25+ T cell populations contained only background or below-detection levels of Foxp3 mRNA. These data confirmed that the CD4−CD25+ T cell populations from wild-type and me/me mice are comparable and also further supported that they are Treg cells.

Functionality of sorted splenic CD4−CD25+ T cells was assessed in suppression assays. CD4−CD25+ T cells (Treg) from both DO11:+/+ and DO11:me/me mice failed to proliferate in response to stimulation with soluble anti-CD3 Ab in the presence of APCs. They also suppressed the proliferation of purified CD4+CD25− cells (Teff cells) upon coculturing, further indicating that these CD4+CD25− cells are functional Treg cells (Fig. 2f).

The data presented above suggest that the lack of SHP-1 results in an increase in the percentage of functional CD4−CD25+ Treg cells, the assays did not address whether there were any differences in the suppressive capabilities of +/+ and me/me Treg cells. Assessment of the expression level of SHP-1 in electronically sorted splenic CD4−CD25+ and CD4−CD25− cells showed that SHP-1 is expressed at comparable levels in CD4−CD25+ Treg cells and CD4−CD25− T cells (Fig. 3f). To examine the respective suppressive potentials, we used decreasing ratios of Treg cells to responder cells (Teff cells) in an in vitro suppression assay. Treg cells from both DO11:+/+ and DO11:me/me mice completely suppressed T eff cell proliferation at a ratio of 1:2, partially suppressed at a ratio of 1:4, and failed to show suppression at dilution of 1:8 (Fig. 4b). At the highest dilution of Treg cells, we noticed that the proliferation was higher than that of Treg cells alone. This may be due to IL-2 produced by the activated Teff cells, which helps to overcome the anergy of the Treg cells in coculture allowing them to also proliferate. Thus, although there are a greater number of Treg cells in the me/me background, the suppressive
The potential of individual cells appears comparable between +/+ and me/me T<sub>reg</sub> cells based on the above assays.

**CD4⁺ CD25⁺ T cells are thymus derived**

It has been reported previously that T<sub>reg</sub> cells are thymus derived and can be earliest detected in the thymus at day 3–4 postnatal, whereas they are still undetectable in the spleen at that time (11, 50). Both +/+ and me/me mice have CD4⁺ CD25⁺ T cells in their thymi by day 4, but me/me mice have a ~2-fold higher percentage of CD4⁺ CD25⁺ T cells within their CD4⁺ SP population (Fig. 5a). However, neither the +/+ nor the me/me mice have detectable CD4⁺CD25⁺ T cells in their spleens. Essentially identical results were obtained when DO11.10 TCR-Tg mice in the +/+ and me/me background were analyzed (Fig. 5b).

To further test the thymic origin of the CD4⁺ CD25⁺ T cells, we used FTOCs. CD4⁺ CD25⁺ T cells are readily detectable within the CD4⁺ SP population in day 7 FTOCs derived from +/+ and me/me DO11.10 mice (Fig. 5c). FTOCs from me/me mice contain 2- to 3-fold higher percentages of CD4⁺ CD25⁺ T cells within their CD4 T cells populations, consistent with the findings in the neonatal and 2- to 3-wk-old mice presented above. Thus, the CD4⁺ CD25⁺ T cells detected in the +/+ and me/me mice are of thymic origin, and the increased percentages detected in me/me mice are already present very early in the life of the mouse. This further supports the hypothesis that the increase in CD4⁺ CD25⁺ T cells in the me/me mice is directly due to the lack of SHP-1 during thymic development, and not a secondary effect in the context of the mouse.

**Selective enrichment of CD4⁺ CD25⁺ T cells upon exposure of cognate Ag**

The above data suggested a model that stronger TCR-mediated signaling, due to the absence of the negative regulator SHP-1, favors the generation of CD4⁺ CD25⁺ T<sub>reg</sub> cells. A logical extension of this hypothesis is that, even in wild-type mice, the percentage of T<sub>reg</sub> cells should be increased when cognate Ag is present during the selection process. Consistent with this hypothesis, transgenic expression of the cognate Ag in the thymus of TCR-Tg mice has been shown to promote the development of T<sub>reg</sub> cells (11–13); however, the design of these in vivo studies did not allow for a careful dose response of the cognate peptide. To directly determine whether a cognate peptide can enhance the appearance of CD4⁺ CD25⁺ T cells and to determine the effect of various peptide concentrations in this process, we set up FTOCs from +/+ me/me DO11.10 mice.
DO11.10 mice and cocultured them with increasing concentrations of OVA peptide (Fig. 6). We made three notable observations. First, we observed an increase in the percentage of CD4<sup>+</sup>/CD25<sup>+</sup> cells within the CD4<sup>+</sup> SP subpopulation at peptide concentrations coincident with negative selection, as manifested by a loss of the DP population (10<sup>μg/ml</sup> OVA peptide; Fig. 6). Second, the absolute cell number of CD4<sup>+</sup>/CD25<sup>+</sup> T cells remained relatively constant at all concentrations of OVA peptide, indicating that increasing the peptide concentration per se (in turn, greater strength of signal) did not change the absolute number of CD4<sup>+</sup>/CD25<sup>+</sup> T cells that emerge under these conditions. Third, even at peptide concentrations 10<sup>3</sup> times required for negative selection, no decrease in numbers of the CD4<sup>+</sup>/CD25<sup>+</sup> T cell population was detectable, suggesting a selective resistance of the Treg cell population to conditions of negative selection. Concurrent analysis of Vα2 usage in the CD4<sup>+</sup>/CD25<sup>+</sup> and CD4<sup>+</sup>/CD25<sup>-</sup> subpopulations demonstrates that, at least at the lower concentrations of peptide, the percentage of Vα2-positive cells are relatively constant (data not shown). This observation indicated that the resulting CD4<sup>+</sup>/CD25<sup>+</sup> cells are T<sub>reg</sub> cells and not activated T cells. The above data finding a constant number of CD4<sup>+</sup>/CD25<sup>+</sup> cells under different peptide concentrations suggests that a fraction of the developing T cells in the thymus are precommitted to become CD4<sup>+</sup>/CD25<sup>+</sup> cells, and that they can withstand an environment/signals strong enough to cause negative selection of non-T<sub>reg</sub> cells (see Discussion).

The relatively constant number of CD4<sup>+</sup>/CD25<sup>+</sup> cells observed in FTOCs is different from what we observed in the whole organism, where the motheaten mice, especially in the DO11.10 background, showed an increase in the absolute number of thymic and splenic CD4<sup>+</sup>/CD25<sup>+</sup> Treg cells. This can be readily reconciled by the fact that no new prothymocytes enter the FTOCs and that the thymus cannot be replenished during changes in thymocyte composition. In contrast, new progenitor cells can replenish the thymus in the mouse, resulting in a higher throughput during states of increased precursor influx, thereby leading to increased accumulation of CD4<sup>+</sup>/CD25<sup>+</sup> T<sub>reg</sub> cells.

Expanding the above study on CD4<sup>+</sup>/CD25<sup>+</sup> T cell development in +/+ :DO11.10 FTOCs, we tested the effect of combining SHP-1 deficiency and addition of cognate peptide during thymic development. We hypothesized that me/me FTOCs should be more sensitive to OVA peptide and that the amount of peptide needed to promote the generation of CD4<sup>+</sup>/CD25<sup>+</sup> T<sub>reg</sub> cells would be decreased in the absence of SHP-1. FTOCs from +/+ and me/me...
DO11.10 mice were cocultured with OVA peptide and analyzed for the presence of CD4^+CD25^+ cells. At low concentrations of OVA peptide (0.5 or 1 μg), the increase in the percentage of CD4^+CD25^+ cells within the CD4^+ SP population of +/- FTOCs was absent or minimal. However, me/me FTOCs contained a readily detectable increase in the percentage of CD4^+CD25^+ cells in response to the same OVA peptide concentration (Fig. 7). The enrichment of CD4^+CD25^+ T cells in the me/me FTOCs was concurrent with extensive deletion.

Taken together, our data suggest that the CD4^+CD25^+ Treg cells are precommitted to the Treg cell lineage before their encounter with the negatively selecting thymic environment. Our findings also highlight the importance of SHP-1-dependent regulation of TCR signal strength as a contributing factor in determining the Treg vs non-Treg cell ratios.

**Discussion**

We and others have previously reported that the tyrosine phosphatase SHP-1 plays a fundamentally important role in regulating TCR signal strength (28, 29). This translates to SHP-1 setting thresholds for positive and negative selection during thymic T cell development (31–34). The presence of high-affinity peptides during thymic selection has been shown to promote Treg cell development (11, 12, 14). It was proposed that this was directly related to heightened signaling via the TCR during the selection process. In this study, we took two different approaches to test this model and to address whether modulation of specific signaling molecules can promote increased Treg development. We analyzed mice lacking SHP-1 for the presence of Treg cells and observed an increase in the percentage of Treg cells in the me/me mice compared with control mice. Based on this finding, we hypothesized that controlled modulation of TCR signal strength through varying peptide concentrations would also affect Treg development. Using a FTOC model system where we added increasing concentrations of cognate peptide, we have presented evidence that Treg cell development is favored under conditions of high signal strength that promote negative selection.

Several lines of evidence confirmed that the CD4^+CD25^+ T cells present in me/me mice were functional Treg cells. These include the following: their anergic phenotype when stimulated in vitro, their ability to suppress the proliferation of Treg cells, their surface marker expression, the presence of Foxp3 mRNA, and the coexpression of a second endogenous TCRα chain in the case of DO11.10 TCR-Tg background. Moreover, these CD4^+CD25^+ T cells seen in the me/me mice were thymus derived as confirmed using the FTOC system.

Our analyses also revealed a relative as well as an absolute increase in numbers of Treg cells in spleens of me/me mice. This is most likely a direct result of the increased output of Treg cells from the thymus, although an additional peripheral expansion is still possible. There is some evidence for Treg cell development in the periphery, although the precise mechanisms are unclear (20, 40, 51). Although all of the spleens analyzed were from 14- to 17-day-old mice due to the limited life span of the me/me mice, environment-driven alterations in Treg numbers in the periphery could not be excluded. Whether defects in other cell types in me/me mice influence peripheral Treg cell numbers remains to be established. Additional studies are needed before making definitive conclusions about thymus-independent Treg cell development in me/me mice.

The increased percentage of thymic CD4^+CD25^+ Treg cells in the me/me background suggested that absence of SHP-1 favored Treg cell development over effector CD4^+CD25^- T cells. There are at least two potential, not mutually exclusive, models that can explain this phenotype (Fig. 8).

**Increased-selection model**

In this model, thymic selection of Treg cells would be limited by a threshold that has to be overcome, similar to the classical positive- and negative-selection criteria. This threshold would, at least in...
part, be regulated by SHP-1. In other words, the positive selection
of Treg cells would be enhanced by the absence of SHP-1. How-
ever, because the loss of SHP-1 would have lowered the threshold
for Treg and non-Treg cells, the me/me mice are selectively
enriched for Treg cells, the model would require that Treg cells be
more affected from lowering the threshold than the non-Treg cells.

Precommitment/selection model

In this model, a set fraction of thymocytes would be precommitted
to go into the Treg lineage before they undergo the regular selection
process. This could either be achieved by a stochastic event before
the selection process or through an encounter of the developing
thymocyte with a special cell type that provides an instructional
signal. Cells thereby committed to the Treg lineage would still re-
quire a strong signal to be positively selected but would be less
susceptible to negative selection. In the context of this model, two
other previous observations are of interest. First, conditions where
one can expect greater negative selection coincide with increased
appearance of Treg cells, suggesting that Treg cells are less suscep-
tible to negative selection (11–13). Second, the higher TCR signal
strength in the absence of SHP-1 leads to enhanced negative se-
lection (31). Therefore, in the me/me background, where there is
greater negative selection of non-Treg cells, this could lead to a
selective enrichment of precommitted Treg cells.

If model 1 were true, conditions of heightened TCR signal
strength would lead to an increase in the absolute number of thymi
cells, which is dictated by the fraction of cells precommitted to this
lineage, would not change, unless there is also an increased ex-
ansion of the Treg population; however, the ratio of Treg cells vs
non-Treg cells would be altered. Therefore, the absence of a change
in the absolute number of Treg cells supports model 2, whereas an
increase in the absolute cell number would require further studies
to distinguish between the two models. Although we did see an
increase in the absolute number of thymic Treg cells in the
me/me DO11.10 TCR-Tg mice, the interpretation of this result was com-
plicated by a potentially elevated influx of progenitors as a
consequence of changes in the thymocyte subpopulations (31). A
recent study addressed the mechanism of controlling progenitor
recruitment/precursor influx into the thymus (52). The authors
found that the size of the double-negative (DN) pool and specifi-
cally the cell number at the DN3 stage control the influx of new
progenitors. In this context, it is of particular interest that thymi
from DO11.10 me/me mice, which have greatly reduced numbers
of DN3 cells (data not shown) and therefore potentially the great-
est influx of thymocyte precursor cells have the greatest increase in
the number of CD4+CD25+ Treg cells. Increased influx would have resulted in
increased absolute numbers independent of which model was op-
erational. To circumvent the complication of changes in influx, we
used the FTOC model system that precludes new influx of pro-
genitors into the thymus. An additional advantage of the FTOC

FIGURE 7. FTOCs derived from me/me embryos are more sensitive to OVA peptide than FTOCs derived from +/+ littermates. Thymi were removed
from embryos at day 15 of gestation (day 0). The two lobes of each thymus were separated, and one was cultured without OVA peptide, whereas the other
was cultured with the indicated amounts of OVA peptide on days 2–5. On day 7 of culture, thymi were removed from culture and stained with CD4-PerCP,
CD8-allophycocyanin, and CD25-PE. Approximately 10^6 live-gated thymocytes were collected for each point. CD8 vs CD4 profiles are shown for each
OVA concentration (top panels). CD4+ SP thymocytes were further analyzed for CD25 surface expression (bottom panels). Percentages of CD25+ cells
within the CD4+ SP subpopulations are indicated. The data shown are derived from matched pairs of thymic lobes isolated from the same thymus and
are representative of at least 13 thymi for each concentration. The data for FTOCs exposed to 500 and 1000 ng OVA peptide, respectively, were derived from
different experiments, which required setting the gates differently between the experiments. However within each concentration, +/+ and me/me FTOCs
were derived from littermates.
system is that it allows the addition of a selecting peptide at controlled concentrations, enabling us to correlate signal strength with the development/enrichment of T\textsubscript{reg} cells.

When FTOCs (derived from +/+ DO11.10 TCR-Tg mice) were treated with increasing amounts of OVA peptide, the absolute numbers of CD4\(^{+}\)CD25\(^{+}\) cells stayed relatively constant throughout the various OVA concentrations, despite the relative increase within the CD4\(^{+}\) population. This argues against model 1, because this should have led to higher absolute numbers of CD4\(^{+}\)CD25\(^{+}\) T cells. However, the data are consistent with the precommitment/selection model (model 2), where a set fraction of developing thymocytes are committed to the T\textsubscript{reg} lineage, yet these cells are less susceptible to negative selection. Moreover, we observed that the CD4\(^{+}\)CD25\(^{+}\) T cell population recovered after OVA peptide treatment was still enriched for V\textsubscript{\textalpha}2 compared with the CD4\(^{+}\)CD25\(^{+}\) T cell population. This is consistent with the CD4\(^{+}\)CD25\(^{+}\) T cell population being precommitted to the T\textsubscript{reg} lineage and not representing CD4\(^{+}\)CD25\(^{-}\) thymocytes newly recruited to this lineage. The selective loss of non-T\textsubscript{reg} lineage cells at deletion-inducing peptide concentrations would lead to a relative increase in T\textsubscript{reg} cells, without a concomitant increase in absolute numbers. An important question is at what stage of thymic T cell development do cells commit to the T\textsubscript{reg} cell lineage. Based on our present knowledge, commitment could occur before any selection event or somewhere during the developmental process guided by extrinsic cues; alternatively, T\textsubscript{reg} cells could also arise from a different precursor pool. Careful future studies are necessary to address this question.

It is interesting that the cells committed to the T\textsubscript{reg} lineage are very resistant to negative selection in the FTOC model system. In fact, we failed to detect deletion of the CD4\(^{+}\)CD25\(^{+}\) population even at \(>10\) times the concentration required to achieve negative selection of CD4\(^{+}\)CD25\(^{+}\) T cells. The CD4\(^{+}\)CD25\(^{+}\) T cells that survived were still KJ1-26 positive, and therefore should have been capable of recognizing OVA peptide. However, it is possible that the CD4\(^{+}\)CD25\(^{+}\) T cells were less sensitive to OVA peptide due to their coexpression of an endogenous TCR\textsubscript{\textalpha} chain. Resulting changes in the expression or signaling of the clonotypic TCR on this T cell population compared with the CD4\(^{+}\)CD25\(^{+}\) T cells might have rendered the CD4\(^{+}\)CD25\(^{+}\) T cells more resistant to deletion. Future studies are needed to better understand the contribution of the endogenous TCR\textsubscript{\textalpha} chain to the development and function of TCR-Tg CD4\(^{+}\)CD25\(^{+}\) T\textsubscript{reg} cells. We also considered the possibility that, in the FTOCs, the OVA presentation occurred through thymic subcompartments that do not support T\textsubscript{reg} deletion; however, this seems unlikely because the me/me mice also show a selective enrichment of the CD4\(^{+}\)CD25\(^{+}\) T cell population.

To further determine the relationship between negative selection and the emergence of T\textsubscript{reg} cells vs non-T\textsubscript{reg} cells, we combined the genetic approach of using me/me mice and the addition of peptides in the FTOC system. In me/me FTOCs, where the negative depletion of non-T\textsubscript{reg} cells occurs at lower OVA peptide concentrations, the relative increase in T\textsubscript{reg} cells was concurrently detected. This further supported our hypothesis that TCR signal strength, and its regulation by SHP-1, influence the ratio of T\textsubscript{reg} and non-T\textsubscript{reg} cells.
In this regard, it is interesting that pharmacological interference with the glucocorticoid/glucocorticoid receptor pathway, thought to lower the threshold for T cell activation, also influences the fraction of Treg/non-Treg cell ratios (53).

Although most of the previous models of Treg cell development suggest that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells are generated in direct response to increased signaling via the TCR, our data support an alternative model where Treg cells are precommitted to this lineage to strong signaling events, which trigger their deletion. Therefore, although the precommitment/selection model proposed here represents an elegant study using triple transgenic mice, they showed that radioresistant stromal cells, expressing the glucocorticoid/glucocorticoid receptor pathway, thought to be selectively enriched under conditions of increased negative selection. Our observations that the percentage of Treg cells can be altered through the modulation of TCR signal strength, either by specific signaling molecules such as SHP-1, or by changing the concentration of selecting peptides, provide an exciting avenue for the manipulation of Treg numbers in vivo. Given the link between Treg cells and autoimmunity, the above studies may have important implications for physiology and pathophysiology of the immune system.

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Disclosures

The authors have no financial conflict of interest.

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