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Antigen-Specific T Cell Repertoire Modification of CD4⁺CD25⁺ Regulatory T Cells

Yuki Hayashi,*† Shin-ichi Tsukumo,* Hiroshi Shiota,† Kenji Kishihara,* and Koji Yasutomo²* 

T cell immune responses are regulated by the interplay between effector and suppressor T cells. Immunization with Ag leads to the selective expansion and survival of effector CD4⁺ T cells with high affinity TCR against the Ag and MHC. However, it is not known if CD4⁺CD25⁺ regulatory T cells (Treg) recognize the same Ag as effector T cells or whether Ag-specific TCR repertoire modification occurs in Treg. In this study, we demonstrate that after a primary Ag challenge, Treg proliferate and TCR repertoire modification is observed although both of these responses were lower than those in conventional T cells. The repertoire modification of Ag-specific Treg after primary Ag challenge augmented the total suppressive function of Treg against TCR repertoire modification but not against the proliferation of memory CD4⁺ T cells. These results reveal that T cell repertoire modification against a non-self Ag occurs in Treg, which would be crucial for limiting excess primary and memory CD4⁺ T cell responses. In addition, these studies provide evidence that manipulation of Ag-specific Treg is an ideal strategy for the clinical use of Treg. The Journal of Immunology, 2004, 172: 5240–5248.

The immune system has evolved several mechanisms to control self-reactive T cells that escape thymic negative selection (1–3). However, T cell nonresponsiveness to self-Ags does not exclusively result from clonal deletion, T cell anergy, or T cell ignorance (4, 5). Recent accumulating evidence has suggested that CD4⁺CD25⁺ regulatory T cells (Treg), which constitute 5–10% of the peripheral CD4⁺ T cells in normal naive mice, play an important role in controlling self-reactive T cells and maintaining immunologic self-tolerance (3, 6, 7). Treg in normal naive mice are nonresponsive to Ag-specific stimulation in vitro, but upon stimulation through the TCR, they potently suppress the activation of other CD4⁺ T cells in an Ag-nonspecific manner (3, 6, 7). Indeed, Treg can suppress autoreactive T cells that cause autoimmune gastritis in neonatal day 3 thymectomized mice (8) and also can suppress allo-MHC-responsive T cells in an allogeneic skin graft model (9). Also, depletion of total Treg has been found to augment tumor-specific (10) or a parasite-specific T cell response (11). Therefore, the application of Treg to clinical medicine (the reduction or increase in function of Treg) such as treatment of tumors, autoimmune diseases, or allogeneic transplantation has been anticipated.

As for the lineage of Treg, several recent papers have revealed that Foxp3 expression is crucial for their development (12–14). According to these results, Foxp3 is expressed in Treg and to a lesser degree in CD25⁺ T cells (14). Furthermore, the ectopic expression of Foxp3 in CD4⁺CD25⁺ T cells allowed such cells to become regulatory T cells that have the ability to suppress the T cell response (12, 14), suggesting that Foxp3 is at least one transcription factor critical for the development of Treg. Conventional CD4⁺CD25⁺ T cells (Tconv) proliferate in response to antigenic stimulation with the selective survival of cells with a high affinity TCR against the Ag (15–23). This Tconv TCR repertoire contraction plays an important role in augmenting primary and secondary immune responses against Ags, thus establishing Ag-specific T cell memory (17, 19). Although the total T cell immune response is thought to be regulated by the interplay between Tconv-derived effector/memory T cells and Treg, it remains unclear if Treg recognize the same Ag as effector T cells seen in vivo.

Thus, in this study we evaluated changes in TCR diversity in both Tconv and Treg during the emergence of the primary and memory responses to pigeon cytochrome c (PCC) in nontransgenic animals to address if Treg really recognize the same Ags as effector T cells do and contract their own repertoire. We found that Treg do recognize the same Ag as Tconv, and expand while contracting their repertoire. Furthermore, Ag-specific Treg suppress TCR repertoire modification but not cell proliferation of memory CD4⁺ T cells.

Materials and Methods

Mice and immunization

The B10.A and B10.BR mice used were from The Jackson Laboratory (Bar Harbor, ME) and were crossed with each other to make F1 mice. A primary immunization of 400 μg of PCC (Sigma-Aldrich, St. Louis, MO) in an emulsion with CFA was s.c. injected into the base of the mouse tail two days after cell transfer. PBS alone was used for the adjuvant only controls. Secondary challenge was a repeat of the primary regimen including adjuvant, also at the base of the tail, 8 wk after the initial priming.

Cell transfer and purification

The protocol of cell transfer is shown (see Fig. 1A). The bone marrow (5 × 10⁶) from 6-wk-old B10.A mice was transferred into 950 rad irradiated (B10.A x B10.BR)F1 mice. Two months after bone marrow transplantation, lymph node cells from chimeric mice were stained with anti-B220, CD8, and CD8 mAbs and positive cells were removed by anti-rat mouse IgG-coated beads (Dynal Biotech, Oslo, Norway). The resultant cells were then stained with anti-CD25 mAb and CD4⁺CD25⁺ or CD4⁺CD25⁺ T cells were separated by negative or positive selection of CD25⁺ cells by
anti-rat IgG-coated beads (Dynal Biotech) or anti-rat IgG-coated beads followed by LS+ magnetic column (Miltenyi Biotec, Cologne, Germany), respectively. The purity of CD4+CD25+ and CD4+CD25− T cells in this preparation is >98% and D+positive cells are <1% and the CD4+CD25+ T cells contaminating the CD4+CD25− T cell population is <1% (see Fig. 1B). Then, CD4+CD25− or CD4+CD25+ purified T cells (1 × 106) were transferred into nonirradiated (B10.A × B10.BR)F1 mice. The mice were immunized with PCC (400 μg) emulsified with CFA 2 days after cell transfer and activated donor-derived cells were purified from lymph nodes several days or weeks after immunization.

For the purification of activated cells for complementarity-determining region 3 (CDR3) analysis, lymph node cells from immunized mice were stained with anti-B220, D3, and CD8 mAbs and positive cells were removed by anti-rat mouse IgG-coated beads (Dynal Biotech). Then resultant T cells were stained with anti-TCR-α/β, CD25, and D126 ligand (CD62L) mAbs, and TCRVβ3 TCRVβ3+CD4+CD62L+ cells were sorted by a cell sorter as described (24). For the analysis of total Ag-specific cell number, after live cell count by trypan blue staining, spleen and lymph node cells were stained with PE-conjugated anti-TCR-α/β, biotin-conjugated anti-TCR-β, allophycocyanin-conjugated anti-CD62L, FITC-conjugated anti-D3, and FITC-conjugated anti-CD8 mAbs followed by streptavidin-CyChrome. Then percentage of TCRVβ3 cells was determined by flowcytometry. The Ag-specific T cells were calculated by multiplying total live cell numbers and relative activated T cells. All Abs were purchased from BD Biosciences (San Francisco, CA). In some experiments, CD25+ cells were depleted by injecting anti-CD25 mAb (7D4, 500 μg) 1 day before PCC immunization.

In some experiments, PCC-activated or naive CD4+CD25− Vα11+Vβ3+ T cells (1 × 107) were transferred into nonirradiated (B10.A × B10.BR)F1 mice, which had been immunized by PCC 8 wk earlier. For the purification of activated and naive CD4+CD25+ T cells, lymph node and spleen cells were first purified from PCC-immunized or unimmunized (B10.A × B10.BR)F1 mice, reconstituted with CD4+CD25+ T cells from 8-wk-old chimeric mice (B10.A bone marrow into irradiated B10.A × B10.BR mice). Then cells were stained with anti-B220, D3, and CD8 mAbs and positive cells were removed by anti-rat mouse IgG-coated beads (Dynal Biotech). These resultant cells were then stained with anti-CD25 mAb, and CD25− T cells were separated by positive selection by anti-rat IgG-coated beads followed by LS+ magnetic column (Miltenyi Biotec). Then, cells were stained with anti-TCR-α/β, Vβ3, CD62L, and CD4+CD25+ or Vα11+Vβ3+CD62L−CD4+T cells (activated CD4+CD25+ T cells) from PCC-immunized mice or Vα11+Vβ3+CD62L+CD4+T cells (naive CD4+CD25− T cells) from PCC-unimmunized mice were sorted by a cell sorter. Each population was transferred into nonirradiated (B10.A × B10.BR)F1 mice that had been immunized with PCC 8 wk earlier.

**PCR products**

Several days after primary and secondary immunization, CD8+D3+–depleted single cells (TCRα/β3+CD62L−CD44high) from the lymph nodes were sorted into single cells and placed in a 5-μl DNA reaction mixture (4 U/ml murine leukemia virus reverse transcriptase with recommended buffer, 0.5 mM spermidine, 100 μg/ml BSA, 10 ng/ml oligo(dT), 200 μM each dNTP, and 1% Triton X-100) and then immediately held at 37°C for 90 min. Aliquots (2 μl) of the cDNA reaction mixture were used for the second PCR reaction (1 U/ml Taq polymerase with the recommended reaction buffer, 0.1 mM each dNTP, 2 mM MgCl2, and 1.2 μM primer), one for the TCR-α/β1 and one for the TCR-β3, using primers specific for both the variable and constant regions of each chain. The following combinations of primers were used: Vα11, 5′-ATGGCAGGGAAAACTTGGGAC-3′ and 5′-ATGGCTCAAGGTGCTCTCCTCGTA-3′; Vβ3, 5′-ATGGCTCAAGGGTCTTGG-3′ and 5′-ATGGCTCAAGGACGGAGAGA-3′. The total of 1 μl of the first PCR product was used for further 25 μl amplification reactions (2 U/ml Taq polymerase with the recommended reaction buffer, 0.1 μM each of dNTP, 2 mM MgCl2, and 0.8 μM primer) for each chain of the TCR, using nested primers for: Vα11, 5′-AATCTTGACGCGCGACATGGCTTGG-3′ and 5′-GAGTCTCAAGGGTCTTGGGAC-3′; Vβ3, 5′-AATCTTGACGCGCGACATGGCTTGG-3′ and 5′-GAGTCTCAAGGGTCTTGGGAC-3′. The PCR product was then blunt-end cloned using a BstXI restriction enzyme.

**T cell proliferation assay**

Total lymph node cells (5 × 105/well) from B10.A × B10.BR mice 10 days after immunization with PCC were cultured with PCC (1 μg/ml) and varying numbers of activated CD4+CD25− Vα11+Vβ3+CD62L−CD44high T cells from PCC-immunized (B10.A × B10.BR)F1 mice reconstituted with CD4+CD25+ T cells from bone marrow chimeric mice or naive CD4+CD25+Vα11+Vβ3+CD62L−CD44high T cells from PCC-immunized (B10.A × B10.BR)F1 mice reconstituted with CD4+CD25− T cells from bone marrow chimeric mice (B10.A bone marrow into irradiated B10.A × B10.BR mice). The Tα1 μCi/ml[^H]thymidine was pulsed during the final 8 of 72-h culture. [^H]Thymidine incorporation was evaluated using an automated beta liquid scintillation counter.

**Results**

**PCC immunization protocol**

The PCC immunization protocol (24) was used to examine Ag-specific CD4+ T cells as this system allowed us to analyze the TCR-β and TCR-β CDR3 sequences critical for binding PCC presented by I[^E]5. The major PCC-responder CD4+ T cell population expresses TCR-α/β and TCR-β CDR3 sequences (24). We first examined the relative number of TCRα/β1+Vβ3+ cells in Tconv and Treg fractions.

**FIGURE 1.** Scheme of experimental system. A. Irradiated (B10.A × B10.BR)F1 mice were reconstituted with bone marrow from B10.A mice, and donor-derived Treg or Tconv collected and purified 2 mo after reconstitution as described in Materials and Methods. Treg from chimeric mice had T cell suppressive activity comparable to Treg from B10.A mice (data not shown). Each cell population was then transferred into nonirradiated (B10.A × B10.BR)F1 mice, and 2 days later mice immunized with PCC emulsified in CFA. Several days or weeks after PCC immunization, single donor-derived activated (CD4+CD8−) B220+Vα11+Vβ3+D3+CD62L−CD44high T cells were sorted from lymph nodes by flow cytometry and CDR3 sequences evaluated by PCR followed by DNA sequencing. B. After purification of CD4+CD25− (left panel) or CD4+CD25+ (right panel) T cells as described in Materials and Methods, cells were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD25 mAbs (7D4) and evaluated by flow cytometry.
FIGURE 2. Ag-driven modification of preferred TCR-Vα11 CDR3 sequence parameters from PCC-specific TCR. The three most-preferred CDR3 sequences for TCR-Vα11 (position α93, position α95, CDR3 length, and Jα usage) observed in PCC-specific TCR are shown. Each number represents the number of cells with the indicated CDR3 parameter from a single cell analysis (after day 0), with the preferred CDR3 sequence parameters (E) presented at the top of each panel and the percentage of single cells expressing this characteristic listed in the next row. Data are shown as a progression over time (left to right) before injection (day 0), after primary immunization (days 4, 8, 10, 14, and 18), and after memory immunization (days 0, 4, 8, and 10). The second immunization was done 8 wk after the primary immunization. The number of sequences used in the analysis (n) is displayed below the days shown on the x-axis. The day 0 group for primary immunization includes Vα11/Vβ3-expressing T cells before injection with resting CD44 low CD62L high phenotypes.
FIGURE 3. Ag-driven modification of preferred TCR-Vβ3 CDR3 sequences of PCC-specific TCR. The TCR-Vβ3 CDR3 regions of PCC-specific TCR were sequenced as in Fig. 2. The three most-preferred TCR-Vβ3 CDR3 (position β100, position β102, CDR3 length, and Vβ usage) sequences are shown. Each number represents the number of cells having the indicated CDR3 characteristic after single cell analysis (from day 0), with data organized as in Fig. 1. The day 0 primary immunization group includes Vα11Vβ3-expressing T cells before injection with a resting CD44<sup>low</sup>CD62L<sup>high</sup> phenotype.
T reg and T conv had similar frequencies of TCR-Vα11 and TCR-Vβ3 CDR3 sequence parameters before PCC immunization (Figs. 2 and 3), which suggested that both populations had similar TCR repertoires in terms of recognition of PCC presented by IEd. PCC immunization led to cell proliferation of both T conv and T reg after primary immunization (Fig. 4A). However, the degree of proliferation of activated cells was lower and the amount of time to reach the peak day was longer for T reg than for T conv after primary immunization (Fig. 4B). For the memory response, peak proliferation occurred earlier for both T reg and T conv (Fig. 4B). These findings indicated that polyclonal T reg proliferated in response to Ag immunization with adjuvant in vivo, although these findings are in contrast with a previous report of Treg anergy after Ag stimulation via TCR ligation in vitro and in vivo (25, 26).

**TCR repertoire modification after primary PCC immunization**

We next analyzed the changes in TCR-Vα11 and TCR-Vβ3 CDR3 sequence parameters using lymph node T cells obtained after primary and memory responses. All preferred TCR-Vα11 or TCR-Vβ3 CDR3 sequence parameters (TCR-Vα11 positions 93 and 95, CDR length, and Jα; TCR-Vβ3 positions 100 and 102, CDR length, and Jβ) from PCC-specific T conv changed during the primary immune response (Figs. 2 and 3). The percentage of PCC-specific T conv with preferred TCR-Vα11 or TCR-Vβ3 CDR3 sequence parameters of TCR-Vα11 positions 93 and 95, TCR-Vβ3 CDR length (Fig. 2), TCR-Vβ3 positions 100 and 102, TCR-Vβ3 CDR length and Jβ (Fig. 3) exceeded 90% during the primary response. However, the percentage of T conv with preferred Jα usage had increased to only 51% (Fig. 2). Using data from Figs. 2 and 3, the percentage of T conv having six or more characteristic TCR-Vα11 or TCR-Vβ3 CDR3 CDR3 sequence parameters was calculated (24). In parallel, total donor-derived activated cell numbers were calculated by multiplying total live cell numbers by the relative proportion of activated T cells. Total cell counts on the y-axis represents the mean of six animals ± SEM. Total cell counts of T conv (■) and T reg (□) are shown. There was no significant difference in the adjuvant-only response across different days (data not shown). B, Relative increases in Ag-specific cell counts against day 0 for primary or secondary Ag challenge were calculated. The y-axis represents the mean of six relative cell counts per animal ± SEM. Total cell counts of T reg (□) and T conv (■) are shown.

T reg proliferation after primary and secondary PCC immunization

T reg and T conv contained ~1% TCRVα11^+Vβ3^+ cells (data not shown). Because CD25, the IL-2R-α chain, can be up-regulated following T conv stimulation (6), we established the following experimental system to clearly discriminate between T reg and activated T conv (Fig. 1A). Although both B10.A and B10.BR mice express the MHC class II k haplotype, B10.A and B10.BR express the MHC class I^d and I^k haplotypes, respectively. Irradiated (B10.A × B10.BR)F 1 mice were reconstituted with bone marrow from B10.A mice to remove donor-derived CD4^+ T cells reactive against B10.BR-derived Ags (Fig. 1A). Donor-derived CD4^+CD25^−D^k^− (T reg) or CD4^+CD25^−D^k^− (T conv) cells were then purified as described in Materials and Methods (Fig. 1B) and transferred into nonirradiated (B10.A × B10.BR)F 1 mice (designated CD25^− chimeric mice or CD25^+ chimeric mice, respectively) (Fig. 1A). Using this protocol, donor- and host-derived cells could be discriminated according to MHC class I haplotype expression.

After immunization of the chimera with PCC emulsified in CFA, single donor-derived activated cells (CD4^+CD8^−B220^−CD11b^−Vα11^+Vβ3^−D^k^−CD62L^low^CD44^high^) from lymph nodes were sorted by flow cytometry. The CDR3 regions of each cell were then amplified by PCR and sequenced as previously described (24). In parallel, total donor-derived activated cell (CD8^−D^k^−Vα11^+Vβ3^−D^k^−CD62L^low^) numbers from the spleen and lymph nodes were counted as described in Materials and Methods. Both the primary and memory responses were examined, we determined eight CDR3 sequence parameters from TCR-Vα11 and TCR-Vβ3 of PCC-specific T cells (Figs. 2 and 3) (24), and measured total activated cell numbers (Fig. 4). The eight CDR3 sequence parameters from the PCC-specific T cells were CDR3 length, DNA sequence at positions 93 and 95, and Jα usage for TCR-Vα11 (Fig. 2), and CDR3 length, DNA sequence at positions 100 and 102 and Jβ usage for TCR-Vβ3 (Fig. 3). Figs. 2 and 3 summarize the results of one representative experiment and show CDR3 sequence information for TCR-Vα11 and TCR-Vβ3 chains from T cells obtained after PCC immunization. A summary of the combined data obtained from individual animals is shown in Fig. 5.
102 (Fig. 3). Further analysis showed that only 30% of PCC-specific T<sub>reg</sub> had six or more preferred of TCR-α11 or TCR-β3 CDR3 sequence parameters during the memory response (Fig. 5B).

**TCR repertoire modification after secondary PCC immunization**

We next examined whether the TCR repertoire altered during the memory response. PCC was administered 8 wk after the primary immunization and CDR3 sequences from T cells obtained (Figs. 2 and 3). Results showed that the PCC-specific T<sub>conv</sub> TCR repertoire was further modified during the memory response in terms of preferred TCR-α11 or TCR-β3 CDR3 sequence parameters, including TCR-α11 positions 93 and 95, TCR-α11 CDR length, and Jα usage (Fig. 2), and TCR-β3 positions 100 and 102, TCR-β3 CDR length, and Jβ usage (Fig. 3). However, the percentage of T<sub>conv</sub> showing the preferred Jα usage was ~60% (Fig. 2) compared with nearly 100% for the other CDR3 sequence parameters (Figs. 2 and 3). This further modification in T<sub>conv</sub> reflected the fact that 90% of the cells had acquired six or more of the preferred PCC-specific TCR-α11 or TCR-β3 CDR3 sequences during the memory response (Fig. 5A). In contrast, whereas the second immunization increased T<sub>reg</sub> cell numbers (Fig. 4A), further modification of TCRα (Fig. 2) and TCRβ (Fig. 3) repertoire was not observed (Fig. 5B). Because >88% of T<sub>reg</sub> before secondary immunization had three preferred CDR3 sequence parameters (TCR-α11 CDR length, TCR-β3 position 102, and TCR-β3 CDR length) (Figs. 2 and 3), it was difficult to determine small changes in these parameters after secondary immunization. However, significant differences in the frequencies of preferred TCR-α11 positions 93 and 95, Jα usage (Fig. 2), TCR-β3 position 100 and Jβ usage (Fig. 3) were observed after secondary responses for both T<sub>conv</sub> and T<sub>reg</sub>. The relative percentages of these five preferred CDR3 sequence parameters did not change in T<sub>reg</sub> after secondary immunization (Figs. 2 and 3). Similar results were observed in terms of TCR repertoire modification both of T<sub>conv</sub> and T<sub>reg</sub> when spleen cells were used instead of lymph node cells (data not shown).

**Low level contamination of T<sub>conv</sub>, in the T<sub>reg</sub> fraction did not greatly affect the repertoire modification of T<sub>reg</sub>**

To negate the possibility that T<sub>reg</sub> proliferation and repertoire modification reflected the proliferation of low numbers of contaminating T<sub>conv</sub>, we used a mixture of 99% T<sub>reg</sub> and 1% T<sub>conv</sub>, or 95% T<sub>reg</sub> and 5% T<sub>conv</sub> instead of purified T<sub>reg</sub> in our proliferation (Fig. 6) and repertoire modification (Fig. 7) assays, and compared the results with those obtained using purified T<sub>reg</sub> (Figs. 4 and 5). Proliferation and repertoire modification assay results were similar between the 1% and 5% T<sub>conv</sub> contamination samples, which were in turn similar to results obtained using purified T<sub>reg</sub> (Figs. 5, 6, and 7). These results suggested that low levels of T<sub>conv</sub> contamination in the T<sub>reg</sub> fraction would not be expected to have a significant impact on T<sub>reg</sub> proliferation and repertoire modification.

**Impact of incomplete T<sub>reg</sub> TCR repertoire modification on the total immune response**

To examine the impact of incomplete T<sub>reg</sub> repertoire modification on effector T cell generation, we first evaluated the repertoire modification of lymph node CD4<sup>+</sup>CD25<sup>−</sup> cells from PCC-immunized and CD25<sup>+</sup>-depleted (B10.A × B10.BR)F<sub>1</sub> mice using anti-CD25 mAb. The kinetics of TCR repertoire modification in activated CD4<sup>+</sup>CD25<sup>−</sup> T<sub>conv</sub> T<sub>α11</sub> V<sub>β3</sub>CD62L<sup>low</sup>CD44<sup>high</sup> cells in response to PCC immunization was similar to that of CD4<sup>+</sup>CD25<sup>−</sup> donor cells (CD4<sup>+</sup>CD25<sup>−</sup>-/ V<sub>α11</sub> V<sub>β3</sub>CD62L<sup>low</sup>CD44<sup>high</sup>) from CD25<sup>+</sup>-chimeric mice (data not shown). For this reason we used (B10.A × B10.BR)F<sub>1</sub> mice instead of CD25<sup>+</sup> or CD25<sup>−</sup> chimeric mice to evaluate the impact of low T<sub>reg</sub> repertoire modification on effector T cell activation or differentiation.

Following primary PCC immunization, (B10.A × B10.BR)F<sub>1</sub> mice that had been T<sub>reg</sub> depleted using anti-CD25 mAb generated higher Ag-specific T cell numbers in the lymph nodes and spleen compared with non-T<sub>reg</sub> depleted (B10.A × B10.BR)F<sub>1</sub> mice (Fig. 8A). The kinetics of lymph node PCC-activated T cell TCR repertoire modification was also faster in the absence of T<sub>reg</sub> after the
primary immune response (Fig. 8C). To evaluate the effect of Treg on memory T cell responses, CD25-positive cells were depleted by anti-CD25 mAb treatment in PCC-immunized (B10.A × B10.BR)F1 mice 8 wk after primary immunization. Mice were then reimmunized with PCC and the TCR repertoire and total cell numbers of CD4+CD8- B220- CD11b+ Vα11+ Vβ3+ D- CD62LlowCD44th T cells were evaluated. Depletion of Treg before secondary immunization induced a faster TCR repertoire modification (Fig. 8D), but did not affect PCC-specific T cell numbers (Fig. 8B).

We then examined whether the inability to inhibit Treg proliferation during the memory response was due to limited Treg expansion or repertoire modification, or to the intrinsic failure of effector/memory T cells to respond to Treg inhibitory activity. Donor-derived PCC-activated Treg were purified from PCC-immunized CD25+ chimeric mice as described in Materials and Methods. Purified Treg were then transferred into PCC-immunized (B10.A × B10.BR)F1 mice 2 days before a second PCC immunization. The addition of purified PCC-activated Treg before the second immunization inhibited both cell proliferation (Fig. 8B) and TCR repertoire modification (Fig. 8D).

These findings suggested that limited expansion or repertoire modification of PCC-activated Treg after secondary immunization was one of the factors that prevented the inhibition of effector T cell proliferation. This PCC-specific inhibitory ability of Treg was confirmed by in vitro experiments. Activated CD4+CD25+D- Vα11+ Vβ3+ CD62LlowCD44th T cells were purified from PCC-immunized CD25+ chimeric mice as described in Materials and Methods. Naive CD4+CD25+D- Vα11+ Vβ3+ CD62LhighCD44low T cells from CD25+ chimeric mice were used as a control. The activated or naive T cells were cocultured with total lymph node cells from PCC-immunized (B10.A × B10.BR)F1 mice in the presence of PCC. The proliferative responses of PCC-specific T cells were inhibited by increasing numbers of activated non-naive regulatory T cells but not control cells (Fig. 8E).

**Discussion**

The adaptive immune response is dependent on the recognition of Ag peptides presented in the context of self-encoded MHC molecules by specific T cells. A broad preimmune TCR repertoire is established via processes involving positive and negative selection on developing T cells in the thymus (4). The particular T cell repertoire that responds to a given Ag is usually quite diverse in terms of TCR β-chain V region usage and fine epitope specificity (18, 19). However, in some cases clonal dominance prevails and T cells with preferred TCR motifs are selectively expanded during primary responses and then appear to be selectively preserved for memory responses (16, 24). Although these studies have tended to focus on the repertoire of effector T cells, the total T cell immune response appears to be regulated by the interplay between effector and suppressor T cells. Therefore, to gain a better understanding of the total adaptive immune response, we attempted to clarify the mode of repertoire modification and proliferation of suppressor/regulatory T cells against a given Ag. Our study revealed an evolving Treg clonal dominance during the primary immune response. Although Tconv were less affected, Treg clonal modification was not observed during the memory response. Experiments using CD25+...
form response. Nonetheless, addition of PCC-activated T<sub>reg</sub> still inhibited memory T cell proliferation.

T<sub>reg</sub> are thought to express high affinity TCRs against self-peptides (27) and suppress effector T cell responses independent of Ag specificity, at least in vitro (28). These findings suggest a role for self-Ag recognition in the acquisition of T<sub>reg</sub> suppressive function. Although T<sub>reg</sub> have very broad TCR Vβ repertoires, similar to T<sub>conv</sub> (data not shown), it remains unclear whether T<sub>reg</sub> actually recognize non-self Ags to acquire suppressive functions in vivo. Recently, several papers have shown that T<sub>reg</sub> suppress murine CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses against parasitic or bacterial infections in vivo (29, 30). This suggests two possibilities regarding the Ag specificity of T<sub>reg</sub> in vivo. First, T<sub>reg</sub> may recognize parasite- or bacteria-derived broad Ags, acquire a suppressive function, and then regulate/suppress effector T cell responses. Second, infections may change the sensitivity of T<sub>reg</sub> TCR signaling via local cytokine bursts or interaction with other activated cells, which then modifies the TCR signaling threshold of T<sub>reg</sub> to allow sufficient response to self-Ags with resultant acquisition of suppressive functions. In this regard, our results indicated that the frequency of preferred TCR-V<sub>α11</sub> or TCR-Vβ CD3<sup>+</sup> sequence parameters of PCC-specific T cells were increased in T<sub>reg</sub> after PCC immunization, which strongly suggested the direct recognition of PCC by T<sub>reg</sub>. In addition, the repertoire modulation of T<sub>reg</sub> increased the total suppressive function of T<sub>reg</sub> toward effector/memory T cell responses against PCC, which demonstrated that T<sub>reg</sub> can suppress effector T cells via recognition of a specific common Ag in vivo. These findings suggested that the regulation of Ag-specific T<sub>reg</sub> rather than total T<sub>reg</sub> may be required for the clinical application of T<sub>reg</sub> in agreement with a number of groups that have tried to regulate effector T cells in an Ag-dependent manner (31).

Previous studies using TCR-transgenic mice have shown that T<sub>reg</sub> are anergic in vitro, but have the potential to respond to lymphoproliferative conditions (25, 26). Recently, it was shown that T<sub>reg</sub> proliferated in vivo (32). Under more physiologic conditions, as in our experimental protocol, polyclonal T<sub>reg</sub> responded to PCC immunization and proliferated in vivo, although the response was less than the T<sub>conv</sub> response. One might argue that the T<sub>reg</sub> proliferation we observed might have at least partly reflected the proliferation of contaminating T<sub>conv</sub> as CD25 is not an exclusive T<sub>reg</sub> marker. However, this is unlikely because the TCR repertoire of all CD25<sup>+</sup>-derived cells was examined 18 days after the first immunization, which would reflect the presence of cell proliferation. We also performed experiments using a mixture of 99% T<sub>reg</sub> and 1% T<sub>reg</sub> or naive T<sub>reg</sub> population and obtained similar findings in terms of cell proliferation and repertoire modulation. If the reduced TCR repertoire seen in T<sub>reg</sub> is due to vigorous expansion of contaminating T<sub>conv</sub>, we should have observed increased repertoire modulation with increasing numbers of contaminating T<sub>conv</sub>. However, the possibility that contaminating T<sub>conv</sub> do contribute to T<sub>reg</sub> result cannot be ruled out as we do not have cell surface markers able to discriminate between T<sub>reg</sub> and activated T cells.

The CD25<sup>+</sup> cell depletion experiments showed that during the memory response, T<sub>reg</sub> inhibited effector T cell TCR repertoire modification, but not T cell-mediated proliferation. However, the addition of PCC-activated T<sub>reg</sub> during the memory response inhibited T cell proliferation. This suggested the inability to inhibit effector T cell proliferation was due to limited T<sub>reg</sub> expansion or TCR repertoire modification after primary immunization rather than the intrinsic failure of effector T cells to respond to the inhibitory activity of T<sub>reg</sub>. Although T<sub>reg</sub> generally inhibit T cell proliferation, the precise regulatory mechanisms of T<sub>reg</sub> remain unknown.
unknown, despite reports suggesting possible roles for TGF-β or CTLA-4 (33, 34). Treg inhibition of T cell repertoire modification, but not proliferation, suggests another regulatory role of Treg in the Tconv response. Furthermore, our findings provide evidence for a distinct regulatory mechanism linking T cell proliferation and TCR repertoire modification, although these have generally been thought to be coordinated processes.

We observed no differences in the frequencies of CDR3 features involved in PCC recognition between Treg and Tconv, before immunization. Nonetheless, it is still possible that Treg and Tconv exhibited different TCR repertoires in terms of PCC recognition that might underlie the different proliferation and TCR repertoire exhibited different TCR repertoires in terms of PCC recognition. This divergence may have occurred to maximize the ability of the organism to mount a protective memory T cell response against bacteria, protozoans, fungi, and viruses.

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References