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Generation of CD4\(^+\)CD25\(^+\) Regulatory T Cells from Autoreactive T Cells Simultaneously with Their Negative Selection in the Thymus and from Nonautoreactive T Cells by Endogenous TCR Expression\(^1\)

Kazuto Kawahata,* Yoshikata Misaki,‡ Michiko Yamauchi,* Shinji Tsunekawa,† Keigo Setoguchi,* Jun-ichi Miyazaki,‡ and Kazuhiko Yamamoto*

Normal T cell repertoire contains regulatory T cells that control autoimmune responses in the periphery. One recent study demonstrated that CD4\(^+\)CD25\(^+\) T cells were generated from autoreactive T cells without negative selection. However, it is unclear whether, in general, positive selection and negative selection of autoreactive T cells are mutually exclusive processes in the thymus.

To investigate the ontogeny of CD4\(^+\)CD25\(^+\) regulatory T cells, neo-autoantigen-bearing transgenic mice expressing chicken egg OVA systemically in the nuclei (Ld-nOVA) were crossed with transgenic mice expressing an OVA-specific TCR (DO11.10). Ld-nOVA × DO11.10 mice had increased numbers of CD4\(^+\)CD25\(^+\) regulatory T cells in the thymus and the periphery despite clonal deletion. In Ld-nOVA × DO11.10 mice, T cells expressing endogenous TCR αβ chains were CD4\(^+\)CD25\(^-\) T cells, whereas T cells expressing autoreactive TCR were selected as CD4\(^+\)CD25\(^+\) T cells, which were exclusively dominant in recombination-activating gene 2-deficient Ld-nOVA × DO11.10 mice. In contrast, in DO11.10 mice, CD4\(^+\)CD25\(^+\) T cells expressed endogenous TCR αβ chains, which disappeared in recombination-activating gene 2-deficient DO11.10 mice. These results indicate that part of autoreactive T cells that have a high affinity TCR enough to cause clonal deletion could be positively selected as CD4\(^+\)CD25\(^+\) T cells in the thymus. Furthermore, it is suggested that endogenous TCR gene rearrangement might critically contribute to the generation of CD4\(^+\)CD25\(^+\) T cells from nonautoreactive T cell repertoire, at least under the limited conditions such as TCR-transgenic models, as well as the generation of CD4\(^+\)CD25\(^-\) T cells from autoreactive T cell repertoire. The Journal of Immunology, 2002, 168: 4399–4405.

N o r m a l T cell repertoire contains CD4\(^+\)CD25\(^+\) regulatory T cells that control autoimmune responses in the periphery (1–4). Impairment of the generation of CD4\(^+\)CD25\(^+\) regulatory T cells results in various organ-specific autoimmune diseases, as demonstrated in mice thymectomized on day 3 of life (5). It has been demonstrated that development of regulatory T cells requires the thymus (6–8) and the presence of the relevant autoantigen in the periphery (9). However, development of CD4\(^+\)CD25\(^+\) regulatory T cells remains poorly understood. Although CD4\(^+\)CD25\(^+\) T cells are anergic to TCR stimulation and suppress the activation of CD4\(^+\)CD25\(^-\) T cells in an Ag-independent manner, it has been demonstrated that regulatory function of CD4\(^+\)CD25\(^+\) T cells requires their activation via TCR in vitro (10–12). Seddon and Mason (9) observed that peripheral autoantigen is responsible for the survival of specific regulatory T cells in vivo. These findings suggest the critical role of TCR specificity of CD4\(^+\)CD25\(^+\) regulatory T cells in their generation, survival, and ability to prevent autoimmunity. Recently, Jordan et al. (13) demonstrated that, in the thymus, self-reactive T cells were positively selected as CD4\(^+\)CD25\(^+\) regulatory T cells and were not deleted. These results suggest that positive selection of CD4\(^+\)CD25\(^+\) regulatory T cells requires higher avidity interactions of their TCRs with self ligands, but that the required avidity must not exceed the threshold of the deletion (14). However, it is unclear whether avidity of autoreactive TCRs that induce positive selection as CD4\(^+\)CD25\(^+\) regulatory T cells is different from avidity of autoreactive TCRs that induce negative selection.

The immune system controls autoreactivity by several mechanisms such as clonal deletion and inactivation. Accumulating evidences suggest that receptor editing or revision, which is induced by autoreactive stimuli and involves endogenous TCR gene rearrangement, is also involved in the generation of nonautoreactive T cell repertoire from autoreactive T cell repertoire (15–17). Moreover, it is suggested that secondary TCR gene rearrangement occurs to escape not only from clonal deletion, but also from death by neglect during thymic selection (18, 19). Interestingly, disturbance of endogenous TCR gene rearrangement seems to be associated with the impaired development of regulatory T cells (20–25). Genetic manipulation of TCR α gene, as in TCR α-chain-deficient (20, 23) and 2B4 TCR α-chain transgenic mice (22), sometimes spontaneously induces organ-specific autoimmune diseases, such as inflammatory bowel disease, autoimmunem gastritis, and thyroiditis. Itoh et al. (7) found that CD4\(^+\)CD25\(^+\) T cells in a
TCR-transgenic model expressed endogenous TCR chains and disappeared in recombination-activating gene 2 (RAG2)-deficient TCR-transgenic mice. These findings have led us to consider how endogenous TCR gene rearrangement controls the generation of CD4\(^+\)CD25\(^+\) T cells or CD4\(^+\)CD25\(^-\) T cells, at least, under certain conditions that CD4\(^+\) T cells lack the CD4\(^+\)CD25\(^+\) T cells or CD4\(^+\)CD25\(^-\) T cells, respectively. However, there are no studies that clearly demonstrate dual roles of endogenous TCR gene rearrangement for the generation of CD4\(^+\) T cell repertoire in one model.

To examine the ontogeny of CD4\(^+\)CD25\(^+\) T cells, we used neo-autointegern-bearing transgenic mice expressing chicken egg OVA systemically in the nuclei (Ld-nOVA) and transgenic mice expressing an OVA-specific TCR (DO11.10). We found that part of autoreactive T cells could be positively selected as CD4\(^+\)CD25\(^+\) T cells in parallel with their deletion in the thymus. We also found that endogenous TCR gene rearrangement generates autoreactive CD4\(^+\)CD25\(^+\) regulatory T cells from nonautoreactive T cells and nonautoreactive CD4\(^+\)CD25\(^-\) T cells from autoreactive T cells.

Materials and Methods

Mice

BALB/c mice were obtained from SLC (Shizuoka, Japan). They were maintained in a temperature- and light-controlled environment with free access to food and water under specific pathogen-free conditions. Female age-matched mice were used in all experiments, and the mice were 7–10 wk old at the start of each experiment. DO11.10 transgenic mice whose T cells express a receptor specific for OVA were kindly provided by T. Watanabe (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). DO11.10 TCR α single transgenic mice were kindly provided by S. Koyasu (Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan). RAG2-deficient BALB/c mice and TCR α-chain-deficient C57BL/6 mice were purchased from Taonomic Farms (Germantown, NY). Generation of Ld-nOVA transgenic mice has been described in another study (26). Briefly, chicken egg OVA cDNA (kindly provided by P. Chambon, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Universite Louis Pasteur, Strasbourg, France) fused with the nuclear localization signal at the 3\(^{rd}\) end was subcloned into pLG-E enhancer into the 5\(^{th}\) end of the L\(^{4}\) class I promoter of pLG-2 plasmid (27). This OVA transgene construct was microinjected into the pronuclei of fertilized eggs from C57BL/6 mice. Ld-nOVA BALB/c mice were produced by crossing Ld-nOVA C57BL/6 mice with normal BALB/c mice for more than six generations. CD25\(^-\) T cells lack the CD4\(^+\)CD25\(^+\) T cells at least, under certain conditions (28, 29).

Preparation of cell populations

Spleen cells were first enriched in T cells by using mouse CD3\(^+\) T cell enrichment columns (R&D Systems, Minneapolis, MN). T cells were then stained with FITC anti-CD4 mAb (PK15.1; BD PharMingen, San Diego, CA) and biotin anti-CD25 mAb (7D4; BD PharMingen), followed by staining with anti-FITC microbeads. CD4\(^+\) T cells were purified with MACS using a positive selection column (Miltenyi Biotec, Bergisch Gladbach, Germany). For the purification of CD4\(^+\)CD25\(^+\) T cells, microbeads of purified CD4\(^+\) T cells were released by FITC MultiSort kit. CD4\(^+\) T cells were stained with streptavidin microbeads, followed by separation with MACS using a positive selection column. The purity of CD4\(^+\)CD25\(^+\) T cells and CD4\(^+\)CD25\(^-\) T cells was ~88%.

In vitro proliferation assay

CD4\(^+\) T cells, CD4\(^+\)CD25\(^-\) T cells, or CD4\(^+\)CD25\(^+\) T cells (2 × 10\(^4\) cells/well) were cultured with irradiated (20 Gy) syngeneic spleen cells (5 × 10\(^4\) cells/well) in the presence of OVA\(_{323-339}\) at 0.5 μM for 3 days, followed by a final 16 h of culture in the presence of 1 μg/mI [\(^{3}H\)ThDPR per well. Suppressor cell activity was assessed by coculturing CD4\(^+\)CD25\(^+\) T cells (2 × 10\(^4\) cells/well) with CD4\(^+\)CD25\(^+\) T cells (2 × 10\(^4\) cells/well) and with irradiated (20 Gy) syngeneic spleen cells (5 × 10\(^4\) cells/well) in the presence of anti-CD3 mAb (145-2C11) at 10 μg/ml or Con A (Sigma-Aldrich, St. Louis, MO) at 1 μg/ml for 3 days, followed by a final 6 h of culture in the presence of 1 μCi [\(^{3}H\)ThDPR per well. In some experiments, anti-CTLA-4 mAb (UC10-4F10-11) (100 μg/ml) was added to the culture. Cells were cultured in 96-well round-bottom plates in RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FCS, and 5 × 10\(^{-3}\) M 2- ME at 37°C, 5% CO\(_{2}\). The incorporated radioactivity was counted with a gamma scintillation counter. The proliferative response was expressed as Δcpm (mean cpm of the culture minus the control cpm without Ag).

Flow cytometry

The following Abs were used for identification and phenotypic analysis of T cell populations: FITC-conjugated or biotinylated KJ1-26; FITC-conjugated or PE-conjugated anti-TCRβ2, anti-TCRVβ6, anti-TCRVβ8, anti-TCRVβ14, anti-TCRαv2; FITC-conjugated or biotinylated anti-TCRB (H57-597); FITC-conjugated anti-CD25; PE-conjugated anti-CD4; PE-conjugated CD8 (all from BD PharMingen); and streptavidin-Triclorol (Caltag Laboratories, Burlingame, CA).

Results

The numbers of CD4\(^+\)CD25\(^+\) regulatory T cells increased in the thymus and the periphery of Ld-nOVA × DO11.10 mice

To investigate immunological tolerance to a systemic nuclear autoantigen, which is perturbed in systemic autoimmune diseases, we generated Ld-nOVA transgenic mice expressing OVA systemically in nuclei. We s.c. immunized Ld-nOVA mice and nontransgenic littermates with 100 μg OVA in CFA at the base of the tail and then cultured the draining lymph node cells with various doses of OVA or OVA\(_{333-339}\) dominant epitope (OVA\(_{p}\) 10 days after the immunization. The proliferative responses were greatly reduced in the Ld-nOVA mice in comparison with nontransgenic littermates, indicating that Ld-nOVA mice are tolerant to OVA (26).

To address the question as to how autoreactive T cells specific for a nuclear autoantigen were rendered tolerized, we mated Ld-nOVA mice with DO11.10 transgenic mice that express a TCR (Vα13.1, Vβ8.2) specific for the OVA\(_{p}\) bound to I-A\(^d\) class II MHC molecules and monitored DOI11.10 TCR-bearing cells using an anti-clonotypic Ab, KJ1-26.

Thymocyte numbers in Ld-nOVA × DOI11.10 mice were significantly reduced to ~55% of those in DOI11.10 mice (1 × 10\(^8\) cells vs 1.8 × 10\(^8\) cells, p < 0.01) (Table I). In comparison with DOI11.10 mice, Ld-nOVA × DOI11.10 mice exhibited a reduction in the percentage of CD4 single-positive (SP) (6.7 ± 0.4% vs 9.7 ± 0.9%, p < 0.01) (Fig. 1A) and CD4 CD8 double-positive thymocytes (61.5 ± 5.9% vs 69 ± 5.8%, p < 0.05), and an increase in the percentage of CD8 SP (27.5 ± 1.1% vs 31.1 ± 1%, p < 0.01) and CD4 CD8 double-negative thymocytes (29.5 ± 5.3% vs 19.8 ± 3.5, p < 0.01). These results indicated autoreactive T cells were negatively selected in the thymus. Spleenic CD4\(^+\) T cell numbers in Ld-nOVA × DOI11.10 mice were reduced to ~45% of those in DOI11.10 mice (1.3 × 10\(^7\) cells vs 2.9 × 10\(^7\) cells, p < 0.01) (Table I) (Fig. 1A).

CD4\(^+\) T cells from the thymus and the spleen of Ld-nOVA × DOI11.10 mice expressed a lower level of clonotypic TCR and Vβ8 than those of DOI11.10 mice (clonotypic TCR in CD4 SP thymocytes, 37 vs 75%; Vβ8 in CD4 SP thymocytes, 81 vs 94%; clonotypic TCR in CD4\(^+\) splenocytes, 26 vs 67%; Vβ8 in CD4\(^+\) splenocytes, 70 vs 88%), despite the same expression level of TCR CB (Fig. 1A). Moreover, mean fluorescence intensity (MFI) of clonotypic TCR and Vβ8 was markedly reduced in Ld-nOVA × DOI11.10 mice compared with DOI11.10 mice (MFI of clonotypic TCR in CD4 SP thymocytes = 9.5 vs 32.5, MFI of Vβ8 in CD4
responses of CD4\(^{+}\) DO11.10 mice had the ability to suppress the proliferative re-
pression of clonotypic T cells in Ld-nOVA
\(\beta\)-chains in DO11.10 mice. This was confirmed by the increased expression of endogenous \(\alpha\) and \(\beta\)-chains in addition to V\(\alpha\)13.1 and V\(\beta\)8.2 in CD4\(^{+}\) T cells of Ld-nOVA \(\times\) DO11.10 mice (Fig. 1B).

We noticed that splenic CD4\(^{+}\) T cells, lymph node CD4\(^{+}\) T cells, and CD4\(^{+}\) SP thymocytes in Ld-nOVA \(\times\) DO11.10 mice contained a higher percentage of CD4\(^{+}\)CD25\(^{+}\) T cells (12.3, 18.2, and 4%, respectively) than those in DO11.10 mice (3.6, 3.3, and 1.2%, respectively) (Table I). Absolute numbers of CD4\(^{+}\)CD25\(^{+}\) T cells were also increased in Ld-nOVA \(\times\) DO11.10 mice. Most of clonotypic T cells in DO11.10 mice were CD4\(^{+}\)CD25\(^{-}\) T cells, whereas most of clonotypic T cells in Ld-nOVA \(\times\) DO11.10 mice were CD4\(^{+}\)CD25\(^{+}\) T cells (Fig. 2A). We next investigated whether these CD4\(^{+}\)CD25\(^{+}\) T cells were regulatory T cells. CD4\(^{+}\)CD25\(^{+}\) T cell-depleted CD4\(^{+}\) T cells from Ld-nOVA \(\times\) DO11.10 exhibited a more vigorous response to OVAp than CD4\(^{+}\) T cells from Ld-nOVA \(\times\) DO11.10, although they exhibited a lower response than CD4\(^{+}\)CD25\(^{+}\) T cell-depleted DO11.10 CD4\(^{+}\) T cells (Fig. 2B). CD4\(^{+}\)CD25\(^{+}\) T cells from Ld-nOVA \(\times\) DO11.10 mice had the ability to suppress the proliferative responses of CD4\(^{+}\)CD25\(^{-}\) T cells not only from the Ld-nOVA \(\times\) DO11.10 mice, but also from nontransgenic BALB/c mice. This inhibitory function was partially blocked by anti-CTLA-4 (Fig. 2C). Because we did not use anti-CTLA4 Fab, the abrogation of inhibition was not so distinguished as demonstrated in the previous reports (28–30). These results indicate that CD4\(^{+}\)CD25\(^{+}\) T cells generated in Ld-nOVA \(\times\) DO11.10 mice are regulatory T cells.

**Clonotypic cells are positively selected into CD4\(^{+}\)CD25\(^{+}\) regulatory T cells in Ld-nOVA \(\times\) DO11.10 \(\alpha\)-chain mice**

To exclude the possibility that the generation of CD4\(^{+}\)CD25\(^{+}\) regulatory T cells in Ld-nOVA \(\times\) DO11.10 mice could be attributed to excessive production of autoreactive T cells beyond the capacity for clonal deletion, we crossed Ld-nOVA mice with DO11.10 \(\alpha\)-chain single transgenic mice and examined CD25 expression of clonotypic T cells in Ld-nOVA \(\times\) DO11.10 \(\alpha\)-chain mice. Although lymph node CD4\(^{+}\) T cells contained the small population of clonotypic T cells in DO11.10 \(\alpha\)-chain and Ld-
nOVA \(\times\) DO11.10 \(\alpha\)-chain mice, we could find clearly different results between these mice. Clonotypic T cells in DO11.10 \(\alpha\)-chain mice were exclusively CD4\(^{+}\)CD25\(^{+}\) T cells, whereas clonotypic T cells in Ld-nOVA \(\times\) DO11.10 \(\alpha\)-chain mice were exclusively CD4\(^{+}\)CD25\(^{+}\) T cells (Fig. 3). These results are consistent with Fig. 2A and indicate that part of autoreactive T cells is positively selected as CD4\(^{+}\)CD25\(^{+}\) regulatory T cells. Although CD4\(^{+}\)CD25\(^{+}\)

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<th>Table I. Ld-nOVA (\times) DO11.10 mice have increased numbers of CD4(^{+})CD25(^{+}) regulatory T cells in the thymus and the periphery</th>
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<td><strong>Organ</strong></td>
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<td>CD4(^{+}) SP ((\times) 10^8)</td>
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<td>CD4(^{+})CD25(^{+}) ((\times) 10^5)***</td>
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<td>Lymph nodes(^a)</td>
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<td>CD4(^{+})CD25(^{+}) ((\times) 10^5)****</td>
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\(^a\) The percentage of CD4\(^{+}\)CD8\(^{-}\) T cells in total thymocytes, splenocytes, or lymph node cells is shown.

\(^b\) The percentage of CD4\(^{+}\)CD25\(^{+}\) T cells in CD4\(^{+}\)CD8\(^{-}\) T cells is shown.

\(^c\) Inguinal, axillary, and para-aorta lymph nodes were collected.

\(^d\) 0.2 > p \(\geq\) 0.05; ***, p \(\geq\) 0.01; **, p \(\leq\) 0.01. Statistical comparison by Student’s t test.

**FIGURE 1.** Expression of endogenous TCR \(\alpha\)- and \(\beta\)-chains in DO11.10 mice and Ld-nOVA \(\times\) DO11.10 mice. A. Thymocyte and splenocyte suspensions prepared from a 2-mo-old DO11.10 mouse and a 2-mo-old Ld-nOVA \(\times\) DO11.10 mouse were stained with anti-CD4, anti-CD8, and KJ1-26, anti-V\(\beta\)8, or anti-TCR\(\beta\). Dot plots showing staining of live gated cells with anti-CD4 and anti-CD8. The percentage of cells falling within the indicated region is shown in each dot plot. Histograms show staining of CD4\(^{+}\)CD8\(^{-}\) cells with KJ1-26, anti-V\(\beta\)8, or anti-TCR\(\beta\). The percentage of cells falling within the indicated marker is shown in each histogram. B, T cell-enriched splenocytes from a 2-mo-old DO11.10 mouse and a 2-mo-old Ld-nOVA \(\times\) DO11.10 mouse were stained with anti-CD4 and anti-TCR\(\beta\), anti-VO2, anti-V\(\beta\)2, anti-V\(\beta\)6, or anti-V\(\beta\)14. The percentage of CD4\(^{+}\) T cells falling within the indicated region is shown in each dot plot. A representative result of seven independent similar experiments is shown. For more information, please refer to the table and figures within the text. The Journal of Immunology

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T cells in Ld-nOVA × DO11.10 α-chain mice contained a higher percentage of clonotypic T cells than CD4⁺CD25⁻ T cells in DO11.10 α-chain mice, as shown in Fig. 3, cell numbers of clonotypic T cells in Ld-nOVA × DO11.10 α-chain mice were lower than those in DO11.10 α-chain mice.

**T cells expressing endogenous TCR chains were differentially recruited into CD4⁺CD25⁺ regulatory T cells or CD4⁺CD25⁻ T cells between Ld-nOVA × DO11.10 mice and DO11.10 mice**

We next investigated the role of endogenous TCR chain expression in the generation of CD4⁺CD25⁺ regulatory T cells and CD4⁺CD25⁻ T cells. In the thymus and spleen of Ld-nOVA × DO11.10 mice, CD4⁺CD25⁻ T cells were clonotypehigh T cells, whereas CD4⁺CD25⁺ T cells were clonotypehigh T cells in the thymus and the spleen of DO11.10 mice, most CD4⁺CD25⁻ T cells were clonotypehigh T cells, whereas CD4⁺CD25⁺ T cells were clonotypelow T cells (Fig. 4A). On the contrary, in the thymus and the spleen of DO11.10 mice, most CD4⁺CD25⁻ T cells were clonotypehigh T cells, whereas CD4⁺CD25⁺ T cells were clonotypelow T cells (Fig. 4A). These clonotypelow T cells in lymph nodes (Fig. 4B) and the thymus (data not shown) expressed not only endogenous Vβs, but also endogenous Vβs. The percentages of CD4⁺ T cells expressing endogenous Vβs in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells from the thymus and lymph nodes of Ld-nOVA × DO11.10 mice and DO11.10 mice are summarized in Fig. 4C. The percentage of T cells expressing endogenous β-chains was defined as the percentage of TCR Cβ-positive cells in CD4⁺ T cells minus the percentage of Vβ8-positive cells in CD4⁺ T cells. These data and the comparison of MFI of Vβ8, as described above, indicate that CD4⁺CD25⁻ T cells of DO11.10 mice preferentially use endogenous Vβs like CD4⁺CD25⁺ T cells of Ld-nOVA × DO11.10 mice. These results imply that CD4⁺CD25⁺ regulatory T cells are derived from autoreactive T cells, which do not undergo clonal deletion, and that CD4⁺CD25⁻ T cells are derived from positively selected nonautoreactive T cells.

**The perturbation of endogenous TCR gene rearrangement exclusively generates CD4⁺CD25⁺ T cells in Ld-nOVA × DO11.10 mice**

The contribution of endogenous TCR gene rearrangement to the generation of CD4⁺CD25⁻ T cells in Ld-nOVA × DO11.10 mice...
and to the generation of CD4+CD25+ T cells in DO11.10 mice suggests that endogenous TCR gene rearrangement is used not only for the avoidance of autoreactivity, but also for the generation of CD4+CD25+ regulatory T cells, probably by creating autoreactive TCRs. To confirm this possibility, we generated Rag2-deficient DO11.10 mice and Rag2-deficient Ld-nOVA × DO11.10 mice, in which endogenous TCR gene rearrangement was impaired. Although the numbers of CD4+ T cells in spleens from Rag2-deficient Ld-nOVA × DO11.10 mice were reduced by clonal deletion, most of the CD4+ T cells were CD4+CD25+ T cells (Fig. 5A), supporting the idea that autoreactive T cells were selected as CD4+CD25+ T cells. We confirmed that these CD4+CD25+ T cells had the ability to suppress the proliferative responses of CD4+CD25+ T cells from nontransgenic BALB/c mice in vitro (Fig. 6). On the contrary, most of CD4+ T cells in spleens of Rag2-deficient DO11.10 mice were CD4+CD25- T cells and lacked CD4+CD25+ T cells. Furthermore, we generated TCR α-chain-deficient DO11.10 mice and TCR α-chain-deficient Ld-nOVA × DO11.10 mice. These mice had almost the same phenotype as Rag2-deficient DO11.10 mice and Rag2-deficient Ld-nOVA × DO11.10 mice, respectively (Fig. 5B). We also confirmed the suppressive activity of CD4+CD25+ T cells in TCR α-chain-deficient Ld-nOVA × DO11.10 mice (data not shown).

These results indicate that endogenous TCR expression, especially endogenous α-chain expression, plays an important role in the generation of regulatory T cells in DO11.10 mice.

Interestingly, although CD4 SP T cells in the thymus of Rag2-deficient Ld-nOVA × DO11.10 mice contained not only CD4+CD25- T cells, but also a large population of CD4+CD25- T cells, splenic CD4+ T cells were exclusively CD4+CD25+ T cells. This finding suggests that there might be another selection mechanism in the periphery that differentially acts on CD4+CD25+ regulatory T cells.

Taken together, these findings indicate that autoreactive T cells are selected as CD4+CD25+ regulatory T cells and that endogenous TCR gene rearrangement plays a critical role in the generation of CD4+CD25+ regulatory T cells from nonautoreactive T cells and in the generation of nonautoreactive T cells from autoreactive T cells.

Discussion

We found that autoreactive T cells were positively selected as CD4+CD25+ regulatory T cells in parallel with clonal deletion in the thymus. These results indicate that part of autoreactive T cells that have a high affinity TCR enough to lead to clonal deletion could be positively selected as CD4+CD25+ T cells in the thymus. We also found that endogenous TCR expression generates CD4+CD25+ regulatory T cells from the nonautoreactive T cells and nonautoreactive CD4+CD25- T cells from the autoreactive T cells.

In Sakaguchi and coworkers’ papers (7, 22), the regulatory T cells in TCR-transgenic models were endogenous TCR α-chain-expressing cells, whereas, in our Ld-nOVA × DO11.10 mice, the regulatory T cells did not express endogenous TCRs. However, these findings do not contradict each other. In the case of CD4+ T cells expressing high affinity TCRs with peripheral autoantigens or nonself ligands, these T cells could be positively selected as CD4+CD25+ T cells without clonal deletion or endogenous TCR chain expression. Because CD4+ T cells expressing TCRs specific for autoantigens in the thymus might be selected as CD4+CD25+ regulatory T cells, in transgenic mice expressing a TCR specific for an exogenous Ag such as DO11.10 mice, CD4+CD25+ regulatory T cells are selected only from CD4+ T cells expressing endogenous TCR chains. Therefore, the impairment of endogenous TCR chain expression led to the disappearance of regulatory T cells. On the contrary, in the case of transgenic mice expressing a TCR specific for a systemic autoantigen such as Ld-nOVA × DO11.10 mice, CD4+CD25+ regulatory T cells are selected from CD4+ T cells expressing a transgenic TCR without endogenous TCR chain expression.

Because endogenous TCR gene rearrangement occurs to escape not only negative selection (15, 16), but also death by neglect during positive selection (18, 19), it is suggested that endogenous TCR gene rearrangement occurs to generate T cells expressing TCRs that have appropriate affinity for a self MHC/self peptide above the selection threshold. Thus, there is a possibility that endogenous TCR gene rearrangement might play an important role in the generation of CD4+CD25+ T cells from nonautoreactive T cells through creating autospecific TCRs and the generation of CD4+CD25+ T cells from autoreactive CD4+ T cells. Therefore, it should be addressed whether second TCR gene rearrangement contributes to the generation of CD4+CD25+ T cells and CD4+CD25+ T cells in nontransgenic mice.

Our experiment revealed that CD4+CD25+ T cells contain a certain T cell repertoire specific for a systemic nuclear autoantigen. Although CD4+CD25+ T cells are anergic to TCR stimulation and suppress the activation of CD4+CD25+ T cells in an Ag-independent manner, it has been demonstrated that regulatory function of CD4+CD25+ T cells requires their activation via TCR in vitro (10–12). Autoreactivity of regulatory T cells increases the chance that they encounter their stimulators in periphery. Thus, it is rational that regulatory T cells are specific for autoantigens in the thymus, which may be systemic autoantigens.

Jordan et al. (13) demonstrated that CD4+CD25+ regulatory T cells are positively selected by a self peptide in the thymus. In their study, autoreactive T cells are positively selected as CD4+CD25+...
regulatory T cells and did not undergo clonal deletion in contrast to our transgenic models. The lack of deletion can probably be attributed either to a lower affinity TCR compared with our transgenic model or to the expression level of the self ligand. These findings suggest that CD4+CD25+ regulatory T cells comprise a broad autoreactive T cell repertoire.

![Figure 4](image)

**FIGURE 4.** Endogenous TCR chain expression in CD4+CD25+ T cells from DO11.10 mice and CD4+CD25+ T cells from LdnOVA × DO11.10 mice. A, CD4+CD25+ T cells were clonotype-low T cells in LdnOVA × DO11.10 mice, whereas CD4+CD25+ T cells were clonotype-high T cells in DO11.10 mice. CD8-depleted thymocytes and lymph node cells from a DO11.10 mouse and a LdnOVA × DO11.10 mouse were stained with anti-CD4, anti-CD25, and KJ1-26. Dot plots show staining of these cells with anti-CD4 and anti-CD25. The percentage of CD25+ cells in CD4+ cells is shown in each dot plot (the mean percentages are shown in Table I). Histograms of staining with KJ1-26 were gated for CD4+CD25+ cells and CD4+CD25- cells. The percentage of cells falling within the indicated marker is shown in each histogram. A representative result of seven independent similar experiments is shown. B, Clonotype-low T cells expressed endogenous Vβ chains. Lymph node cells from a DO11.10 mouse and a LdnOVA × DO11.10 mouse were stained with anti-CD4, anti-CD25, and KJ1-26, anti-Vβ8, or anti-TCRβ. Histograms of staining with KJ1-26, anti-Vβ8, or anti-TCRβ were gated for CD4+CD25+ cells and CD4+CD25- cells. The percentage of cells falling within the indicated marker is shown in each histogram. A representative result of seven independent similar experiments is shown. C, The percentages of T cells expressing endogenous Vβ chains in CD4+CD25+ T cells and CD4+CD25- T cells from the thymus and lymph nodes of DO11.10 mice and LdnOVA × DO11.10 mice are shown. Seven LdnOVA × DO11.10 mice and seven DO11.10 mice were analyzed. The mean percentage of T cells expressing endogenous Vβ chains was defined as the mean percentage of TCRβ-positive cells in CD4+ T cells minus the mean percentage of Vβ8-positive cells in CD4+ T cells. Statistical comparison is by Student’s t test (*, p < 0.01). DBL, LdnOVA × DO11.10 double-transgenic mice.

![Figure 5](image)

**FIGURE 5.** Perturbation of endogenous TCR gene rearrangement exclusively generated CD4+CD25+ T cells and CD4+CD25- T cells in LdnOVA × DO11.10 mice and DO11.10 mice, respectively. Thymocytes and splenocytes of RAG2-deficient LdnOVA × DO11.10 mice and RAG2-deficient DO11.10 mice were analyzed for the expression of CD4 and CD25. B, Thymocytes and splenocytes of TCR α-chain-deficient LdnOVA × DO11.10 mice and TCR α-chain-deficient DO11.10 mice were analyzed for the expression of CD4 and CD25. A representative result of three independent similar experiments is shown.
DO11.10 mice were regulatory T cells. CD4\(^{+}\)H11001 BALB/c mice and irradiated (20 Gy) syngeneic spleen cells (5 x 10\(^5\))

Our results provide new insight into the ontology of regulatory T cells. The thymus has the ability to generate regulatory T cells from autoreactive T cells simultaneously with negative selection, sometimes actively generating autoreactive T cells by endogenous TCR gene rearrangement. The critical contribution of endogenous TCR gene rearrangement to the control of autoreactivity is that it reduces self-reactivity in effector precursor T cells by generating autoreactive CD4\(^{+}\)T cells in RAG2-deficient mice. Means and SDs of triplicate are shown. The data were representative of three independent similar experiments.

FIGURE 6. CD4\(^{+}\)CD25\(^{+}\) T cells in RAG2-deficient Ld-nOVA \(\times\) DO11.10 mice were regulatory T cells. CD4\(^{+}\)CD25\(^{+}\) T cells (2 x 10\(^6\)) from RAG2-deficient Ld-nOVA \(\times\) DO11.10 mice were cultured with CD4\(^{+}\)CD25\(^{+}\) T cells (2 x 10\(^6\)) from nontransgenic BALB/c mice. Means and SDs of triplicate are shown. The data were representative of three independent similar experiments. DBL, Ld-nOVA \(\times\) DO11.10 double-transgenic mice.

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References


