# CD4$^+$CD25$^+$ T-cell development is regulated by at least 2 distinct mechanisms

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It has recently been shown that CD4$^+$CD25$^+$ T cells are immunoregulatory T cells that prevent CD4$^+$ T-cell–mediated organ-specific autoimmune diseases. In this study, the regulatory mechanism of CD4$^+$CD25$^+$ T-cell development were investigated using T-cell receptor (TCR) transgenic mice. It was found that CD4$^+$CD25$^+$ T cells preferentially expressed the endogenous TCRα chain in DO10$^+$ TCR transgenic mice compared with CD4$^+$CD25$^-$ T cells. Moreover, it was found that CD4$^+$CD25$^+$ thymocytes were severely decreased in DO10$^+$ TCR-α$^{-/-}$ mice in positively selecting and nega-tively selecting backgrounds, whereas CD4$^+$CD25$^-$ thymocytes efficiently developed by transgenic TCR in DO10$^+$ TCR-α$^{-/-}$ mice in positively selecting backgrounds, indicating that the appropriate affinity of TCR to major histocompatibility complex (MHC) for the development of CD4$^+$CD25$^+$ thymocytes is different from that of CD4$^+$CD25$^-$ thymocytes and that a certain TCR–MHC affinity is required for the development of CD4$^+$CD25$^+$ thymocytes. Finally, it was found that, in contrast to thymus, CD4$^+$CD25$^+$ T cells were readily detected in spleen of DO10$^+$ TCR-α$^{-/-}$ mice in positively selecting backgrounds and that splenic CD4$^+$CD25$^+$ T cells, but not CD4$^+$CD25$^+$ thymocytes, were significantly decreased in B-cell–deficient mice, suggesting that B cells may control the peripheral pool of CD4$^+$CD25$^+$ T cells. Together, these results indicate that the development of CD4$^+$CD25$^+$ T cells in thymus and the homeostasis of CD4$^+$CD25$^+$ T cells in periphery are regulated by distinct mechanisms. (Blood. 2002;99:555-560)

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## Introduction

Immunologic tolerance is a feature of the immune system essential for discrimination between self and nonself. Recent data suggest that in addition to clonal deletion and anergy, regulatory T cells play a significant role in the generation and maintenance of tolerance.[1-4] The term regulatory T cells is used for a variety of immunoregulatory cells that can be subdivided into a number of subsets based on expression of cell surface proteins and pattern of cytokine production.[1-4] Because these subsets of regulatory T cells have been characterized in experimental models using different assays, the interrelationship between the subsets is difficult to understand.[1-4]

One of the best-characterized subsets of CD4$^+$ regulatory T cells is defined by its constitutive expression of interleukin-2 receptor (IL-2R)–α chain (CD25) (CD4$^+$CD25$^+$ T cells).[3,4] CD4$^+$CD25$^+$ T cells constitute approximately 10% of peripheral CD4$^+$ T cells in nonimmunized naive mice and exhibit a broad spectrum of autoimmunity-preventive activity.[5-8] CD4$^+$CD25$^+$ T cells are naturally anergic and, on T-cell receptor (TCR)–mediated activation, potently suppress the proliferation of CD4$^+$CD25$^-$ T cells by an antigen-nonspecific mechanism.[9-11] Molecular mechanisms by which CD4$^+$CD25$^+$ T cells mediate suppression are unclear but seem to be independent of cytokine production[9,10] and dependent on cell contact[9,10] and to require constitutive expression of CTLA-4.[12,13]

CD4$^+$CD25$^+$ T cells can be subdivided by the expression pattern of CD45RB$^{high}$ or CD45RB$^{low}$, CD38$^+$ or CD38$^-$, CD69$^+$ or CD69$^-$, or CD62L$^{high}$ or CD62L$^{low}$.[5,9,11,14] Although these findings suggest that CD4$^+$CD25$^+$ T cells might be heterogeneous and composed of a mixture of regulatory T cells and activated conventional T cells, it has recently been shown that the anergic and suppressive properties of CD4$^+$CD25$^+$ T cells cannot be subdivided to a smaller subpopulation defined by the expression levels of CD45RB, CD62L, CD38, or CD69.[11,14] Therefore, CD4$^+$CD25$^+$ T cells may represent a relatively homogeneous population of regulatory T cells.

Precise signals that promote the development of CD4$^+$CD25$^+$ T cells remain elusive, but considerable evidence suggests that costimulatory molecules and cytokines play important roles. It has been shown that CD4$^+$CD25$^+$ T cell levels are severely decreased in mice lacking CD28[8] or CD40L,[15] indicating that signaling by CD28 and by CD40L regulates the number of CD4$^+$CD25$^+$ T cells in periphery. In addition, CD4$^+$CD25$^+$ T-cell levels are decreased in mice lacking IL-2[16] or IL-2R component,[17] indicating that IL-2 signaling also regulates the number of CD4$^+$CD25$^+$ T cells.

The CD25$^+$ population constitutes approximately 5% of CD4$^+$CD8$^-$CD3$^{high}$ mature thymocytes in normal mice (CD4$^+$CD25$^+$ thymocytes), and the CD4$^+$CD25$^+$ thymocytes exhibit a similar functional property to that found in peripheral CD4$^+$CD25$^+$ T cells.[18] Moreover, thymectomy on day 3 of life decreases the number of CD4$^+$CD25$^+$ T cells from the peripheral lymphoid organ.[6] Furthermore, CD4$^+$CD25$^+$ T cells cannot be produced by in vitro culture of CD4$^+$CD25$^-$ T cells.[7,9,14] Taken together, these findings suggest that CD4$^+$CD25$^+$ T cells arise in the thymus and migrate to the periphery.

Recently, Itoh et al[18] have shown that CD4$^+$CD25$^+$ T cells can develop in TCR transgenic mice of wild-type background but not of RAG-2–deficient background. Thus, it is possible that B cells, γδ T cells, or natural killer (NK) T cells, all of which are absent in TCR transgenic RAG-2–deficient mice but are present in TCR transgenic mice, are required for the development of CD4$^+$CD25$^+$ T cells. It is also possible that the expression of endogenous TCR and the subsequent interaction with self-major histocompatibility complex (MHC) at a certain affinity is required for the development of these cells. However, a great deal of uncertainty remains about differentiation factors and antigen specificity of CD4$^+$CD25$^+$ T cells.

In the current study, to determine whether positive or negative selection in thymus regulates the development of CD4$^+$CD25$^+$ T cells, we investigated CD4$^+$CD25$^+$ T-cell development in mice expressing TCR transgene in positively selecting and negatively selecting MHC backgrounds. We also investigated the requirement of B cells and NK T cells for the development of CD4$^+$CD25$^+$ T cells. Our results indicate that the size of the CD4$^+$CD25$^+$ T-cell pool in periphery is regulated at least 2 distinct levels. First, in the thymus, where CD4$^+$CD25$^+$ T cells are produced, a certain range of TCR–MHC affinity is required for their development. In addition, B cells, but not NK T cells, regulate the expansion and/or survival of CD4$^+$CD25$^+$ T cells in periphery.

## Materials and methods

### Mice

BALB/c and C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Immunoglobulin μ-chain–deficient mice[19] and NK T-cell–deficient mice[20] of C57BL/6 background were previously described. Ovalbumin-specific DO11.10 (DO10$^+$) TCR transgenic mice[21] were backcrossed to BALB/c mice for more than 10 generations. BALB/c RAG-2$^{-/-}$ mice[22] were a kind gift from Dr T. Saito (Chiba University). TCR-α$^{-/-}$ mice[23] (H-2$^{b/b}$) were crossed with DO10$^+$ mice (H-2$^{d/d}$), and then offspring DO10$^+$ TCR-α$^{+/-}$ H-2$^{d/b}$ mice were crossed with TCR-α$^{+/-}$ H-2$^{d/b}$ mice to develop DO10$^+$ TCR-α$^{-/-}$ mice in H-2$^{d/d}$, H-2$^{d/b}$, or H-2$^{b/b}$ background. Expressions of DO10$^+$ transgene and H-2 haplotypes were determined by fluorescence-activated cell sorter analysis (FACS), as described below. The genotype of TCR-α allele was determined by polymerase chain reaction using the following primer pairs: to detect TCR-α wild-type allele, 5′-aagatcctcggtctcaggacagc-3′ and 5′-ggtaggtggcgttggtctctttg-3′; to detect TCR-α mutant allele, 5′-attcgcagcgcatcgccttctatcg-3′ and 5′-ggtaggtggcgt-tggtctctttg-3′. Homozygosity for the TCR-α mutant allele was confirmed, using FACS, by the absence of T cells that express endogenous TCR V-α2. Mice were housed in micro-isolator cages under pathogen-free conditions; 8- to 10-week-old mice were used in all experiments.

### Flow cytometric analysis

Cells from thymus and spleen were stained and analyzed on a FACScalibur (Becton Dickinson, San Jose, CA) using CELLQuest software. For direct staining, the following conjugated antibodies were purchased from PharMingen (San Diego, CA): anti-CD3 fluorescein isothiocyanate (FITC) (145-2C11), anti-CD4 FITC, phycoerythrin (PE), PerCP, allophycocyanin (APC) (H129.19), anti-B220 FITC (RA3-6B2), anti-CD8 FITC, APC (53.6.7), anti-CD25 PE (PC61), anti-CD25 FITC (7D4), anti-CD45RB FITC (16A), anti–NK-1.1 PE (PK136), anti-TCR V-α2 FITC (B20.1), anti-TCR V-α11 FITC (RR8-1), anti-TCR Vβ8 FITC (F23.1), anti–H-2K$^b$ PE (AF6-88.5), anti–H-2K$^d$ FITC (SF1-1.1), anti–I-A$^b$ PE (AF6-120.1), and anti–I-A$^d$ FITC (AMS-32.1). KJ1-26 monoclonal antibody, anti-idiotype for DO10 TCR,[24] was purified from supernatants of hybridoma cells using protein G columns (Pharmacia, Uppsala, Sweden) and conjugated to FITC or biotin. Before staining, Fc receptors were blocked with anti-CD16/32 antibody (2.4G2; PharMingen).

### Annexin V staining

After cells were stained with anti-CD4 APC and anti-CD25 PE and washed twice with phosphate-buffered saline–1% bovine serum albumin, cells were stained with annexin V–FITC (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cells were then analyzed on a FACScalibur.

### Data analysis

Data are summarized as mean ± SD. Statistical analysis of the results was performed by the unpaired $t$ test. $P < .05$ was considered significant.

## Results

### CD4$^+$CD25$^+$ T cells preferentially express the second TCR-α chain in TCR transgenic mice

It is well established that allelic exclusion of the TCR-β chain is almost perfect, whereas that of the TCR-α chain is incomplete.[25] Thus, a considerable fraction of T cells express 2 different TCR-αβ pairs.[26] Because it has been shown that CD4$^+$CD25$^+$ T cells can develop in TCR transgenic mice of wild-type background but not of RAG-2–deficient background,[18] it is possible that CD4$^+$CD25$^+$ T cells preferentially express the endogenous TCR-α chain and that signaling through the endogenous TCR-α chain coupled with the transgenic TCR-β chain is involved in the development of CD4$^+$CD25$^+$ T cells in TCR transgenic mice. To examine this possibility, we first investigated the frequency of CD4$^+$ T cells that express both transgenic TCR (recognized by anti-idiotypic monoclonal antibody, KJ1-26) and one of the endogenous TCR V-α chains, V-α2, in the CD25$^+$ or CD25$^-$ population in ovalbumin-specific TCR transgenic (DO10$^+$) mice. As shown in Figure 1A, splenic CD4$^+$ T cells that express transgenic TCR and TCR V-α2 chain were found at a higher frequency in CD4$^+$CD25$^+$ T cells than in CD4$^+$CD25$^-$ T cells (CD4$^+$CD25$^+$ T cells 12.0% ± 1.6% versus CD4$^+$CD25$^-$ T cells 2.5% ± 1.0%, mean ± SD; n = 5; $P < .001$). Moreover, CD4$^+$ T cells that express both transgenic TCR and TCR V-α11 were also found at a higher frequency in CD4$^+$CD25$^+$ T cells (data not shown). These results suggest that CD4$^+$CD25$^+$ T cells preferentially express the second TCR-α chain in DO10$^+$ mice. For controls, we performed the same
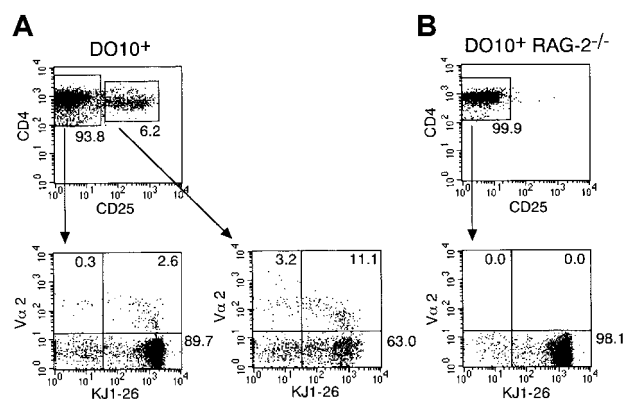


**Figure 1. CD4$^+$CD25$^+$ T cells preferentially express the second TCRα chain in DO10$^+$ TCR transgenic mice.** Single-cell suspension of splenocytes from DO10$^+$ mice (A) and DO10$^+$ RAG-2$^{-/-}$ mice (B) were stained with anti-TCR Vα2 FITC, anti-CD25 PE, anti-CD4 PerCP, and KJ1-26 biotin. After biotinylated antibody was visualized with streptavidin APC, cells were analyzed on FACScalibur. Shown are representative FACS profiles of CD4 versus CD25 of CD4$^+$ splenocytes (upper panels) and KJ1-26 versus Vα2 (lower panels) on either CD4$^+$CD25$^+$ T cells or CD4$^+$CD25$^-$ T cells from 5 mice in each group.

analysis for splenocytes in DO10+ RAG-2–deficient mice. We found that, consistent with the previous report by Itoh et al,[18] CD4+CD25+ T cells were almost absent in these mice (Figure 1B) and that no CD4+CD25− T cell expressed TCR V-α2 (Figure 1B) or TCR V-α11 (data not shown) in these mice.

### Dual TCR-α expression is not essential for the development of CD4+CD25+ T cells

CD4+CD25+ T cells preferentially expressed dual TCR-α chains in DO10+ mice (Figure 1A). To determine whether dual TCR-α expression is required for the development of CD4+CD25+ T cells, we investigated the development of CD4+CD25+ T cells in TCR-α heterozygous mice, in which only one allele of TCR-α was available for the expression. As shown in Figure 2, the number of CD4+CD25+ T cells in thymus and in spleen was normal in TCR-α heterozygous mice, suggesting that though CD4+CD25+ T cells preferentially expressed the second TCR-α chain in TCR transgenic mice, dual TCR-α expression was not essential for the development of CD4+CD25+ T cells.

### CD4+CD25+ thymocytes are severely decreased in DO10+ TCR-α−/− mice in positively selecting and negatively selecting backgrounds

Because CD4+CD25+ T cells are absent in DO10+ RAG-2−/− mice (Figure 1B) and because CD4+CD25+ T cells preferentially express the second TCR-α chain in DO10+ mice (Figure 1A), the expression of endogenous TCR-α chain and subsequent interaction with self-MHC at an appropriate affinity may be required for the development of CD4+CD25+ T cells. To test this possibility, we investigated the development of CD4+CD25+ T cells in DO10+ TCR-α–deficient (TCR-α−/− ) mice (Figure 3). Indeed, the frequency of the CD25+ population in CD4+CD8− mature thymocytes was severely decreased in DO10+ TCR-α−/− mice with positively selecting H-2$^d$ background compared with that in DO10+ TCR-α+/+ mice (Figure 3B). In contrast, no significant difference was observed in the number of conventional CD4+CD25− mature thymocytes between DO10+ TCR-α−/− mice and DO10+
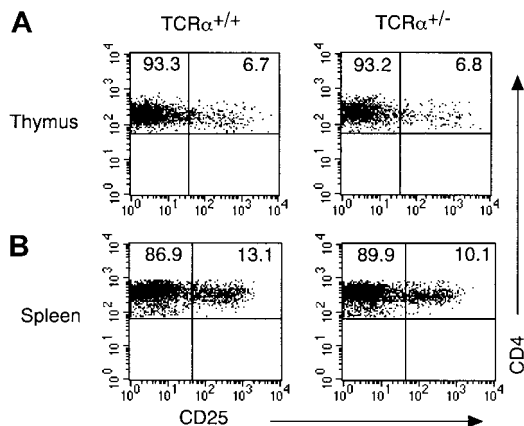


**Figure 2. Dual TCRα expression is not essential for the development of CD4+CD25+ T cells.** (A) Thymocytes from TCRα+/− mice and TCRα+/+ mice were stained with anti-CD3 FITC, anti-CD25 PE, anti-CD8 APC, and anti-CD4 PerCP. Shown are representative FACS profiles of CD4 versus CD25 on a CD4+ CD8−CD3$^{high}$ population. FACS profile of CD4 versus CD8 and cell number of thymocytes was indistinguishable between TCRα+/− mice and TCRα+/+ mice (data not shown). (B) Splenocytes from TCRα+/− mice and TCRα+/+ mice were stained with anti-CD25 PE and anti-CD4 FITC. Shown are representative FACS profiles of CD4 versus CD25 on the CD4+ population from 5 mice in each group.
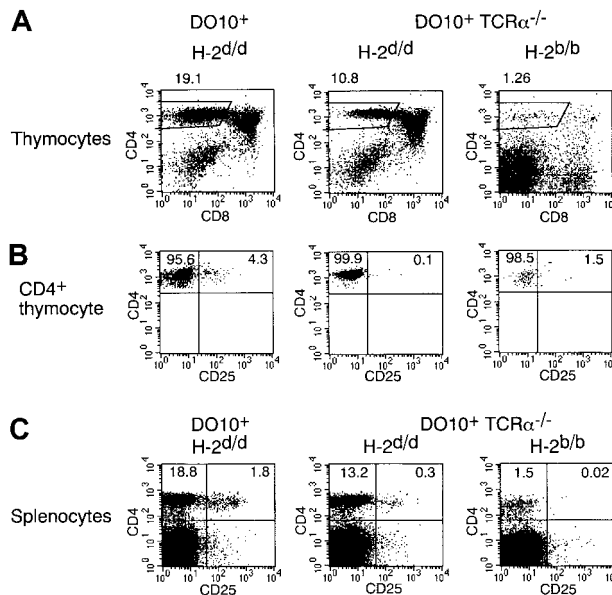


**Figure 3. CD4+CD25+ thymocytes are severely decreased in DO10+ TCRα−/− mice in positively selecting and negatively selecting backgrounds.** (A, B) Thymocytes from DO10+ mice (H-2$^{d/d}$), DO10+ TCRα−/− mice (H-2$^{d/d}$), and DO10+ TCRα−/− mice (H-2$^{b/b}$) were stained with KJ1-26 FITC, anti-CD25 PE, anti-CD8 APC, and anti-CD4 PerCP. Shown are representative FACS profiles of CD4 versus CD8 on total thymocytes (A) and CD4 versus CD25 on CD4+ CD8− KJ1-26$^{high}$ thymocytes (B) from 5 mice in each group. The numbers of thymocytes are as follows: DO10+ TCRα+/+ H-2$^{d/d}$ mice (10.8 ± 2.6 × 10⁷), DO10+ TCRα−/− H-2$^{d/d}$ mice (13.2 ± 3.0 × 10⁷), and DO10+ TCRα−/− H-2$^{b/b}$ mice (0.6 ± 0.2 × 10⁷) (n = 5 mice each; $P < .01$). (C) Splenocytes from DO10+ H-2$^{d/d}$ mice, DO10+ TCRα−/− H-2$^{d/d}$ mice, and DO10+ TCRα−/− H-2$^{b/b}$ mice were stained with anti-CD25 PE and anti-CD4 FITC. Shown are representative FACS profiles of CD4 versus CD25 on splenocytes from 5 mice in each group.

TCR-α+/+ mice (Figure 3B). These results indicate that expression of the endogenous TCR-α chain is required for the development of CD4+CD25+ T cells in DO10+ mice. In addition, they indicate that the appropriate affinity of TCR to self-MHC for the development of CD4+CD25+ thymocytes is different from that of CD4+CD25− thymocytes and that the interaction of TCR with self-MHC at a certain affinity is essential for the development of CD4+CD25+ thymocytes.

We next analyzed CD4+CD25+ T-cell development by transgenic TCR in DO10+ mice in a negatively selecting H-2$^b$ background.[27,28] For this purpose, we investigated DO10+ TCR-α−/− mice to exclude the effect of endogenous TCR-α expression. Interestingly, the number of total thymocytes in DO10+ TCR-α−/− mice was severely decreased in H-2$^b$ background (DO10+ TCR-α−/− H-2$^b$ mice compared with DO10+ TCR-α−/− H-2$^d$ mice; 0.6 ± 0.2 × 10⁷ versus 13.2 ± 3.0 × 10⁷; n = 5 each; $P < .001$). Most thymocytes were within the CD4−CD8− stage (Figure 3A); consequently, the number of CD4+CD8− thymocytes was severely decreased in these mice. When gated on the CD4+CD8− thymocytes that could escape from negative selection in DO10+ TCR-α−/− mice in H-2$^b$ background, the frequency of the CD25+ population was still severely decreased (Figure 3B). These results suggest that CD4+CD25+ T cells could not develop when the affinity between TCR and MHC was so high.

Interestingly, though the number of CD4+CD25+ thymocytes was severely decreased in DO10+ TCR-α−/− mice, CD4+CD25+ T cells were readily observed in the spleens of DO10+ TCR-α−/− mice in H-2$^d$ background (Figure 3C). The population of CD4+CD25+ T cells was also approximately 2-fold in the spleens

of DO10^{+} TCR-α^{+/+} mice compared with that of CD4^{+}CD25^{+} thymocytes (Figure 3B-C). Most splenic CD4^{+}CD25^{+} T cells in DO10^{+} TCR-α^{−/−} mice and DO10^{+} TCR-α^{+/+} mice were of the CD45RB^{low} CD69^{−} phenotype, similar to that in wild-type mice (data not shown). These results suggest that there existed some homeostatic machinery that regulated the size of the CD4^{+}CD25^{+} T-cell pool in periphery.

## B cells but not natural killer T cells regulate the peripheral pool of CD4^{+}CD25^{+} T cells

CD4^{+}CD25^{+} T cells exist in the spleens of DO10^{+} TCR-α^{−/−} H-2[d] mice (Figure 3C) but not in spleens of DO10^{+} RAG-2^{−/−} mice (Figure 1B). Thus, it is possible that a certain population of cells absent in RAG-2–deficient mice regulates the size of the CD4^{+}CD25^{+} T-cell pool in periphery. The possibility that B cells might affect CD4^{+}CD25^{+} T-cell development was examined by flow cytometric analyses of CD4^{+}CD25^{+} T cells in μ-chain–deficient mice, which lack mature B cells.[19] Interestingly, a profound decrease of splenic CD4^{+}CD25^{+} T cells was observed in μ-chain–deficient mice (Figure 4A). Less than 7% of CD4^{+} T cells in μ-chain–deficient mice expressed CD25 compared with 13% in wild-type mice (n = 5 each) (Figure 4A). Because the absolute number of splenic T cells was decreased in μ-chain–deficient mice, the number of splenic CD4^{+}CD25^{+} T cells was significantly decreased in μ-chain–deficient mice (approximately 20% of wild-type levels; $P < .001$; Figure 4B). Interestingly, in contrast to splenic CD4^{+}CD25^{+} T cells, the number of CD25^{+} population in CD4^{+} CD8^{−} mature thymocytes was normal in μ-chain–deficient mice (Figure 5). Taken together, these results indicate that though the development of CD4^{+}CD25^{+} T cells in thymus does not require help from B cells, the size of CD4^{+}CD25^{+} T-cell pool in periphery is regulated by B cells. In contrast, the number of splenic CD4^{+}CD25^{+} T cells (Figure 4) and CD4^{+}CD25^{+} thymocytes (Figure 5) was normal in NK T cell-deficient mice, indicating that NK T cells are not essential for the development of CD4^{+}CD25^{+} T cells.
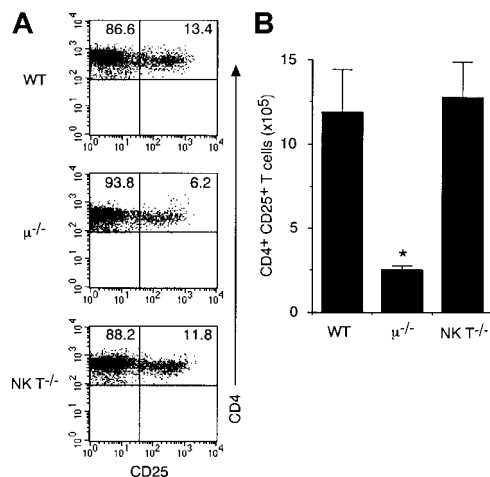


**Figure 5. CD4^{+}CD25^{+} thymocytes can develop normally in μ-chain–deficient mice and NK T-cell–deficient mice.** Thymocytes from μ-chain–deficient mice, NK T-cell–deficient mice, and wild-type (WT) mice were stained with anti-CD3 FITC, anti-CD25 PE, anti-CD8 APC, and anti-CD4 PerCP. Shown are representative FACS profiles of CD4 versus CD25 on CD4^{+}CD8^{−}CD3^{high} thymocytes from 4 to 5 mice in each group. FACS profile of CD4 versus CD8 and cell number of thymocytes was indistinguishable among these mice (data not shown).

# Discussion

In this study, we show that the size of the peripheral CD4^{+}CD25^{+} T-cell pool is regulated by at least 2 distinct mechanisms. First, in the thymus, where CD4^{+}CD25^{+} T cells are produced, a certain TCR–MHC affinity is required for the development of CD4^{+}CD25^{+} T cells. We found that CD4^{+}CD25^{+} T cells preferentially expressed the second TCR-α chain in DO10^{+} TCR transgenic mice (Figure 1A) and that the development of CD4^{+}CD25^{+} thymocytes was severely impaired in DO10^{+} TCR-α^{−/−} mice (Figure 3B), indicating that the expression of the endogenous TCR-α chain is required for the development of CD4^{+}CD25^{+} T cells in TCR transgenic mice. Moreover, we found that CD4^{+}CD25^{+} thymocytes were severely decreased in DO10^{+} TCR-α^{−/−} mice in the positively selecting background and the negatively selecting background, whereas CD4^{+}CD25^{−} thymocytes effectively developed by transgenic TCR in DO10^{+} TCR-α^{−/−} mice in the positively selecting background (Figure 3B). Taken together, these results indicate that the appropriate affinity of TCR to self-MHC for the development of CD4^{+}CD25^{+} thymocytes is different from that of CD4^{+}CD25^{−} thymocytes and that the interaction of TCR with self-MHC at a certain affinity is essential for the development of CD4^{+}CD25^{+} thymocytes. Second, in the periphery, B cells regulate the expansion and/or survival of the developed CD4^{+}CD25^{+} T cells. We found that, in contrast to the thymus, CD4^{+}CD25^{+} T cells were readily detected in the spleens of DO10^{+} TCR-α^{−/−} mice in positively selecting background (Figure 3C), suggesting that a small number of CD4^{+}CD25^{+} T cells that developed in the thymus might have expanded in the periphery. In addition, we found that the number of splenic CD4^{+}CD25^{+} T cells, but not of CD4^{+}CD25^{+} thymocytes, was decreased in B-cell–deficient mice (Figures 4, 5). Therefore, B cells may control the peripheral pool of CD4^{+}CD25^{+} T cells, possibly by inducing the expansion or prolonging the survival of these cells.

Although it is accepted that CD4^{+}CD25^{+} T cells need activation by TCR for regulatory function, the antigen specificity of CD4^{+}CD25^{+} T cells is unknown. We found that CD4^{+}CD25^{+} thymocytes were severely decreased in DO10^{+} TCR-α^{−/−} mice in negatively selecting H-2[b] background (Figure 3). Moreover, we found that Mtv-9–induced clonal deletion of TCR V-β5 cells proceeded normally in CD4^{+}CD25^{+} T cells in BALB/c mice (data not shown). Furthermore, Papiernik et al[29] have shown that Mls-1[a]–induced clonal deletion of TCR V-β6 cells is also normal in CD4^{+}CD25^{+} T cells. These findings indicate that CD4^{+}CD25^{+} T cells are not resistant to clonal deletion in thymus. Recently, an altered negative-selection model of CD4^{+}CD25^{+} T-cell development was proposed.[3] In this model, the suboptimal stimulation of thymocytes with low-affinity self-antigens would result not in



**Figure 4. Splenic CD4^{+}CD25^{+} T cells are decreased in μ-chain–deficient mice but not in NK T-cell–deficient mice.** Splenocytes from μ-chain–deficient mice, NK T-cell–deficient mice, and wild-type (WT) mice were stained with anti-CD25 PE and anti-CD4 FITC. Shown are representative FACS profiles of CD4 versus CD25 on the CD4^{+} population (A) and the absolute number of CD4^{+}CD25^{+} T cells in spleen (B) from 4 to 5 mice in each group. *Significantly different from the mean value of wild-type mice; *$P < .001$.
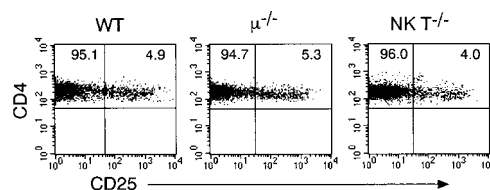
deletion but in a permanent change in TCR signaling. Although we could not identify the TCR–MHC affinity that specifically induced the development of CD4+CD25+ T cells, identification will extend the understanding of the nature of CD4+CD25+ T cells.

Although the expression of dual TCR-α chain is not essential for the development of CD4+CD25+ T cells (Figure 2), CD4+CD25+ T cells preferentially express the second TCR-α chain in DO10+ mice (Figure 1). In addition, though transgenic TCR-α expression is not sufficient for the development of CD4+CD25+ T cells in DO10+ mice (Figure 3), the ligation of transgenic TCR has been shown to be sufficient for the regulatory function of CD4+CD25+ T cells.[10,11] These findings suggest that both TCRs on dual TCR-α–expressing cells are functional for their regulatory activity of CD4+CD25+ T cells. Therefore, dual TCR-α chain expression may increase the chance for TCR signaling of CD4+CD25+ T cells.

We found that B cells played a significant role in the regulation of the CD4+CD25+ T-cell pool in the spleen (Figure 4) but not in the development of CD4+CD25+ T cells in the thymus (Figure 5). Because it has been shown that B7/CD28 interaction[8] and CD40/CD40L interaction[15] regulate the CD4+CD25+ T-cell pool in periphery, B cells may regulate the CD4+CD25+ T-cell pool through B7/CD28 interaction or CD40/CD40L interaction. Recently, it has been shown that the number of dividing cells is modestly increased in CD4+CD25+ T cells compared with that in CD4+CD25− T cells in periphery.[29] Moreover, we found that apoptotic cells, which were assessed as annexin V binding cells, were not increased in CD4+CD25+ T cells (data not shown). Thus, it is likely that B cells regulate the cell expansion of CD4+CD25+ T cells in periphery.

Another subset of CD4+ regulatory T cells, isolated after T cells were activated with alloantigens in the presence of IL-10, was termed type 1 T regulatory (Tr1) cells.[30] Tr1 cells are distinct from classical Th1 or Th2 cells in that they produce large amounts of IL-10 and moderate amounts of transforming growth factor-β.[30] Significantly, Tr1 cells suppress immune responses in vitro and in vivo through a mechanism dependent on the production of the immunoregulatory cytokine IL-10.[31] In contrast, the regulatory function of CD4+CD25+ T cells is independent of IL-10.[9] Although it is still possible that they are in fact the same subset of regulatory T cells in different stages of differentiation, the observation that CD4+CD25+ T cells cannot be differentiated from naive CD4+CD25− T cells in vitro,[7,9,14] whereas Tr1 cells can be differentiated from naive cells,[30] favors the hypothesis that CD4+CD25+ T cells and Tr1 cells are 2 distinct regulatory T cells with similar functions. Moreover, though NK T cells have been shown to be involved in the development of IL-10–dependent regulatory T cells,[32] NK T cells were not required for the development of CD4+CD25+ T cells (Figures 4, 5). These observations also support the above hypothesis.

In the past 10 years, a great deal has been learned about the regulation of the CD25 gene. It is known that the CD25 gene has at least 3 important elements for regulation, denoted positively regulatory regions (PRR) I, II, and III.[33] NF-κB and SRF bind PRRI, Elf-1 and HMG-I (Y) bind PRRII, and Stat5 and Elf-1 bind PRRIII.[33] Although PRRI is essential for mitogen–antigen-mediated induction of the gene and PRRIII is essential for IL-2–induced gene expression, PRRII is essential for transcription in response to either mitogen or IL-2. Thus, the CD25 gene can be controlled not only by activation-dependent signals (NF-κB and Stat5) but also by lineage-specific signals (Elf-1). Consistent with these in vitro findings, we found that the CD4+CD25+ T cells were decreased in both DO10+ TCR-α−/− mice (Figure 3) and DO10+ Stat5a−/− mice,[34] suggesting that both TCR-mediated signaling and Stat5-mediated signaling are required for the development of CD4+CD25+ T cells in vivo. More detailed mechanisms will be uncovered by promoter analyses of the CD25 gene in CD4+CD25+ T cells.

In summary, we have shown that the peripheral pool of CD4+CD25+ T cells is regulated at least 2 distinct levels. First, a certain TCR–MHC affinity is required for the development of CD4+CD25+ T cells in thymus. Second, B cells are required for the expansion of CD4+CD25+ T cells in periphery. The identification of TCR–MHC affinity that preferentially induces the development of CD4+CD25+ T cells will extend our understanding of the nature of CD4+CD25+ T cells.

*Note added in proof.*   After our submission of this paper to *Blood,* Jordan et al reported that selection of CD4+CD25+ thymocytes required a TCR with high affinity for a self-peptide.[35]

## Acknowledgments

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