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Article

Control of Immune Responses by Antigen-Specific Regulatory T Cells Expressing the Folate Receptor

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SUMMARY

Immune responses can be enhanced or dampened by differential manipulation of Foxp3-expressing $CD25^+CD4^+$ natural regulatory T (Treg) cells versus other naive or activated T cells. By searching for a molecule capable of distinguishing these populations, we here found that natural Treg cells constitutively expressed high amounts of folate receptor 4 (FR4). The expression of FR4 and CD25 also separated antigen-stimulated $CD4^+$ non-Treg cells into the $FR4^{hi}CD25^-$ and $FR4^{lo}CD25^+$ populations, which were different in proliferation and cytokine secretion upon restimulation. These distinctions showed that antigenic stimulation activated and expanded antigen-specific natural Treg cells as well as effector and memory T cells. Accordingly, $FR4^{hi}CD25^+CD4^+$ T cells enriched from alloantigen-stimulated T cells suppressed graft rejection. Administration of FR4 monoclonal antibody specifically reduced Treg cells, provoking effective tumor immunity in tumor-bearing animals, whereas similar treatment of normal young mice elicited autoimmune disease. Thus, specific manipulation of $FR4^{hi}CD25^+CD4^+$ Treg cells helps control ongoing immune responses.

INTRODUCTION

The immune system endogenously produces a $CD4^+$ T cell subpopulation that is highly specialized to suppress aberrant or excessive immune responses (Sakaguchi, 2000). A cardinal feature of such $CD4^+$ naturally occurring regulatory T (Treg) cells is that the majority of them constitutively express CD25 (the interleukin-2 receptor α chain) and the transcription factor Foxp3 that specifically controls their development and function (Fontenot and Rudensky, 2005; Malek and Bayer, 2004; Sakaguchi, 2004). The Foxp3-expressing $CD25^+CD4^+$ Treg cells are apparently reactive with a broad spectrum of self and nonself antigens and able to expand upon in vivo and in vitro strong antigenic stimulation (Hsieh et al., 2004; Jordan et al., 2001, Nishimura et al., 2004). They are engaged in suppressing the development of immunological diseases such as autoimmune disease and allergy. They can also be exploited to establish transplantation tolerance through their antigen-specific expansion and to provoke effective immunity against autologous tumor cells or enhance immune responses to invading microbes through their reduction in number or attenuation of their suppressive activity.

An important feature of natural Treg cells is that they are phenotypically in an activated or antigen-primed state (Sakaguchi, 2004). This makes it difficult to phenotypically distinguish natural Treg cells from other activated effector or memory T cells. To further determine the role of natural Treg cells in controlling immune responses, it is therefore necessary to identify a cell-surface molecule that can specifically distinguish them from other T cells, in particular from activated effector or memory T cells. There are several cell-surface molecules that are predominantly expressed in natural Treg cells. CD25, for example, is indispensable for the maintenance of natural Treg cells as an essential component of the high-affinity interleukin-2 (IL-2) receptor because they are highly dependent on exogenous IL-2 for their peripheral survival (D'Cruz and Klein, 2005; Fontenot et al., 2005; Setoguchi et al., 2005). Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor family-related gene or protein (GITR) are expressed constitutively and at high amounts in natural Treg cells and involved in Treg cell-mediated suppression (McHugh et al., 2002; Read et al., 2000; Salomon et al., 2000; Shimizu et al., 2002; Takahashi et al., 2000). Although these molecules are useful for operationally differentiating natural Treg cells from other T cells in immunologically naive animals, they are unable to fully distinguish natural Treg cells from activated T cells because every T cell expresses them upon activation (Ono et al., 2006).

We show in this report that natural Treg cells constitutively express high amounts of the folate receptor

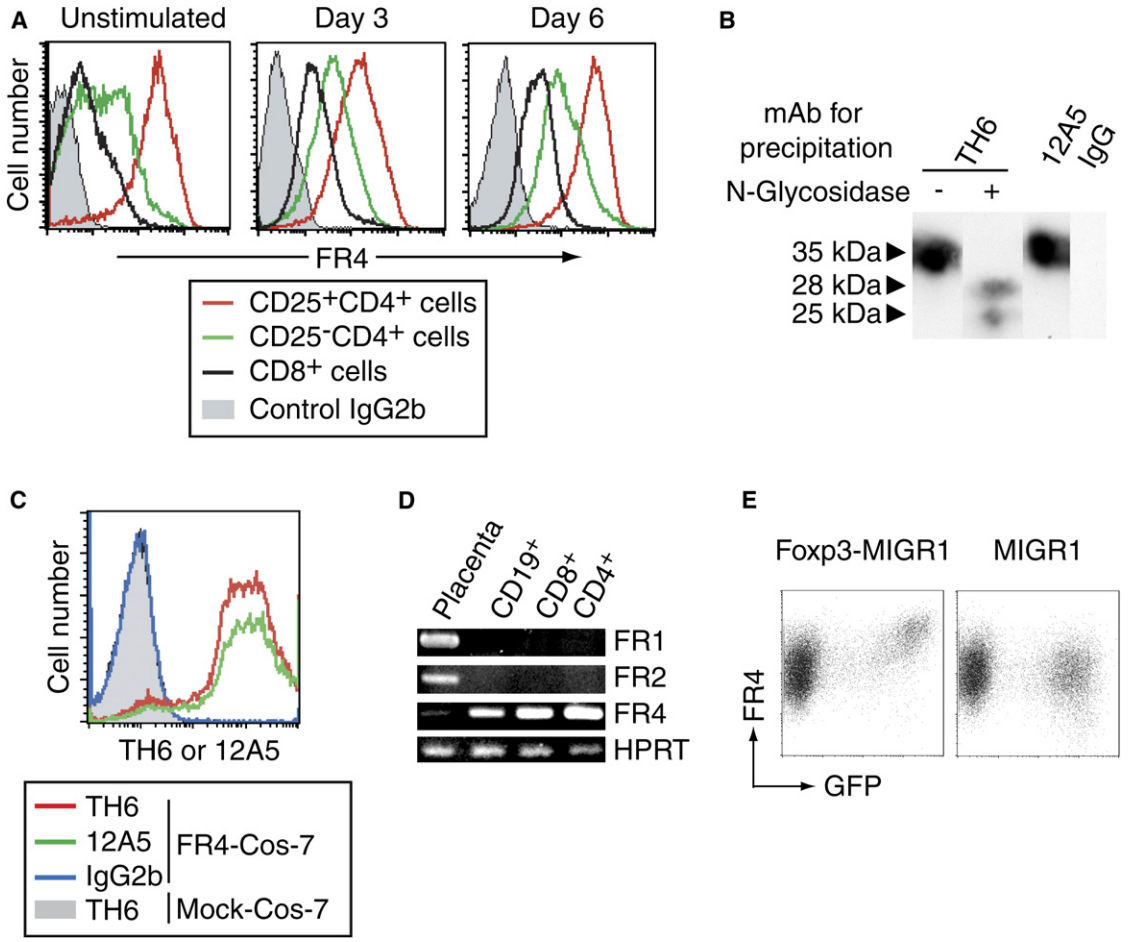


Figure 1. High Expression of FR4 in Natural CD25$^+$CD4$^+$ Treg Cells

(A) CD25$^+$CD4$^+$, CD25$^-$CD4$^+$, and CD8$^+$ T cells before and after stimulation for 3 and 6 days with 0.5 μg/ml of anti-CD3 (2C11), 50 U/ml IL-2, and irradiated spleen cells were stained with TH6 (lines) or rat IgG2b (shaded).

(B) Immunoblotting of normal spleen T cells with 12A5 mAb after immunoprecipitation with TH6, 12A5, or normal rat IgG. An aliquot of the immunoprecipitate with TH6 was treated with N-glycosidase before blotting (Shimizu et al., 2002). Molecular size is shown on the left side.

(C) Cos-7 cells transfected with the mouse FR4 gene (solid line) or nontransfected (shaded) were stained with TH6 and 12A5.

(D) Specific fragments of cDNA for the FR1, FR2, FR4, and HPRT genes were amplified by RT-PCR with cDNA of placenta, CD19$^+$, CD8$^+$, and CD4$^+$ spleen cells.

(E) CD25$^-$CD4$^+$ cells were infected with the bi-cistronic retroviral vector MIGR1 coding for GFP and *Foxp3* or GFP alone (Hori et al., 2003), were stained with TH6. Shown are representative of three independent experiments.

4 (FR4), a subtype of the receptor for the vitamin folic acid, and this high expression of FR4 can distinguish them from other naive or activated T cells. In addition, combinations of high or low expression of FR4 and CD25 can distinguish four functionally different CD4$^+$ T cell subpopulations; i.e., natural Treg cells, effector T cells, memory-like T cells, and naive T cells. With FR4 as a specific marker for natural Treg cells, we address the questions of whether natural Treg cells become antigen activated and clonally expand in physiological and pathological immune responses, how immune responses are controlled by the dynamic balance between Treg cells and effector or memory T cells, and how ongoing immune responses can be altered for the benefit of the host by differential manipulation of antigen-activated Treg cells and effector or memory T cells.

RESULTS

High Expression of FR4 in CD25$^+$CD4$^+$ Treg Cells

By immunizing rats with activated CD25$^+$CD4$^+$ T cell suspensions from normal mice, we obtained two rat monoclonal antibodies (mAbs), designated TH6 and 12A5 of rat IgG2b and IgG1 isotype, respectively. Both mAbs stained CD25$^+$CD4$^+$ T cells at a higher level than other CD4$^+$ or CD8$^+$ T cells even after activation (Figure 1A) and recognized a highly glycosylated protein of 35 kDa in normal spleen cells by immunoprecipitation (Figure 1B). Mass-Fingerprint analysis of the amino acid composition of the precipitate revealed the molecule as mouse FR4, also called folate receptor δ and folate binding protein 3 (Elnakat and Ratnam, 2004; Spiegelstein

et al., 2000). The identity of the protein was confirmed by specific staining of monkey Cos-7 cells transfected with the *Folr4* gene that encodes mouse FR4 (Figure 1C). FR4 is known to be expressed exclusively in lymphoid tissue but has been little characterized in contrast to the well-studied FR1 and FR2, which are mainly expressed in epithelial cells and hematopoietic cells other than lymphocytes, respectively (Elnakat and Ratnam, 2004; Spiegelstein et al., 2000). Among three known subtypes of mouse FRs, splenic lymphocytes, T cells (especially), and mature thymocytes expressed mRNA and protein for FR4 but not for FR1 or FR2 (Figure 1D; Figure S1 in the Supplemental Data available online). Human Treg cells also expressed a homolog of mouse FR4 (Figure S2). Retroviral transduction of *Foxp3*, which can functionally and phenotypically convert normal T cells to natural Treg-like cells (Fontenot et al., 2003; Hori et al., 2003), revealed that the FR4 expression was proportional to that of *Foxp3* in *Foxp3*-transduced $CD25^{-}CD4^{+}$ T cells, suggesting that *Foxp3* directly or indirectly controls the expression of FR4 in natural Treg cells (Figure 1E).

Separation of Treg Cells from Activated T Cells after In Vitro Stimulation

Staining of $CD4^{+}$ T cells in normal naive mice for CD25 and FR4 revealed two discrete populations (fractions a and b in Figure 2A). Notably, stimulation of BALB/c $CD4^{+}$ T cells with allogeneic B6 spleen cells gave rise to three populations well demarcated by high (hi), intermediate (int), or low (lo) expression of FR4 and CD25; i.e., the $FR4^{hi}CD25^{hi}$, $FR4^{int}CD25^{int\text{-}hi}$, and $FR4^{lo}CD25^{-}$ populations (fractions c, d, and e, respectively, in Figure 2A), which constituted ~5%, ~35%, and ~60%, respectively, of $CD4^{+}$ T cells. Allogeneic (B6) stimulation of BALB/c $CD8^{+}$ T cells generated $FR4^{int}CD25^{hi}$ cells and $FR4^{lo}CD25^{-}$ cells, which constituted ~25% and ~75%, respectively, but not $FR4^{hi}CD25^{hi}$ cells. When $CD25^{+}CD4^{+}$ T cells prepared from normal Thy1.2-BALB/c mice were mixed with $CD25^{-}CD4^{+}$ T cells from Thy1.1-BALB/c congenic mice and similarly stimulated with B6 spleen cells, the majority of stimulated $Thy1.2^{+}CD25^{+}CD4^{+}$ T cells differentiated to $FR4^{hi}CD25^{hi}$ cells as a phenotypically discrete population (Figure 2B). Foxp3-expressing $CD4^{+}$ T cells, including $CD25^{+}$ and $CD25^{-}$ cells, were $FR4^{hi}$ before and after allogeneic stimulation (Figure 2C; Figure S3). The $FR4^{hi}CD25^{hi}CD4^{+}$ T cells indeed expressed Foxp3 mRNA at equivalent amounts as naive $CD25^{+}CD4^{+}$ T cells, whereas other populations did not (Figure 2D). In addition, $Foxp3^{+}CD4^{+}$ T cells induced in vitro from naive T cells by allogeneic stimulation in the presence of high-dose transforming growth factor-β (TGF-β) were also $FR4^{hi}CD25^{hi}$ (Figure S4; Chen et al., 2003). Thus, Foxp3-expressing $CD4^{+}$cells, whether they are naturally arising or induced from naive T cells, are persistently $FR4^{hi}$ before and after antigenic stimulation.

Functionally, the $FR4^{hi}CD25^{hi}$ population in alloantigen-stimulated $CD4^{+}$ T cells (fraction c in Figure 2A) were hypoproliferative to in vitro restimulation (Figure 2E) and suppressed the proliferation of $CD25^{-}CD4^{+}$ T cells (Figure 2F), being similar to the $CD25^{+}CD4^{+}$ Treg cell population in naive mice (fraction a in Figure 2A; Takahashi et al., 1998; Thornton and Shevach, 1998). Importantly, these antigen-stimulated $FR4^{hi}CD25^{hi}$ cells were more potent in in vitro suppression than $FR4^{hi}CD25^{hi}$ cells from naive mice and showed antigen specificity: B6-stimulated BALB/c $FR4^{hi}CD25^{hi}CD4^{+}$ T cells were more potent in suppressing anti-B6 response than $CD25^{+}CD4^{+}$ naive or C3H-stimulated $FR4^{hi}CD25^{hi}CD4^{+}$ T cells (Figure 2F). Likewise, C3H-stimulated $FR4^{hi}CD25^{hi}$ BALB/c T cells were more potent than B6-stimulated or their nonstimulated counterpart in suppressing anti-C3H response. The alloantigen-stimulated $FR4^{hi}CD25^{hi}$ cells also strongly suppressed rejection of allogeneic skin graft (Figures 2G and 2H): although BALB/c athymic nude mice rejected B6 skin grafts within 30 days after transfer of BALB/c naive T cells, cotransfer of in vitro B6-stimulated $FR4^{hi}CD25^{hi}$ cells and BALB/c naive T cells significantly prolonged the graft survival to average 45 days, whereas cotransfer of naive $CD25^{+}$ cells was much less effective in prolongation. When nude mice with prior transplantation of both B6 and C3H skins were transferred with the mixture of BALB/c naive T cells and B6-stimulated BALB/c $FR4^{hi}CD25^{hi}$ T cells, the cell transfer led to significantly longer survival of B6 grafts than C3H grafts, while cotransfer with C3H-stimulated BALB/c $FR4^{hi}CD25^{hi}$ T cells significantly prolonged the survival of C3H grafts compared with B6 grafts (Figure 2H).

In contrast with $FR4^{hi}CD25^{hi}$ T cells, which were suppressive, antigen-stimulated $FR4^{int}CD25^{int\text{-}hi}CD4^{+}$ T cells (fraction d in Figure 2A) appeared to be antigen-primed effector T cells because they showed earlier and more vigorous proliferation to allostimulation compared with $FR4^{lo}CD25^{lo}$ (fraction e) or naive $CD25^{-}CD4^{+}$ T cells (fraction b) (Figure 2E). In addition, transfer of these B6-stimulated BALB/c $FR4^{int}CD25^{int\text{-}hi}CD4^{+}$ T cells resulted in significantly shorter survival of B6 skin grafts in BALB/c nude mice compared with transfer of BALB/c naive T cells (Figure 2G).

These results collectively indicate that antigen-stimulated $CD4^{+}$ T cells can be differentiated by the expression of FR4 and CD25 into activated Treg cells, activated effector T cells, and naive T cells as $FR4^{hi}CD25^{hi}$, $FR4^{int}CD25^{int\text{-}hi}$, and $FR4^{lo}CD25^{lo}$ cells, respectively. In addition, this demarcation reveals that antigen stimulation not only activates and expands antigen-reactive effector T cells but also confers antigen-specific suppressive activity to the $FR4^{hi}CD25^{hi}$ fraction. Thus, antigen-reactive $CD4^{+}$ T cells with opposite functions can be well separated by expression levels of FR4.

In Vivo Distinction of Treg Cells from Effector and Memory T Cells

We next examined whether Treg cells could be differentiated from effector or memory T cells after in vivo antigen stimulation. We immunized ovalbumin (OVA) in complete Freund's adjuvant (CFA) to DO11.10 transgenic mice, which express a transgenic T cell receptor (TCR) specific for an OVA peptide (Murphy et al., 1990), or to

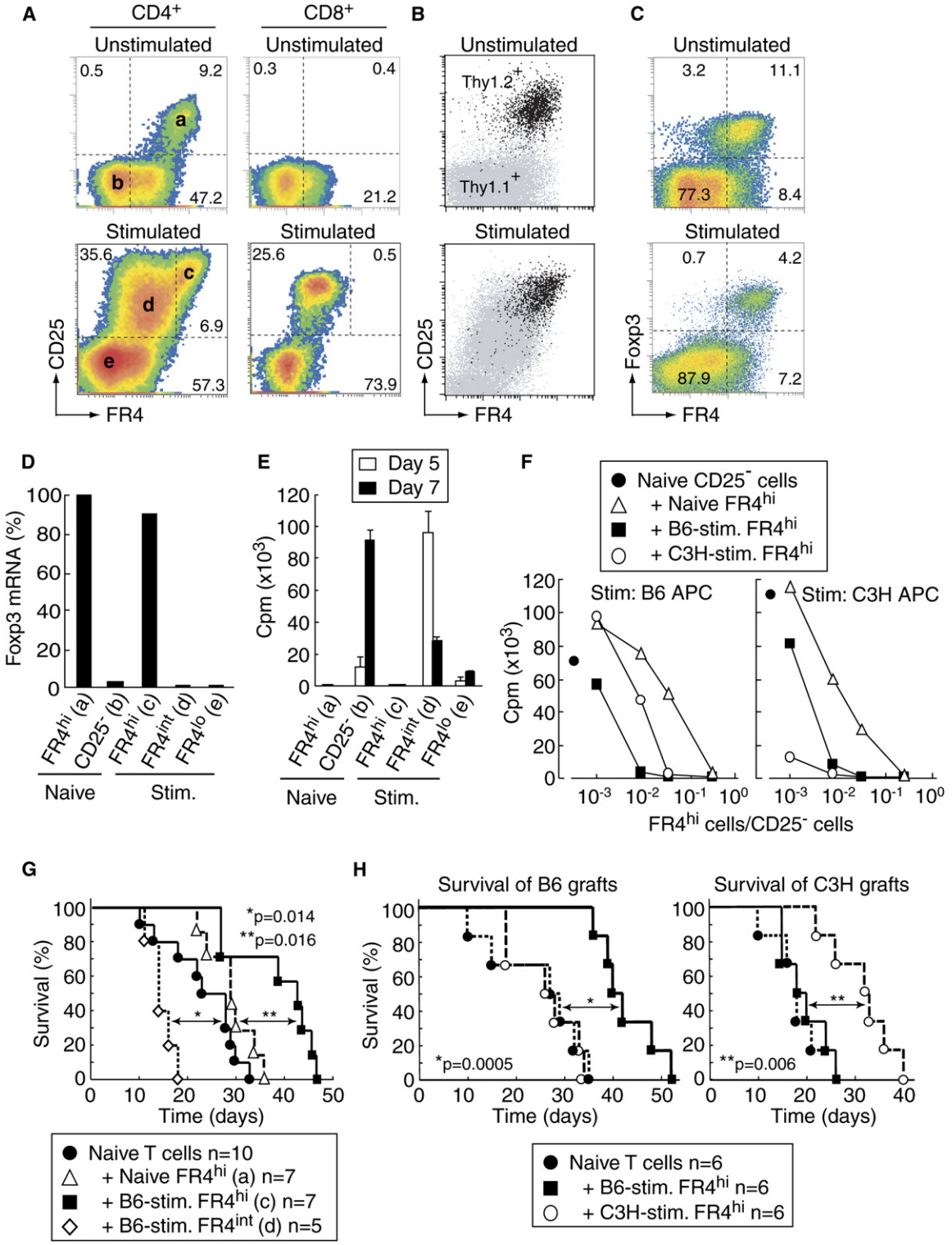


Figure 2. Antigen-Specific Suppression of Allogeneic Immune Responses by In Vitro Stimulated Treg Cells

(A) FR4 and CD25 expression of BALB/c $CD4^+$ and $CD8^+$ T cells before and after allogeneic stimulation with B6 irradiated splenocytes for 6 days. Numbers indicate the percentages of gated cells.

(B) FR4 and CD25 expression by BALB/c $Thy1.2^+CD25^+CD4^+$ T cells (black dots) and BALB/c $Thy1.1^+CD25^-CD4^+$ T cells (gray dots) mixed at 1:5 ratio and stimulated as in (A).

(C) FR4 and intracellular Foxp3 expression of BALB/c $CD4^+$ T cells before and after B6 stimulation as in (A).

RAG2-deficient (*Rag2*$^{-/-}$) DO11.10 transgenic mice, which lack natural CD25^{+}CD4^{+} Treg cells (Figure 3A; Itoh et al., 1999). FR4^{hi}CD25^{hi}CD4^{+} T cells increased in the regional lymph nodes of immunized DO11.10 mice, in which the majority of Foxp3^{+} T cells were FR4^{hi}CD25^{hi} as shown by intracellular staining of Foxp3, whereas *Rag2*$^{-/-}$ DO11.10 mice failed to develop FR4^{hi}CD25^{hi} or Foxp3^{+}CD4^{+} T cells (Figure 3A). Intracellular staining of Bcl-2, an antiapoptotic protein, showed that the FR4^{hi}CD25^{hi} fraction in naive mice included apoptosis-prone Bcl-2^{lo} cells at a higher ratio than other T cell fractions and that Bcl-2^{lo} cells increased among FR4^{hi}CD25^{hi}CD4^{+} T cells and Foxp3^{+} cells after antigen stimulation (Figure 3B; Figure S5). Similarly, CTLA-4^{hi} cells increased among FR4^{hi}CD25^{hi} cells after antigen stimulation (Figure S5). The results indicate that at least some antigen-stimulated Treg cells, which express CD44, CD69, CD103, IL-7Rα (CD127), and CTLA-4 at higher amounts than unstimulated Treg cells (Figure S5), become Bcl-2^{lo} and may die by apoptosis.

Functionally, FR4^{hi}CD25^{hi} cells in OVA-CFA-immunized BALB/c mice 1 week or 3 months after immunization (Figures 3D and 3E, respectively) did not proliferate nor produce IL-2 or interferon-γ (IFN-γ) upon in vitro OVA restimulation. They suppressed the proliferation of *Rag2*$^{-/-}$ DO11.10 T cells in a dose-dependent fashion and more potently than FR4^{hi}CD25^{hi} cells from BALB/c mice treated with CFA alone, whereas the suppressive activities of FR4^{hi}CD25^{hi} cells from the OVA-immunized or -nonimmunized BALB/c mice were equivalent when polyclonally stimulated with CD3 mAb (Figure 3F).

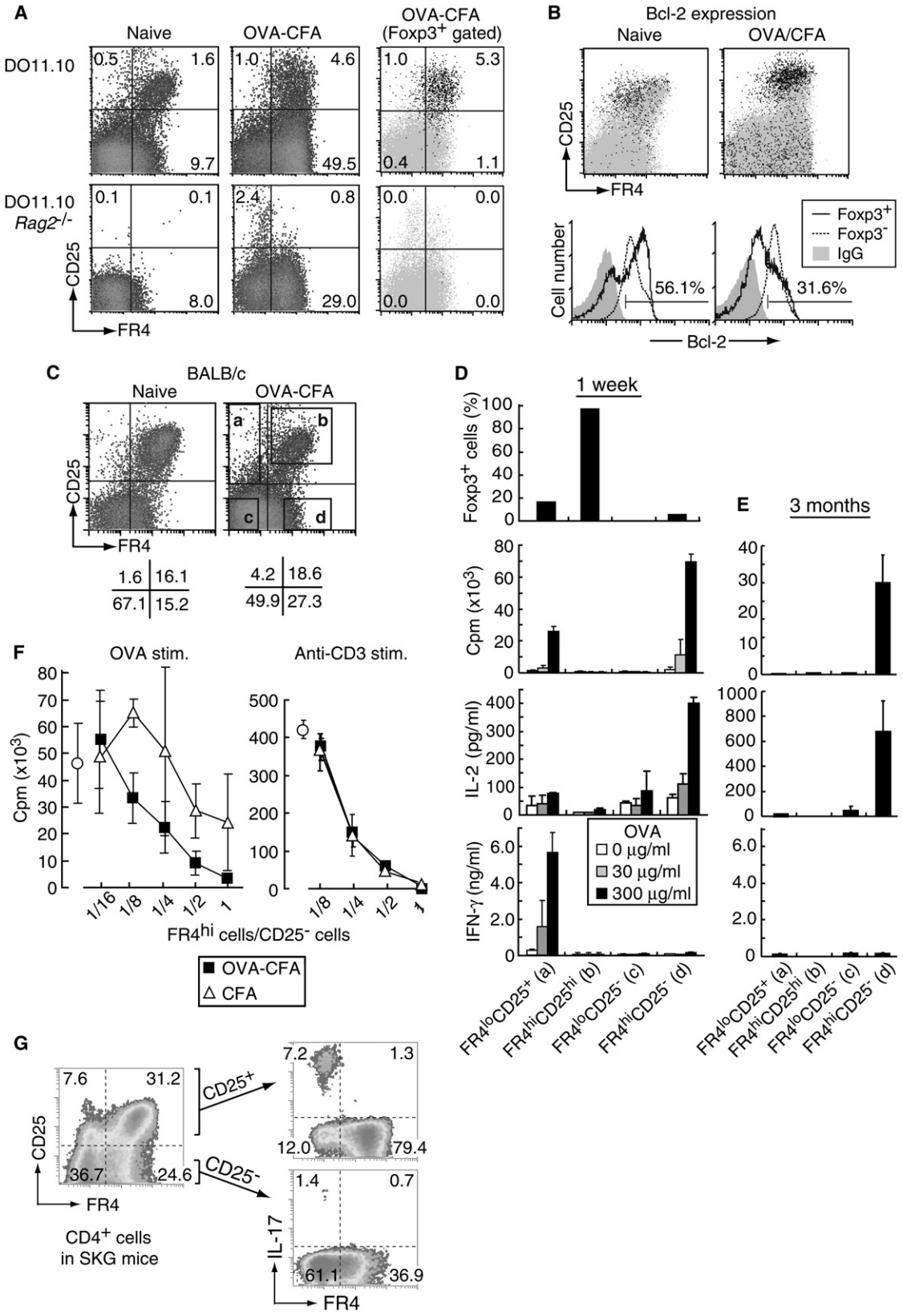
In addition to the increase in the number of FR4^{hi}CD25^{hi} cells after OVA-CFA immunization, the immunization resulted in a marked and slight increase in the number of FR4^{hi}CD25^{-}CD4^{+} T cells and FR4^{lo}CD25^{+}CD4^{+} T cells, respectively, in the regional lymph nodes of both RAG2-intact and -deficient DO11.10 mice (Figure 3A). FR4^{hi}CD25^{-} and FR4^{lo}CD25^{+}CD4^{+} T cells similarly increased in the regional lymph nodes of normal BALB/c mice immunized with OVA-CFA, although less conspicuous than DO11.10 mice (Figure 3C). Interestingly, upon in vitro OVA restimulation 1 week after OVA immunization, BALB/c FR4^{hi}CD25^{-}CD4^{+} cells vigorously proliferated and produced IL-2, but scarcely produced IFN-γ, whereas FR4^{lo}CD25^{+}CD4^{+} cells proliferated and produced IL-2 to a lesser degree but almost solely produced a large amount of IFN-γ (Figure 3D). Even 3 months after immunization, lymph node FR4^{hi}CD25^{-} cells proliferated and produced IL-2 but not IFN-γ in response to in vitro OVA restimulation, whereas other fractions failed to proliferate or produce cytokines (Figure 3E). Furthermore, T cells secreting IL-17 in chronic inflammation, for example, in spontaneous T cell-mediated autoimmune arthritis in SKG mice, were confined to FR4^{lo}CD25^{+}CD4^{+} T cells in lymph nodes (Figure 3G; Hirota et al. 2007). Phenotypically, both FR4^{hi}CD25^{-} and FR4^{lo}CD25^{+} cells in naive DO11.10 and normal BALB/c mice expressed various activation markers (e.g., CD69, CD44, IL-2Rβ [CD122], GITR, and IL-7Rα) differently (Figures S5 and S6).

These functional and phenotypic characteristics of the FR4-CD25 subpopulations indicate that, in addition to FR4^{hi}CD25^{hi} cells as natural Treg cells, activated non-Treg CD4^{+} cells can be differentiated into two phenotypically and functionally discrete populations; i.e., FR4^{lo}CD25^{+} effector (and presumably effector memory) T cells and FR4^{hi}CD25^{-} cells including memory (especially central memory) T cells (Lanzavecchia and Sallusto, 2005; Reinhardt et al., 2001). Furthermore, this distinction of Treg cells from activated non-Treg cells reveals that not only in vitro allogeneic stimulation (Figure 2) but also in vivo stimulation with protein antigen enhances antigen-specific suppressive activity of the Foxp3^{+}FR4^{hi}CD25^{hi} Treg cell population.

In Vivo Antigen-Specific Clonal Expansion of FR4^{hi}CD25^{hi} Treg Cells

To determine then whether the enhancement of alloantigen-specific in vivo and in vitro suppression by FR4^{hi}CD25^{hi}CD4^{+} T cells is due to expansion of antigen-specific Treg cell clones in the FR4^{hi}CD25^{hi} T cell fraction, we inoculated DBA/2 spleen cells into BALB/c mice and assessed possible expansion of each FR4-CD25 subpopulation expressing TCR Vβ6 subfamily, which specifically respond to Mls1^{a} antigen (Mtv-7 superantigen) expressed by DBA/2 spleen cells (Figure 4; Nishimura et al., 2004). Whereas the percentages of FR4-CD25 subpopulations among CD4^{+} T cells did not markedly change 1 week after immunization, the percentages of FR4^{hi}CD25^{+} and FR4^{hi}CD25^{-} cells among Vβ6^{+}CD4^{+} cells increased 2-fold after DBA/2 stimulation; the percentages of these populations among Vβ6^{-}CD4^{+} T cells did not change and were similar to those among unstimulated CD4^{+} T cells (Figure 4A). The percentages of Vβ6^{+} cells among more strictly gated FR4-CD25 fractions (fraction a–d in Figure 4A) similarly changed 1 week after stimulation: they significantly increased in the FR4^{hi}CD25^{hi} fraction, decreased in the FR4^{lo}CD25^{-} fraction, and greatly increased in the FR4^{hi}CD25^{-} fraction (Figure 4B). The

(D) Amounts of Foxp3 mRNA measured by real-time quantitative PCR before and after stimulation. a–e correspond to the populations shown in (A).
(E) Proliferative responses of T cell subpopulations (a–e in [A]) to restimulation with B6 splenocytes for 5 or 7 days. The means and SDs of triplicates are shown. Cpm, counts per minute.
(F) Antigen-specific suppressive activity of B6- or C3H-stimulated BALB/c FR4^{hi}CD25^{hi} cells on BALB/c CD25^{-}CD4^{+} T cells at various ratios in 6-day culture with B6 or C3H splenocytes (APC, antigen-presenting cells).
(G) B6 skin graft survival on BALB/c nude mice after transfer of 2 × 10^{5} BALB/c naive T cells with 1 × 10^{5} sorted CD4^{+} T cell subpopulations shown in (A).
(H) B6 and C3H skin graft survival in the same BALB/c nude mice after transfer of 2 × 10^{5} BALB/c T cells with 1 × 10^{5} alloantigen-stimulated FR4^{hi}CD25^{hi} cells. The results in (A)–(F) are representative of more than three independent experiments; those in (G) and (H) are the total of three separate experiments.


A
Naive
OVA-CFA
OVA-CFA (Foxp3+ gated)
DO11.10
DO11.10 Rag2-/-
CD25
FR4
B
Bcl-2 expression
Naive
OVA/CFA
CD25
FR4
Cell number
56.1%
31.6%
Foxp3+
Foxp3-
IgG
Bcl-2
C
BALB/c
Naive
OVA-CFA
CD25
FR4
D
1 week
Foxp3+ cells (%)
Cpm (x10^3)
IL-2 (pg/ml)
IFN-γ (ng/ml)
OVA
0 μg/ml
30 μg/ml
300 μg/ml
E
3 months
FR4loCD25+ (a)
FR4hiCD25hi (b)
FR4loCD25- (c)
FR4hiCD25- (d)
F
OVA stim.
Anti-CD3 stim.
Cpm (x10^3)
FR4hi cells/CD25- cells
OVA-CFA
CFA
G
CD25+
CD25-
CD25
FR4
CD4+ cells in SKG mice
IL-17

percentage of Vβ6$^+$ cells among Foxp3$^+$ cells also increased to a similar extent as among Foxp3$^-$ cells, in contrast with no significant changes in Foxp3$^+$ or Foxp3$^-$ cells expressing Mls1^a nonreactive TCR Vβ10 (Figure 4C). The results collectively support the hypothesis that antigen-specific natural Foxp3$^+$ Treg cells in the FR4^{hi}CD25^{hi} fraction clonally expand after antigen stimulation. In addition, after in vivo antigen stimulation, a great number of antigen-specific naive T cells, which are FR4^{lo}CD25$^-$, differentiate to FR4^{hi}CD25$^-$ cells.

Treatment of Tumors by Depleting FR4^{hi} T Cells

Based on the above results indicating high expression of FR4 as a marker for Foxp3$^+$ natural Treg cells regardless of whether they are activated or not, we attempted to control ongoing immune responses by the use of FR4 mAb. In vivo injection of TH6 in a range of 1 to 25 μg reduced the number of CD25$^+$CD4$^+$ T cells and CD25$^-$CD4$^+$ T cells in the peripheral blood to ~20% and 70%, respectively, of control mice and, at a large amount (100 μg) of injected TH6, to 15% and 50%, respectively (Figures 5A and 5B). Importantly, injection of Fab fragment of TH6 also reduced the number of CD25$^+$CD4$^+$ cells to a similar extent (Figure 5A), indicating that blockade of FR4 suffices to deplete CD25$^+$CD4$^+$ natural Treg cells.

To examine then whether depletion of FR4-expressing cells can enhance antitumor immune responses in tumor-bearing mice, we assessed tumor growth after TH6 administration to BALB/c mice bearing Meth A fibrosarcoma or Colon 26 colorectal adenocarcinoma cells, both of which are BALB/c derived and neither of which expressed FR4 (data not shown). The percentages of FR4^{hi}CD25^{hi} T cells among CD4$^+$ T cells infiltrating into Meth A tumor masses were higher (2- to 3-fold) than that in lymph nodes or spleen of the tumor-bearing hosts; the majority of such FR4^{hi}CD25^{hi}CD4$^+$ T cells were Foxp3$^+$ (Figure 5C). When BALB/c mice were intradermally inoculated with Meth A and i.v. injected with 100 μg of TH6 on the same day, all the mice rejected tumors and survived more than 3 months (Figure 5D). When mice bearing advanced Meth A or Colon 26 tumors larger than 4 mm in diameter 8 days after tumor inoculation were treated 3 times with i.v. injection of a small dose (10 μg) of TH6 every 4 days, 60% of the mice rejected tumors and survived more than 3 months (Figures 5E and 5F). The majority of control-treated mice died of tumor progression by 40 days in both experiments.

Next, as an adaptive cell therapy for tumors, lymph nodes and spleen cells from BALB/c mice with advanced Meth A tumors were stimulated in vitro with tumor cells and IL-2 and then transferred to tumor-inoculated BALB/c nude mice (Figure 6). The stimulated CD4$^+$ T cells were composed of FR4^{hi}CD25^{hi}, FR4^{int}CD25$^{int\text{-}hi}$, and FR4^{lo}CD25^{lo} cells, whereas CD8$^+$ cells contained FR4^{int}CD25$^{int\text{-}hi}$ and FR4^{lo}CD25^{lo} cells, as observed in in vitro allogeneic stimulation (Figures 2A and 6A). Transfer of these stimulated CD4$^+$ and CD8$^+$ T cells led to tumor rejection only in 17% of Meth A-inoculated BALB/c nude mice. In contrast, when FR4^{hi} cells were removed prior to cell transfer, a substantially larger number of nude mice (46%) rejected tumors; they rejected earlier and nonrejecting mice exhibited retarded tumor growth compared with those transferred with nondepleted cell suspensions (Figure 6B).

This transfer of FR4^{hi} cell-depleted T cell suspensions also induced autoimmune disease in nude mice that had rejected tumors (Sakaguchi et al., 1995; Shimizu et al., 1999). Histologically evident autoimmune gastritis (see below) developed in 20% of them (Figures 6B and 6C). In contrast, TH6 mAb treatment of tumor-bearing euthymic BALB/c mice hardly elicited autoimmunity (Figures 5D–5F and 6C).

Thus, in vivo depletion of activated Treg cells as FR4^{hi} T cells can provoke effective tumor immunity even in advanced stages of tumor progression. Furthermore, depletion of FR4^{hi} cells from tumor-stimulated T cell suspensions enhances tumor-killing activity of the cell suspensions when adoptively transferred to tumor-bearing hosts.

Figure 3. In Vivo Antigen-Specific Activation of Treg Cells

(A) Expression of FR4, CD25, and Foxp3 by CD4$^+$ T cells in regional lymph nodes of DO11.10 and *Rag2*$^{-/-}$ DO11.10 mice before and 1 week after immunization with OVA-CFA. FR4-CD25 expression by Foxp3$^+$ (black dots) and Foxp3$^-$ cells (gray dots) after intracellular staining of Foxp3 is shown (right) with percentages of Foxp3$^+$ cells in each fraction among CD4$^+$ cells.

(B) Expression of Bcl-2 by CD4$^+$ lymph node T cells in DO11.10 mice before and 1 week after OVA immunization. Upper panels show FR4-CD25 expression by Bcl-2^{lo} (black dots) and Bcl-2^{hi} (gray dots) cells. Lower histograms show Bcl-2 expression by Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells in DO11.10 mice before and after OVA immunization with percentages of Bcl-2^{hi} cells in Foxp3$^+$ cells.

(C) FR4-CD25 expression by CD4$^+$ T cells in the regional lymph nodes of OVA-CFA-immunized or control BALB/c mice. Numbers indicate the percentages in each quadrant.

(D) Function of CD4$^+$ T cell subpopulations from BALB/c mice immunized with OVA-CFA 1 week before. Top panel shows the percentages of intracellular Foxp3$^+$ cells in T cell subpopulations (a–d in [C]). Lower 3 panels show proliferation, IL-2 and IFN-γ production by T cell subpopulations (a–d in [C]) after in vitro stimulation with graded concentrations of OVA.

(E) Proliferation and cytokine (IL-2 and IFN-γ) production by T cell subpopulations (a–d in [C]) from BALB/c mice immunized with OVA-CFA 3 months before.

(F) Suppressive activity of OVA-CFA- or CFA-immunized FR4^{hi}CD25^{hi}CD4$^+$ T cells on in vitro proliferation of *Rag2*$^{-/-}$ DO11.10 T cells in the presence of 300 μg/ml OVA or 0.5 μg/ml CD3 mAb. The open circles represent proliferation of *Rag2*$^{-/-}$ DO11.10 T cells alone. The means and SDs of triplicate cultures are shown.

(G) Intracellular staining of IL-17 in FR4-CD25 subsets in lymph nodes of 10-month-old SKG mice. IL-17 was stained as previously described (Hirota et al., 2007). Shown are representative of three independent experiments.

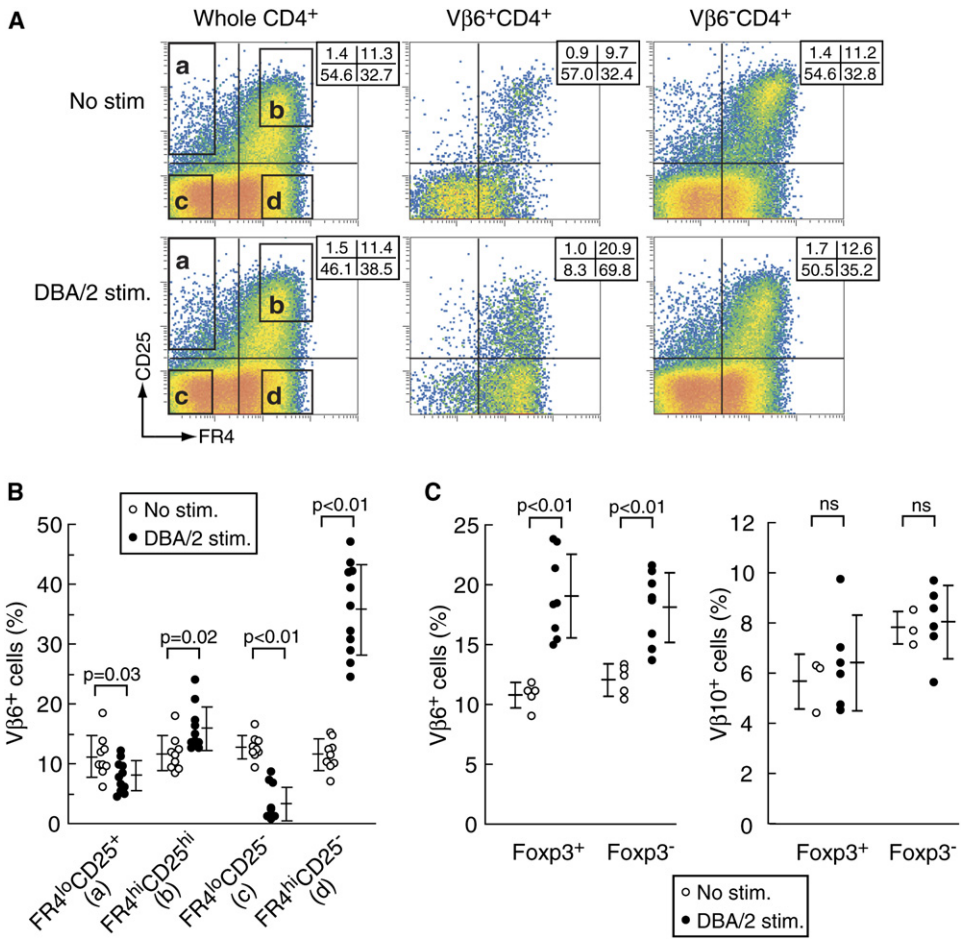


Figure 4. In Vivo Antigen-Specific Expansion of Treg Cells

(A) Expression of FR4, CD25 by $CD4^+$, and $TCRV\beta6^+CD4^+$ lymph node T cells in BALB/c mice immunized with DBA/2 spleen 1 week before. Numbers show percentages of cells in each quadrant.

(B) Proportion of $TCRV\beta6^+$ cells in $CD4^+$ T cell subpopulations (a–d in [A]) from BALB/c mice before and 1 week after in vivo stimulation with DBA/2 spleen.

(C) Proportion of $TCRV\beta6^+$ and $TCRV\beta10^+$ cells in $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cell subpopulations from BALB/c mice before and 3 days after stimulation with DBA/2 spleen cells. Circles indicate individual mice, and the means and SDs are shown. Student's t tests were performed for statistical analyses.

Development of Autoimmune Diseases after FR4 mAb Treatment

Although the administration of TH6 to adult BALB/c mice at a single high dose or several times at a low dose failed to produce histologically evident autoimmune disease (Figure 6C), we attempted to determine whether treatment of young mice with a large dose of TH6 for a limited period would elicit autoimmune disease. With administration of 100 μg TH6 mAb to BALB/c mice on day 10 and 20 after birth, all the mice developed macroscopically evident gastritis with parietal cell autoantibody (Figures 7A–7C). Notably, many mice (50%) developed hydronephrosis (Figures 7D and 7E). They showed histologically evident inflammatory damage of urinary bladder and ureter, accompanied by the development of circulating autoantibody specific for the epithelial cells of these organs, indicating that the hydronephrosis was due to inflammatory narrowing of the lumen of the organs (Figures 7F–7K). Similar treatments of young NOD mice, which are genetically diabetes prone, enhanced the development of diabetes: the treated mice started to develop the disease before 3 months of age and all the mice developed overt diabetes by 5 months of age (Figure 7L) with severe insulitis (Figure 7M), whereas none of the control NOD mice developed the disease by then and they had only mild insulitis (Figures 7L and 7N).

In the pancreatic lymph nodes of NOD mice with insulitis, whether they had developed overt diabetes or not, $FR4^{hi}CD25^+$ cells were $Foxp3^+$ (Figures 7O and 7P and data not shown). Notably, a discrete population of $FR4^{lo}CD25^{hi}$ cells (about 1% of $CD4^+$ cells) developed in the pancreatic lymph nodes, but not in the inguinal lymph nodes, of such NOD mice (fraction a in Figure 7O). The population was $CD45RB^{lo}$, $CD44^{hi}$, $CD62L^{lo}$, $IL\text{-}7R\alpha^{hi}$,

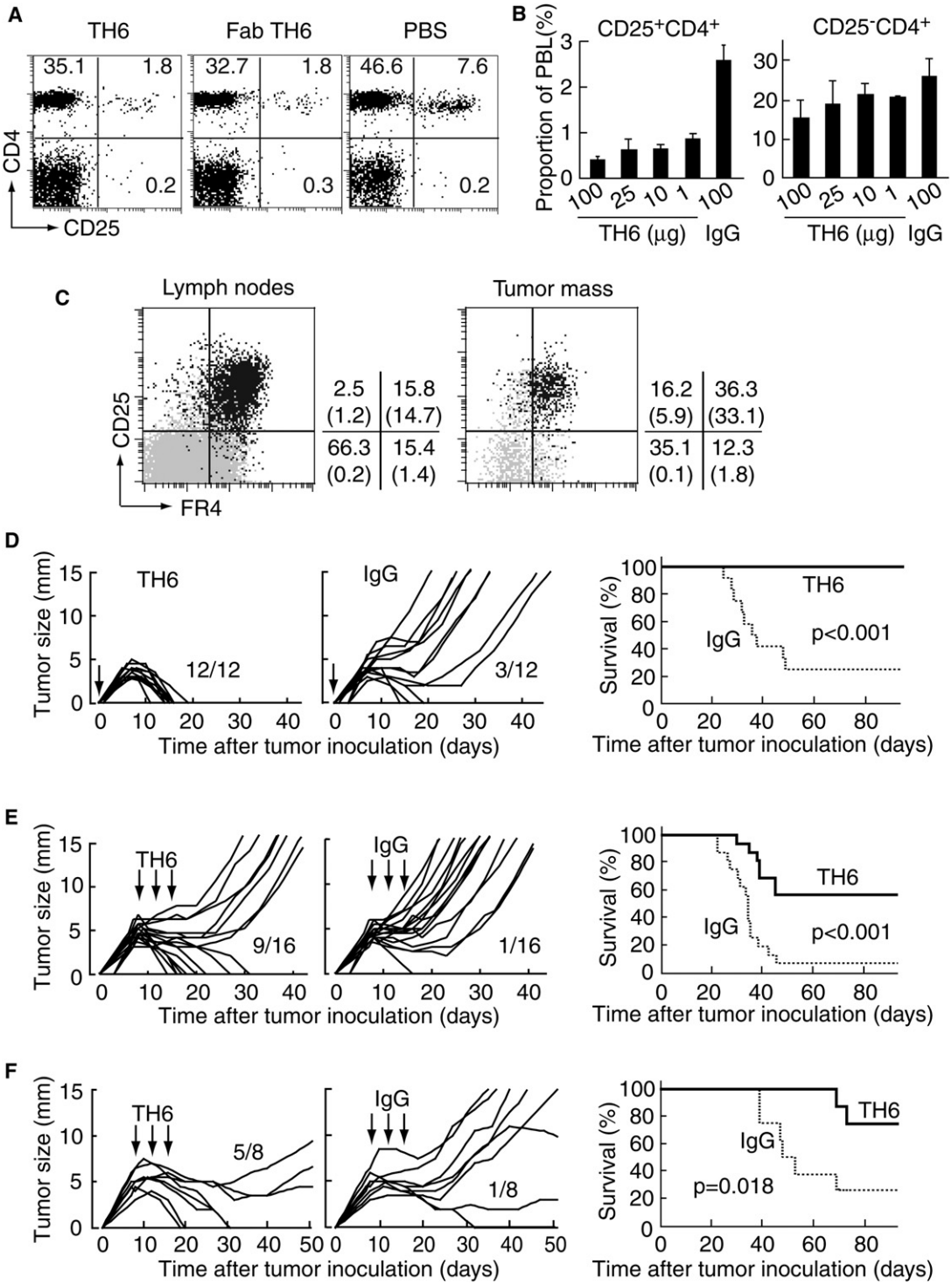


Figure 5. Induction of Tumor Immunity by FR4 mAb Treatment

(A) Depletion of $CD25^+CD4^+$ lymph node T cells 4 days after i.v. injection of 10 μg TH6 or its Fab fragment.

(B) Reduction of $CD25^+$ or $CD25^-CD4^+$ T cells in peripheral blood lymphocytes (PBLs) 4 days after i.v. injection of graded doses of TH6 or control rat IgG. The mean and SD of three mice is shown for each dose.

(C) Expression of FR4-CD25 by $CD4^+$ T cells in an inguinal lymph node and the tumor mass from BALB/c mice with Meth A tumor on the back. Numbers indicate percentages of $CD4^+$ cells in each quadrant; numbers in blankets indicate percentages of $Foxp3^+$ cells in each fraction.

(D–F) Tumor diameter of each mouse and Kaplan-Mayer survival curve of each group. Meth A-inoculated BALB/c mice were treated with 100 μg TH6 i.v. on day 0 (D). Meth A- or Colon 26-inoculated BALB/c mice ([E] and [F], respectively) were treated with 10 μg TH6 i.v. on day 8, 12, and 16. Arrows show the days of antibody injection. The results are the total of 3 or 4 separate experiments. Logrank test was performed for statistical analyses.

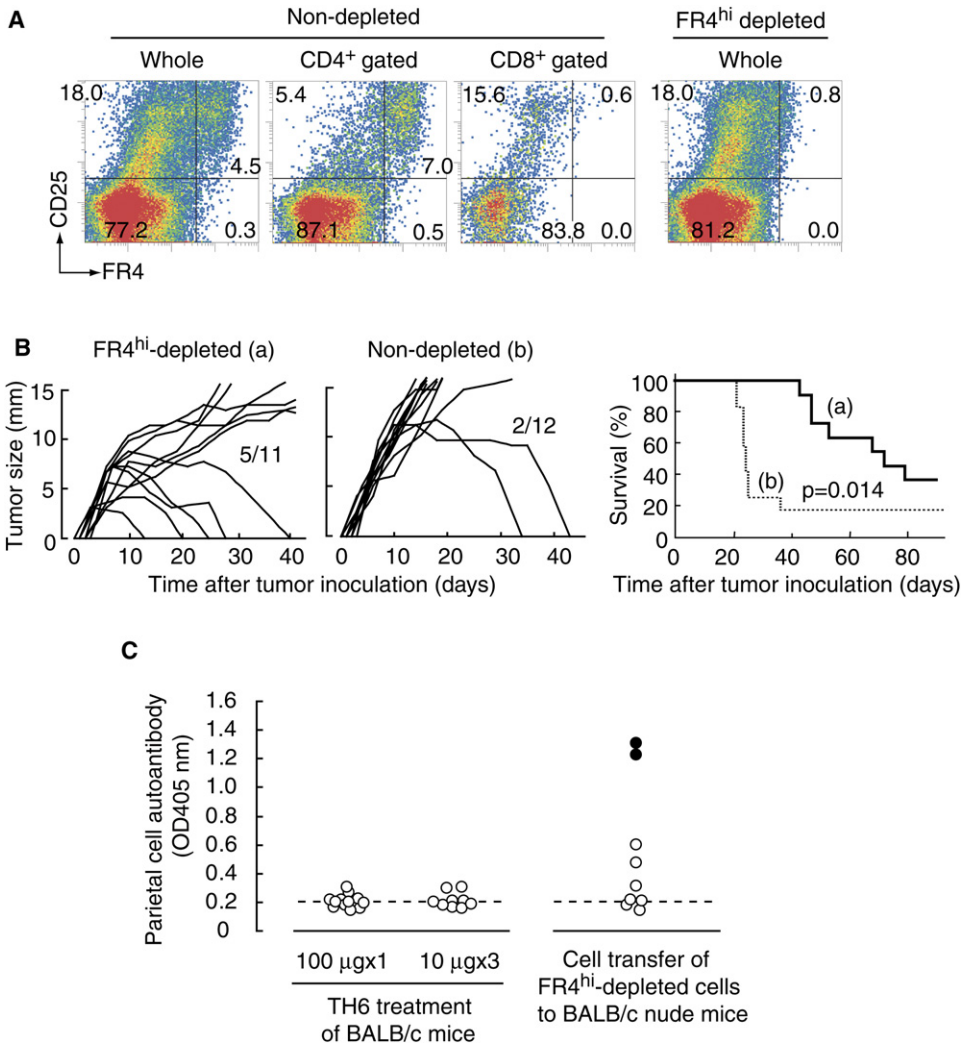


Figure 6. Adaptive Cell Therapy with Antigen-Stimulated T Cells after Depletion of FR4^{hi} Cells

(A) FR4 and CD25 expression of whole, CD4$^+$, or CD8$^+$ T cells after in vitro stimulation with Meth A cells for 9 days. Right panel shows FR4-CD25 expression of FR4^{hi}-depleted cells.

(B) Stimulated lymphocytes with or without depletion of FR4^{hi} cells were transferred to Meth A-inoculated BALB/c nude mice. Tumor diameter of each mouse and survival curves of treated mice are shown. The results are the total of four separate experiments with Logrank test for statistical estimation.

(C) Autoimmune gastritis in treated mice. Autoantibodies against gastric parietal cells in the sera from FR4 mAb-treated BALB/c mice (mice in Figures 5D–5F) or BALB/c nude mice transferred with FR4^{hi} cell-depleted lymphocytes were assessed by ELISA (Sakaguchi et al., 1995). Closed circles represent macroscopically and histologically evident autoimmune gastritis; open circles represent histologically intact gastric mucosa (Sakaguchi et al., 1995).

IL-2Rβ$^+$, Foxp3$^-$, and Bcl-2^{hi} (Figure 7P), corresponding to effector or effector memory T cells (Lanzavecchia and Sallusto, 2005).

Thus, expression of FR4 and CD25 can separate Treg cells from effector and memory-like T cells. Treg deletion by FR4 mAb in young mice can elicit a variety of autoimmune diseases depending on the genetic background of the hosts.

DISCUSSION

We have shown in this report that FR4 is constitutively expressed in Treg cells at a higher amount compared with other activated or naive T cells before and after antigenic stimulation. FR4 is not a mere marker for natural Treg cells but also is functionally essential for their maintenance because blockade of FR4, for example by administration of Fab fragment of TH6 mAb, was sufficient for reducing natural Treg cells in vivo. It has been shown that despite in vitro hypoproliferation upon TCR stimulation, natural Treg cells are more proliferative in vivo than other T cells in normal naive mice, presumably by responding to self-antigens or commensal microbes (Fisson et al., 2003; Setoguchi et al., 2005; Walker et al., 2003). It is therefore likely that natural Treg cells are highly dependent on folic acid for their maintenance in the periphery and that high

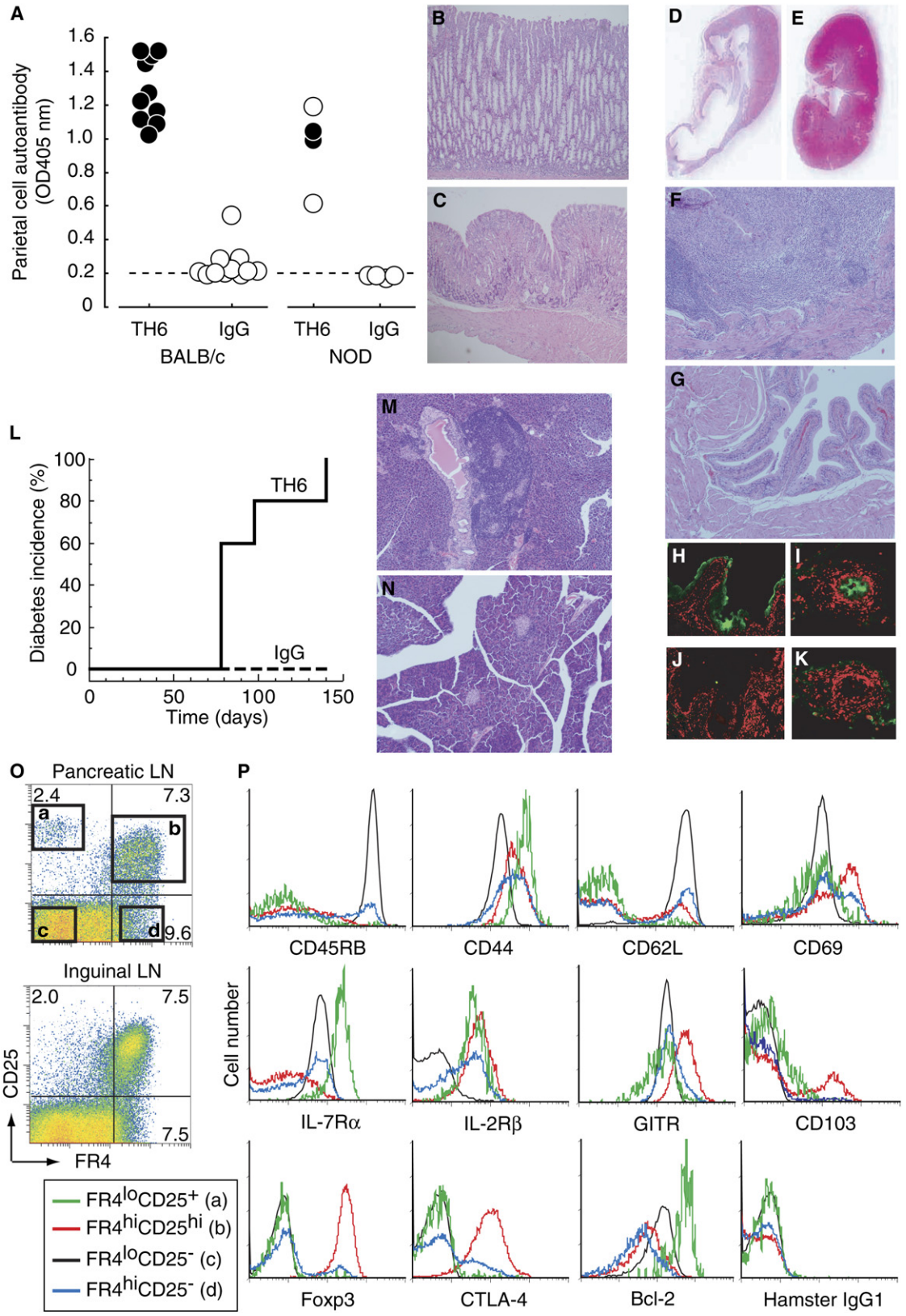
expression of FR4 may enable them to bind and incorporate folic acid efficiently. This is similar to the fact that natural Treg cells are highly dependent on IL-2 for their peripheral survival, and mere blockade of IL-2 binding can reduce the number of natural Treg cells (Setoguchi et al., 2005). Small compounds capable of blocking the uptake of folic acid or altering its cellular metabolism could selectively control the number of natural Treg cells.

With FR4 as a Treg cell marker, we have shown here that strong in vivo or in vitro antigenic stimulation activates and expands not only antigen-specific nonregulatory T cells but also antigen-reactive Treg cells, leading to an enhancement of antigen-specific suppression. This enhancement of antigen-specific suppressive activity is mainly due to clonal activation and expansion of antigen-reactive Treg cells, as indicated by the following findings. First, Foxp3-expressing Mls1^a-reactive BALB/c Vβ6$^+$ T cells expanded in the FR4^{hi}CD25^{hi} population after DBA/2 stimulation whereas Mls1^a-nonreactive Foxp3$^+$Vβ10$^+$ cells did not. This in vivo expansion of Vβ6$^+$ cells correlates with our previous finding that in vitro stimulation of BALB/c T cells with DBA/2 antigen-presenting cells resulted in a substantial increase of the number of TCR Vβ6$^+$ T cells in the Foxp3$^+$ Treg population (Nishimura et al., 2004). Second, not only the number of FR4^{hi}CD25$^+$ T cells but also that of Foxp3$^+$ cells increased 3-fold in the regional lymph nodes of OVA-CFA-immunized DO11.10 mice compared with nonimmunized mice. Third, FR4^{hi}CD25$^+$ T cells increased the expression of CTLA-4, CD69, and CD103 in DO11.10 mice after OVA-CFA immunization. Previous studies by others showed that antigen-specific Treg cells isolated from TCR transgenic mice exhibited in vivo and in vitro clonal expansion upon antigenic stimulation (Klein et al., 2003; Tang et al., 2004; Walker et al., 2003). Also, antigen-pulsed mature DCs were able to induce proliferation of TCR-transgenic natural Treg cells in vitro and in vivo (Fehervari and Sakaguchi, 2004; Yamazaki et al., 2003). Together with these findings, our results with nontransgenic T cells demarcated by FR4 and CD25 expression indicate that the clonal activation and expansion of antigen-specific Treg cells physiologically occurs upon strong antigenic exposure, such as immunization with protein antigen in CFA or allogeneic grafts. This may form the cellular basis of antigen-specific downregulation or tolerance mediated by natural Treg cells.

The combination of FR4 and CD25 can define two functionally distinct subpopulations of Foxp3$^-$ non-Treg CD4$^+$ T cells. FR4^{hi}CD25$^-$ cells in BALB/c mice after OVA-CFA immunization vigorously proliferated and abundantly produced IL-2 but scarcely produced IFN-γ, whereas FR4^{lo}CD25$^+$ cells from the same mice produced a large amount of IFN-γ but showed much less proliferation and production of IL-2. FR4^{hi}CD25$^-$ cells retained this response pattern when restimulated in vitro with OVA 3 months after initial immunization. Phenotypically, FR4^{lo}CD25$^+$ cells and FR4^{hi}CD25$^-$ cells in antigen-primed mice were similarly Bcl-2^{hi}, CD44^{hi}, largely CD69^{hi}, largely CD62L^{lo}, whereas the former were higher in IL-7Rα expression than the latter. Notably, in NOD mice with insulitis, pancreatic lymph nodes but not inguinal lymph nodes contained a larger number of FR4^{lo}CD25^{hi} cells as a phenotypically discrete population. Furthermore, FR4^{lo}CD25$^+$ cells predominantly secrete proinflammatory cytokines such as IL-17 and IFN-γ. These functional and phenotypic differences, when taken together, indicate that FR4^{lo}CD25$^+$ cells contain effector and effector memory T cells, whereas FR4^{hi}CD25$^-$ cells, which enormously expand after antigen stimulation, include central memory T cells. Distinction between effector memory and central memory T cells by cell-surface markers has not been clear in murine CD4$^+$ T cells compared with the human counterparts (Lanzavecchia and Sallusto, 2005; Reinhardt et al., 2001; Sallusto et al., 1999; Seder and Ahmed, 2003; Sprent and Surh, 2002). The combination of FR4 and CD25 may help phenotypically distinguishing these T cell subsets.

Differentiation of activated Treg cells from activated non-Treg cell effector or memory T cells by FR4 mAb makes it possible to specifically manipulate Treg cells in ongoing immune responses. For example, FR4 mAb (TH6) treatment can preferentially reduce natural Treg cells, enhance antitumor immune responses, leading to eradication of even advanced tumors, whereas anti-CD25 treatment or IL-2 neutralization by IL-2 mAb affects both Treg cells and effector T cells and therefore may fail to induce effective tumor immunity against advanced tumors (Ko et al., 2005, and data not shown). Although high-dose TH6 mAb treatment may reduce FR4^{hi}CD25$^-$ memory-like T cells to a certain extent, it apparently spares FR4^{lo}CD25$^+$CD4$^+$ and CD25$^+$CD8$^+$ activated effector T cells. In addition to such TH6 mAb-mediated in vivo depletion, in vitro complement-dependent cell lysis of FR4-expressing cells enhanced antitumor activity of T cell suspensions from tumor-bearing mice in adoptive immunotherapy. In contrast, use of FR4 as a marker for activated Treg cells enables us to isolate antigen-activated and clonally expanded Treg cells as FR4^{hi}CD25^{hi} cells, for example, from the host undergoing graft rejection, to expand them ex vivo polyclonally or antigen specifically, and to transfer them back for suppressing graft rejection. A similar procedure can also be of use for the treatment of autoimmune disease by enriching antigen-expanded Treg cells from the site or regional lymph nodes of autoimmune tissue damage.

Our study shows the possibility that depletion of FR4-expressing T cells, especially in young mice, can evoke severe immune responses to self antigens, in addition to enhancing immune responses to nonself antigens (such as allotransplantation antigens) or quasi-self tumor antigens. For example, TH6 mAb treatment of young BALB/c mice produced not only gastritis, to which BALB/c mice are genetically susceptible upon depletion or reduction of Treg cells (Sakaguchi et al., 1995), but also autoimmune cystitis, which can be adoptively transferred to BALB/c nude mice by CD4$^+$ T cells (data not shown). This autoimmune cystitis can be a good model for human chronic interstitial cystitis associated with other


A
Parietal cell autoantibody (OD405 nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
TH6
IgG
TH6
IgG
BALB/c
NOD
B
C
D
E
F
G
H
I
J
K
L
Diabetes incidence (%)
100
80
60
40
20
0
TH6
IgG
0
50
100
150
Time (days)
M
N
O
Pancreatic LN
2.4
7.3
a
b
c
d
9.6
Inguinal LN
2.0
7.5
7.5
CD25
FR4
FR4loCD25+ (a)
FR4hiCD25hi (b)
FR4loCD25- (c)
FR4hiCD25- (d)
P
CD45RB
CD44
CD62L
CD69
Cell number
IL-7Rα
IL-2Rβ
GITR
CD103
Foxp3
CTLA-4
Bcl-2
Hamster IgG1

autoimmune diseases (Alarcon-Segovia et al., 1984; Oravisto, 1980). In contrast to young mice, however, TH6 mAb treatment of adult mice at a dose sufficient to provoke effective tumor immunity failed to elicit histologically evident autoimmune disease. This indicates that tissue-damaging autoimmune responses are more tightly regulated than antitumor immune responses, especially in adult animals. Nevertheless, the two-sided effect of Treg depletion on tumor immunity and autoimmunity needs to be considered in its clinical application for immunotherapy of cancer, in particular in individuals genetically susceptible to autoimmune disease.

In conclusion, FR4 is a functionally essential molecule for Treg cells and also a useful marker for distinguishing functionally different T cell subpopulations after antigenic stimulation, in particular between activated Treg cells and other T cells, and for monitoring immune responses involving various T cell subpopulations. It needs to be determined whether FR4 is a useful molecular marker for manipulating natural Treg cells and other T cell subpopulations in humans as well.

EXPERIMENTAL PROCEDURES

Mice

BALB/c, C57Bl/6 (B6), C3H, DBA/2, and BALB/c nude mice of 6 to 10 weeks of age were purchased from Japan SLC (Shizuoka, Japan), and NOD mice were purchased from Japan Clea (Tokyo, Japan). RAG2-deficient DO11.10 TCR transgenic mice were bred in our animal facility. They were maintained in our animal facility and treated in accordance with the guidelines for animal care in the Institute for Frontier Medical Sciences, Kyoto University.

Preparation of mAbs

Wistar rats from Charles River Japan (Yokohama, Japan) were three times i.p. immunized with 5×10^6 cells of CD3 mAb-activated $CD25^+CD4^+$ T cells, and spleen cells were fused with P3U1 myeloma cells 3 days after the final immunization (Shimizu et al., 2002). Hybridoma cells secreting mAbs that stained $CD25^+CD4^+$ T cells in flow cytometry were selected and cloned. Purified mAbs were conjugated with biotin (GE Healthcare Bio-Sciences Co., Piscataway, NJ), Alexa Fluor 455, or FITC (Invitrogen Co., Carlsbad, CA). Fab fragments were prepared by as previously described (Shimizu et al., 2002).

Immunoprecipitation

Lymph nodes and spleen cells were lysed, incubated with protein G Sepharose prereacted with antibodies, and subjected to SDS-PAGE (Shimizu et al., 2002). Protein extracted from bands in SDS-PAGE visualized by silver staining (DAIICHI PURE Chemicals, Tokyo, Japan) was subjected to Mass-Fingerprint analysis (Henzel et al., 1993).

Transfection of FR4 and Foxp3

Cos-7 cells were transfected with the pMXS-IG expression vector (Kitamura et al., 2003) with or without *Folr4* cDNA with Lipofectamine2000 (Invitrogen). For the retroviral transduction of *Foxp3*, CD3 mAb-activated $CD25^-CD4^+$ T cells were infected with MIGR1 expressing GFP (Kitamura et al., 2003) with or without *Foxp3* cDNA (Hori et al., 2003).

Flow Cytometry and Cell Sorting

$CD4^+$ T cells were stained with Alexa Fluor 488- or FITC-conjugated FR4, Phycoerthrin-conjugated CD25 (BD Biosciences, San Jose, CA), and CyChrome-conjugated CD4 (BD Biosciences) mAbs, and fractionated to each population by a MoFlo cell sorter (DAKO, A/S, Denmark) to more than 97% purity of each fraction. For intracellular Foxp3 staining, cells were fixed after cell-surface staining of FR4, CD25, and CD4, permeabilized, and stained with Foxp3 mAb (eBioscience, San Diego, CA).

RT-PCR

Total cellular RNA was extracted with Isogen (Nippon Gene, Tokyo, Japan) from sorted lymphocytes or mouse placenta. The total amount of RNA was reverse transcribed with Superscript II reverse-transcriptase and oligo(dT)12-18 primer (Invitrogen). PCR consisted of a denaturation step at 94°C for 2.5 min, followed by 25–40 cycles (40 cycles for FR1 and FR2, 30 cycles for FR4, and 25 cycles for HPRT), at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 45 s, with following primer sequences: *FR1*, gggttgtattttacccagtaggagt and aggtaggaaatgtcctta tgtgctt; *FR2*, gctggaagactgaactaagacagaa and ggagtcttggatgaagtg actctta; *FR4*, cactgtggactgctga and ggctcaaaccacttctg; *HPRT*, tgaag agctactgtaatgatcagtcaac and agcaagcttgcaaccttaacca. Real-time quantitative PCR analysis of *Foxp3* was performed as described previously (Hori et al., 2003).

In Vivo T Cell Activation and In Vitro Assay for Proliferation and Cytokine Production

For in vivo stimulation, 25 μg of OVA emulsified in 50 μl of CFA was injected into footpads, and 8 days later, popliteal lymph node cells were collected. *Rag2*$^{-/-}$ DO11.10 T cells (5×10^4 per well) were cultured with a graded number of $FR4^{hi}CD25^+CD4^+$ cells for 5 days in the presence of 300 μg/ml OVA and 15 Gy-irradiated BALB/c spleen cells (1×10^5) in U-bottomed 96-well plates; incorporation of ^{3}H-thymidine (1 μCi/well) (Du Pont/NEN, Boston, MA) during the last 6 hr of culture was measured (Takahashi et al., 1998). In allogeneic mixed lymphocytes reactions, $CD4^+$ or $CD8^+$ cells were stimulated with allogeneic 15 Gy-irradiated spleen cells in 1:1 ratio with 50 U/ml IL-2 (Shionogi, Osaka, Japan) for 3–9 days. In suppression assay, $CD25^-CD4^+$ cells (2.5×10^4) were stimulated with irradiated allogeneic spleen cells (1×10^5) and graded numbers of $FR4^+CD25^+CD4^+$cells for 5 days,

Figure 7. Autoimmune Diseases in Mice Treated with TH6 mAb on Day 10 and 20 after Birth

(A) Autoantibodies against gastric parietal cells in the sera from TH6-treated BALB/c and NOD mice were assessed as in Figure 6C. Closed or open circles, see Figure 6C.

(B and C) Gastric mucosa from BALB/c mice treated with TH6 (B) or control rat IgG (C). Hematoxilin and eosin (HE) staining (×50 as original magnification).

(D and E) Section of a kidney of a BALB/c mouse treated with TH6 (D) or control rat IgG (E). HE staining (×1).

(F and G) Bladder of BALB/c mice treated with TH6 (F) or control rat IgG (G). HE staining (×50).

(H–K) Indirect immunofluorescence staining of normal bladder (H and J) (×50) and ureter (I and K) (×100) with the sera from TH6- or control rat IgG-treated mice (H) and (I) or control rat IgG (J) and (K). Autoantibody against the epithelia of normal bladder and ureter were detected with Alexa 488-conjugated anti-mouse IgG antibody as green with counter nuclear staining (POPO-3 iodide) as red.

(L) Incidence of diabetes in NOD mice after treatment with TH6 or control rat IgG.

(M and N) Histology of islets from NOD mice treated with TH6 (M) or control rat IgG (N). HE staining (×50).

(O) Expression of FR4 and CD25 by $CD4^+$ T cells in pancreatic or inguinal lymph nodes from NOD mice without overt diabetes.

(P) Pancreatic lymph node $CD4^+$ T cells (gated as shown in [O]) from NOD mice were stained for FR4, CD25, and indicated molecules. Foxp3, CTLA-4, Bcl-2, and Hamster IgG were stained intracellularly. Shown in (O) and (P) are representative of three independent experiments.

as previously described (Nishimura et al., 2004). Concentrations of IL-2 and IFN-γ in the culture supernatants were determined by ELISA (eBioscience).

Tumor Inoculation

BALB/c mice were intradermally inoculated with Meth A (2×10^5) or Colon 26 cells (1×10^5) and i.v. injected with TH6 mAb (Ko et al., 2005). Tumor diameter in two directions was measured for each mouse every 4 days, and mice were sacrificed when the average of the tumor diameter exceeded 15 mm. For in vitro stimulation with tumor cells, draining lymph nodes and spleen cells from Meth A-bearing mice were cultured with mitomycin C-treated Meth A cells and 50 U/ml IL-2 for 9 days, treated in vitro with TH6 and rabbit complement, and 2×10^6 remaining T cells were i.v. transferred to nude mice that were inoculated with Meth A cells on the same day.

Histology and Serology

Autoantibodies specific for the gastric parietal cells were determined by ELISA (Sakaguchi et al., 1995). Urinary or blood glucose levels were measured with Testape or the Medisafe reader (TERUMO, Tokyo, Japan), respectively. Mice with blood glucose levels higher than 300 mg/dl were considered diabetic. Histological and immunohistochemical examination was performed as previously described (Ono et al., 2006).

Statistical Methods

Logrank or Student's t test was used for statistical analyses.

Supplemental Data

Six figures are available at http://www.immunity.com/cgi/content/full/27/1/145/DC1/.

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