

Complementary role of CD4⁺CD25⁺ regulatory T cells and TGF- β in oral tolerance

Yeonseok Chung, Seung-Ho Lee, Dong-Hyeon Kim, and Chang-Yuil Kang¹

Laboratory of Immunology, Institute of Pharmaceutical Sciences, College of Pharmacy,
Seoul National University, Korea

Abstract: CD4⁺CD25⁺ regulatory T cells are thought to be generated in the periphery as well as in the thymus. We sought to determine the roles played by CD4⁺CD25⁺ T cells and transforming growth factor- β (TGF- β) in the induction and maintenance of tolerance generated by oral antigens in BALB/c mice. We found that oral administration of a high dose of ovalbumin (OVA) suppressed OVA-specific proliferation and antibody production in BALB/c mice depleted of CD25⁺ cells. In contrast, the unresponsiveness induced by lower doses of OVA was only partially blocked by CD25 depletion prior to feeding. Depletion of CD4⁺CD25⁺ cells after mice were orally tolerized did not reverse the tolerant status, indicating that these cells were not required to maintain the established tolerance. Furthermore, the induction of oral tolerance was not hampered by the administration of TGF- β -neutralizing antibodies. However, in mice depleted of CD25⁺ cells, anti-TGF- β -neutralizing antibodies blocked the induction of tolerance, regardless of whether the mice followed the high- or low-dose regimens of oral OVA. CD25 depletion together with TGF- β neutralization led the expansion of OVA-specific CD4 T cells against the subsequent antigen challenge, and each treatment alone did not. Our findings indicate that CD4⁺CD25⁺ T cells and TGF- β play a complementary role in the induction of oral tolerance, at least in part, by regulating the expansion of antigen-specific CD4 T cells. *J. Leukoc. Biol.* 77: 906–913; 2005.

Key Words: Tr depletion · neutralization

INTRODUCTION

Studies in experimental models have demonstrated that a number of CD4⁺ T cell subpopulations possess the capacity to regulate responses by other T cells [1, 2]. One of the most useful markers of regulatory T cells (Tr) is CD25. The CD4⁺CD25⁺ T cell population has been shown to be responsible for the prevention of autoimmunity [3]. Indeed, transfer of this subset of T cells has proven effective in the prevention of transplant rejection [4, 5] and inflammatory diseases [6, 7]. Furthermore, depletion of CD25⁺ T cells in vivo has been

shown to enhance antitumor immunity [8, 9]. These findings clearly point to an immunoregulatory role in vivo for CD4⁺CD25⁺ T cells.

CD4⁺CD25⁺ T cells were originally thought to be generated in the thymus [3]. However, several recent studies have reported the generation of Tr cells in the periphery. For example, Thorstenson and Khoruts [10] showed that this population of Tr cells was also generated in the periphery in response to soluble antigens using recombination activating gene-deficient mice. Zhang et al. [11] also showed that antigen-specific CD4⁺CD25⁺ T cells were activated, and their numbers were increased by oral antigen and that transfer of these cells prevented the development of delayed-type hypersensitivity (DTH) in vivo. Thus, it is reasonable to assume that CD4⁺CD25⁺ T cells are generated in the periphery as well as in the thymus.

The mechanism by which Tr cells regulate the responses of other T cells is still under debate. Although a number of in vitro studies have described Tr cells as anergic, several recent studies demonstrated that Tr cells proliferate in response to antigen-specific stimuli in vivo as well as antigen-specific, non-Tr cells [12, 13]. A number of these in vitro studies demonstrated that Tr cells suppress the activation of other T cells via a cell-to-cell, contact-dependent mechanism, and CD4⁺ T cells become anergic after interaction with CD25⁺ Tr cells [14]. Furthermore, two recent studies demonstrated that when cocultured with CD4⁺CD25⁺ T cells, CD4⁺ T cells gain suppressive activity in a process termed infectious tolerance [15, 16]. Whether transfer of suppressor properties also occurs in vivo remains to be clarified. Cytotoxic T-lymphocyte antigen-4 is highly expressed on Tr cells and may contribute to the immunosuppressive property of these cells. Similarly, transforming growth factor- β (TGF- β) was found on the surface of Tr cells. Nakamura et al. [17] have shown that the blocking of TGF- β on the Tr cells abrogated the suppressive property of these cells. However, another study has argued that Tr cell-mediated immunosuppression does not require this cytokine [18].

The oral administration of soluble antigen is the classical method of inducing peripheral tolerance in vivo. High doses of

¹ Correspondence: Laboratory of Immunology, College of Pharmacy, Seoul National University, Shillimdong, Kwanakgu, Seoul 151-742, Korea. E-mail: cykang@snu.ac.kr

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oral antigen resulted in tolerance characterized by anergy or deletion, whereas low doses of antigen favored active suppression by the generation of suppressor T cells, T helper cell type 3 (Th3) cells [19]. However, recent studies have shown that CD4⁺CD25⁺ Tr cells were generated by high doses of oral antigen [10, 11], suggesting that clonal anergy or deletion may not be the exclusive mechanism behind high-dose oral tolerance. The regulatory properties of the Th3 cells, induced by low doses of oral antigen, were mediated by TGF- β [20, 21]. Nevertheless, oral tolerance could be induced in TGF- β null mice, suggesting that multiple mechanisms could be involved in tolerance induction [22].

As CD4⁺CD25⁺ T cells are generated by exogenous antigen and have suppressor properties *in vitro* and *in vivo*, it seemed reasonable to assume that CD4⁺CD25⁺ T cells are crucial for the induction and maintenance of peripheral tolerance to that antigen. Indeed, a recent report by Dubois et al. [23] has shown that CD4⁺CD25⁺ T cells are critical for hapten-specific CD8 T cell tolerance induced by oral administration of the hapten. In the present study, we asked whether the depletion of CD4⁺CD25⁺ T cells *in vivo* affected the peripheral tolerance induced by oral administration of protein antigens. We also sought to determine the role of TGF- β in the induction of oral tolerance. Our study suggests that CD4⁺CD25⁺ T cells are dispensable for the induction and maintenance of oral tolerance; however, these cells play a mutually compensatory role with TGF- β in the induction of oral tolerance.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from Charles River (Biogenomics, Seoul, Korea) and used at 6–10 weeks. DO11.10 mice [specific for ovalbumin (OVA)_{323–337} peptide] were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our animal center. Offspring were identified as transgenic by polymerase chain reaction (PCR) of genomic DNA. Mice were housed at Seoul National University (Korea) until use and were kept in specific pathogen-free conditions during the entire period.

Experimental designs

CD25 depletion study

We used two oral tolerance models that varied by antigen dose and frequency. To establish OVA-specific tolerance, groups of BALB/c mice were given a single 20-mg dose (high dose) or three daily 1 mg doses (low dose) of oral OVA (Grade V, Sigma Chemical Co., St. Louis, MO), respectively. Positive control mice were fed phosphate-buffered saline (PBS). To deplete CD25⁺ cells *in vivo*, anti-CD25 monoclonal antibody (mAb; PC61, 400 μ g) was injected before or after oral administration of antigens. This mAb has been shown to deplete CD25-positive lymphocytes [24]. To test the role of CD4⁺CD25⁺ T cells in the “induction” of tolerance, anti-CD25 mAb was intraperitoneally (i.p.) injected at 14 days before antigen administration. To test the role of these cells in the “maintenance” of tolerance, anti-CD25 mAb was i.p.-injected at 7 days after the last antigen administration. Two weeks after the last treatment, mice were primed and boosted with 50 μ g OVA emulsified in complete and incomplete Freund’s adjuvant (CFA and IFA, respectively) at 2-week intervals. Ten days later, sera and spleens were obtained from the mice, and OVA-specific immunoglobulin G (IgG) and proliferation were measured.

TGF- β -neutralizing study

Anti-TGF- β (1D11), described as a neutralizing antibodies for TGF- β 1, was prepared as ascites (~4 mg/ml). Groups of BALB/c mice were given a single

20-mg (high) dose at day 0, or three daily 1 mg (low) doses of oral OVA from day 0, respectively, and received anti-TGF- β antibody (500 μ g per injection) at days 0–2. In some groups, mice were additionally given anti-CD25 antibody at day –14 to deplete CD25⁺ cells. Then all mice were immunized by the basic prime-boost schedule described above.

OVA-specific enzyme-linked immunosorbent assay (ELISA) and proliferation assay

OVA-specific IgG in serum was measured using a standard, indirect ELISA. Concentration of OVA-specific IgG in tested serum was determined from standard curves constructed using anti-OVA mAb (AO3, IgG) developed in our laboratory.

To measure OVA-specific proliferation, lymphoid cells from the spleen of BALB/c mice were plated at 5×10^5 cells per well in 96-well, round-bottomed microtiter plates and cultured for 4 days with 5, 50, and 500 μ g/ml OVA. After 90 h of incubation, including a final 18-h pulse with [³H]thymidine (1 μ Ci per well), cells were harvested, and the level of label incorporation was measured.

Cytokine ELISA

Interleukin (IL)-2 was quantified from splenocyte culture supernatant. Cells were plated at 8×10^6 cells per well in 1 ml aliquot. Cells were cultured for 48 h with 50 μ g/ml OVA or alone. Purified rat anti-mouse IL-2 mAb (clone JES6-1A12), biotinylated rat anti-mouse IL-2 mAb (clone JES6-5H4), and recombinant mouse IL-2 (PharMingen, San Diego, CA) were used for IL-2 sandwich ELISA.

Reverse transcriptase (RT)-PCR

For detection of mRNA expression, DO 11.10 mice received 400 μ g rat IgG or anti-CD25 antibody at day –14, and then the mice were given 1 mg and 20 mg oral OVA or PBS. Cells from Peyer’s patches (PP) were isolated 6 h after the oral administration [25]. Total RNA was isolated from the cells using the RNeasy mini-kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using the SuperScript II RNaseH-RT (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. Amplification was conducted as follows: an initial heating step of 10 min at 95°C, followed by 94°C for 45 s, 52°C for 45 s, 72°C for 1 min, and a final synthesis of 10 min at 72°C. Specific primer sequences were designed from available GenBank sequences: TGF- β 1 sense primer, 5′-CTT TAG GAA GGA CCT GGG TT-3′, and TGF- β 1 antisense primer, 5′-CAG GAG CGC ACA ATC ATG TT-3′. The amount of each cDNA was standardized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers: GAPDH sense primer, 5′-TTA GCA CCC CTG GCC AAG G-3′, and GAPDH antisense primer, 5′-CTT ACT CCT TGG AGG CCA TG-3′.

OVA-specific CD4 T cell adoptive transfer study

CD4⁺ T cells were isolated from spleens and lymph nodes of DO11.10 mice by magnetic cell sorter using CD4-microbeads and transferred into BALB/c mice intravenously (i.v.; 1.5×10^7 per transfer) at day 0. The recipient mice received anti-CD25 mAb (day –14), anti-TGF- β mAb (days 1–3), or both mAb. The mice were given 20 mg oral OVA or PBS at day 1 and then primed with 50 μ g OVA in IFA at day 14. Five days after priming, lymphoid cells from mesenteric lymph nodes (MLNs) and spleen were stained with phycoerythrin-conjugated KJ1.26 antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibodies and were then analyzed by FACSCalibur (Becton Dickinson, San Jose, CA).

Statistics

Results are expressed as the means $\pm$ SE. Statistical analyses were performed upon comparisons made between the treated groups and the control group using the Student’s *t*-test. Each experiment was repeated at least twice.

RESULTS

In vivo depletion of CD4⁺CD25⁺ T cells does not reverse the induction of oral tolerance in BALB/c mice

Recent studies demonstrated that oral administration of antigens results in the expansion of antigen-specific CD4⁺CD25⁺

T cells [10, 11]. We also observed a significant increase in the percentage of CD25⁺ cells within the CD4⁺ subset in OVA-T cell receptor (TCR) transgenic mice (DO11.10) by oral OVA (data not shown). Therefore, it seemed reasonable to assume that CD4⁺CD25⁺ T cells might play a role in the immune tolerance induced in the periphery by exogenous antigen. To test this hypothesis, we used oral tolerance models in BALB/c mice to investigate whether depletion of CD4⁺CD25⁺ T cells affected peripheral tolerance induced by exogenous antigen. CD25-specific mAb was used to deplete CD4⁺CD25⁺ T cells in vivo. A single injection of 400 μ g anti-CD25 mAb yielded complete depletion of CD4⁺CD25⁺ T cells in secondary lymphoid organs, including gut-associated lymphoid tissues such as MLN and PP (**Fig. 1A**). Consistent with the findings of a previous study [26], depletion of this subset lasted at least 35 days after mAb treatment (**Fig. 1B**).

To test the role of CD4⁺CD25⁺ T cells in induction of oral tolerance in vivo, groups of BALB/c mice were first given anti-CD25 mAb i.p. and then fed high (20 mg once) or low doses (1 mg for 3 consecutive days) of OVA. As expected, OVA-specific antibody production and lymphocyte proliferation were completely suppressed in all OVA-fed, normal mice, indicating the induction of OVA-specific tolerance in these mice (**Fig. 2**, left panels). Of note, CD25-depleted groups of mice given a high dose of oral OVA showed similar suppression to that seen in normal mice (**Fig. 2**, right panels). The production of IL-2 was also significantly decreased in OVA-fed mice in normal and CD25-depleted mice (**Fig. 2C**).

In the low-dose group, however, the suppression of antibody production in anti-CD25 mAb-pretreated mice was substantially reduced. In this group, the production of OVA-specific antibody was significantly higher than it was in 1 mg-fed, normal mice ($P=0.03$). OVA-specific proliferation of lymphocytes was significantly reduced in this group, although the suppression was less than that observed for the high-dose group (**Fig. 2**, right panels). These observations suggest that CD4⁺CD25⁺ T cells play a role in the induction of "low-dose" oral tolerance.

In the absence of OVA feeding, depletion of T cells did not enhance the response to the immunization protocol used in our model, as the production of OVA-specific antibody and lymphocyte proliferation was comparable between normal, PBS-fed and CD25-depleted, PBS-fed groups (**Fig. 2**).

Collectively, these data demonstrate that in vivo depletion of CD4⁺CD25⁺ T cells fails to block the induction of high-dose oral tolerance but partially reverses the induction of low-dose oral tolerance in our models.

In vivo depletion of CD4⁺CD25⁺ T cells does not reverse the established OVA-specific tolerance induced by oral OVA

We next asked whether the depletion of CD4⁺CD25⁺ T cells affected the maintenance of oral tolerance in vivo. We established oral tolerance in mice using a high-dose or low-dose OVA-feeding regimen prior to CD25⁺ T cell depletion. Seven days after the last feeding, these mice were given anti-CD25 mAb followed by immunization with OVA. Whether fed high or low doses of OVA, CD25-depleted mice showed an identical decrease in the production of OVA-specific antibodies compared with OVA-fed, normal mice (**Fig. 3A**). Although OVA-fed, CD25-depleted groups produced a weak OVA-specific antibody response, the suppression was still profound when compared with the PBS-fed, CD25-depleted group. The suppression of OVA-specific proliferation of lymphocytes was observed in the OVA-fed, CD25-depleted groups as well as nondepleted groups, regardless of feeding regimen (**Fig. 3B**). Similarly, the suppression of IL-2 production by oral OVA was maintained in the CD25-depleted groups, although the levels of IL-2 in OVA-fed groups were slightly higher compared with those of the control, IgG-treated group.

In summary, our results indicate that CD4⁺CD25⁺ T cells are not essential for maintenance of oral tolerance in vivo.

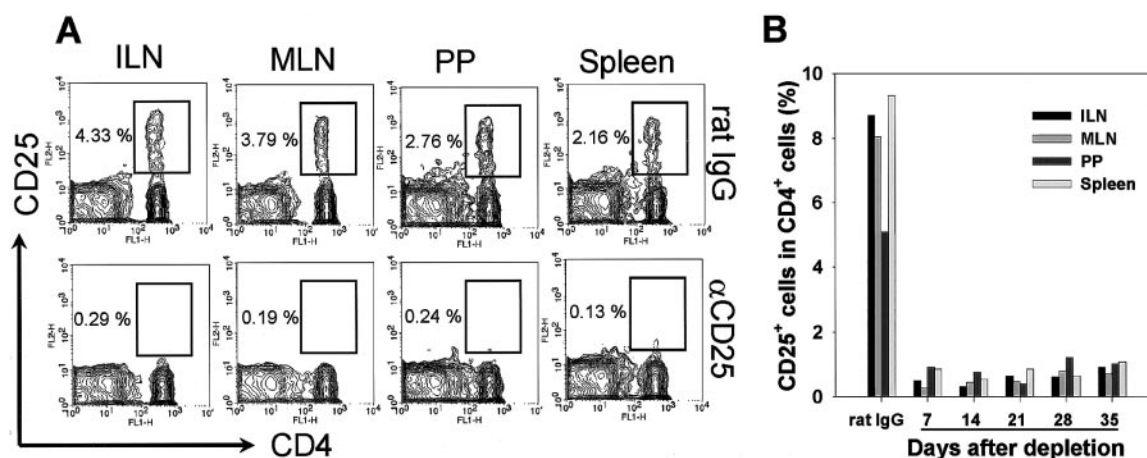


Fig. 1. Anti-CD25 mAb completely depletes CD4⁺CD25⁺ T cells in secondary lymphoid organs. BALB/c mice were injected i.p. with 400 μ g anti-CD25 mAb (PC61) or rat IgG as a control. (A) Ten days later, lymphoid cells in inguinal lymph node (ILN), MLN, PP, and spleen were stained with anti-CD25 (7D4) and FITC-conjugated anti-CD4 and analyzed by flow cytometer. (B) Lymphoid cells from the indicated organs were isolated at 7, 14, 21, 28, or 35 days after anti-CD25 antibody, i.p., and then were analyzed by fluorescein-activated cell sorter for the presence of CD4⁺CD25⁺ T cells. Data are the means of two mice per each group.

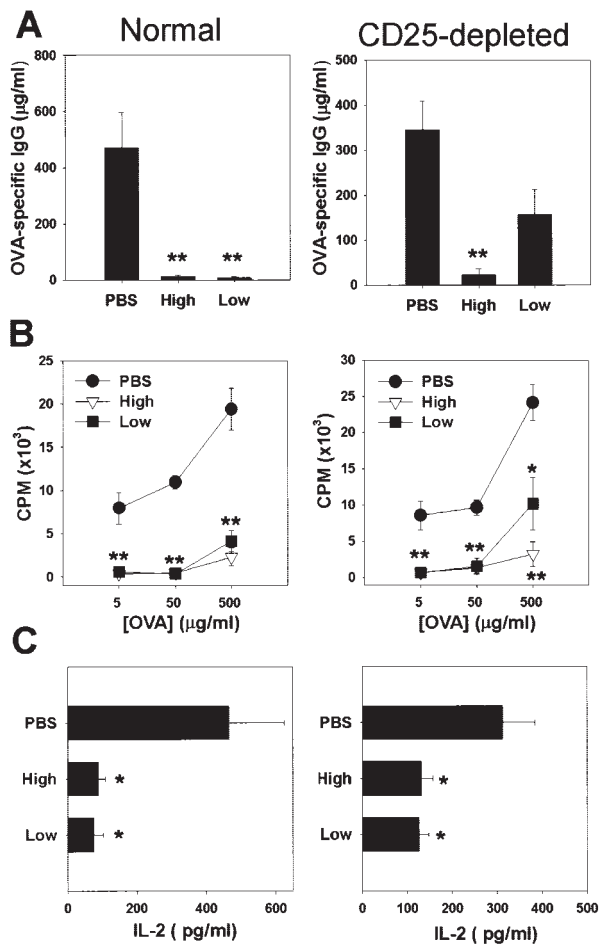


Fig. 2. Effects of CD4⁺CD25⁺ T cell depletion on the induction of oral tolerance in vivo. Groups of BALB/c mice were fed a single 20-mg dose (high dose) or 3 consecutive daily 1 mg doses (low dose) of OVA or PBS as a control. A 400-μg anti-CD25 mAb or rat IgG was injected i.p. at 14 days before oral administration. Two weeks after the last treatment, all mice were primed twice with OVA in CFA or IFA. Ten days after the last priming, sera and lymphoid cells from spleen were obtained, and the concentration of OVA-specific IgG (A) and OVA-specific proliferation (B) and the content of IL-2 in the culture supernatant (C) were analyzed as described in Materials and Methods. *, $P < 0.05$, and **, $P < 0.01$, in comparison with the PBS group. CPM, Counts per minute.

TGF-β neutralization does not block the induction of oral tolerance in vivo

T cells secreting TGF-β, a known immunosuppressive cytokine, were generated by oral administration of antigens and were then observed to suppress an antigen-specific response [19–21]. To test whether oral antigen affected the expression of TGF-β, we used a transgenic mouse model. DO11.10 mice were fed 1 or 20 mg OVA or PBS alone, and then the expression of TGF-β mRNA in PP was analyzed. As shown in **Figure 4**, we observed an up-regulation of TGF-β1 mRNA in 1 mg and 20 mg OVA-fed mice at 6 h after the oral administration, and the increase was more significant in 1 mg OVA-fed mice. Therefore, we next addressed the involvement of TGF-β in vivo using our models of oral tolerance. BALB/c mice were first given a high dose (20 mg once) or low dose (1 mg for 3 days) of oral OVA or PBS as a control from day 0 and then were administered anti-TGF-β-neutralizing mAb i.p. at days 0–2. Mice were primed with OVA in adjuvant and tested.

Again, oral OVA induced the suppression of OVA-specific antibody production and OVA-specific proliferation (**Fig. 5A**). As depicted in **Figure 5B**, the suppression of OVA-specific antibody production was also significant in anti-TGF-β, mAb-treated mice, although the level of suppression was lower than that seen in the control, antibody-treated group (90.1% vs. 77.3% suppression, respectively, after a high dose), suggesting a partial role for TGF-β in the suppression of OVA-specific antibody production by oral OVA. Consistently, with no variation as a result of feeding regimen, the OVA-specific proliferation of splenocytes was significantly lower in OVA-fed mice treated with anti-TGF-β antibody (**Fig. 5B, middle**). We observed similar results for IL-2 production (**Fig. 5B, right**).

Collectively, these results demonstrate that neutralization of TGF-β does not affect the induction of OVA-specific tolerance by oral antigens, whether administered using the high- or low-dose feeding regimen.

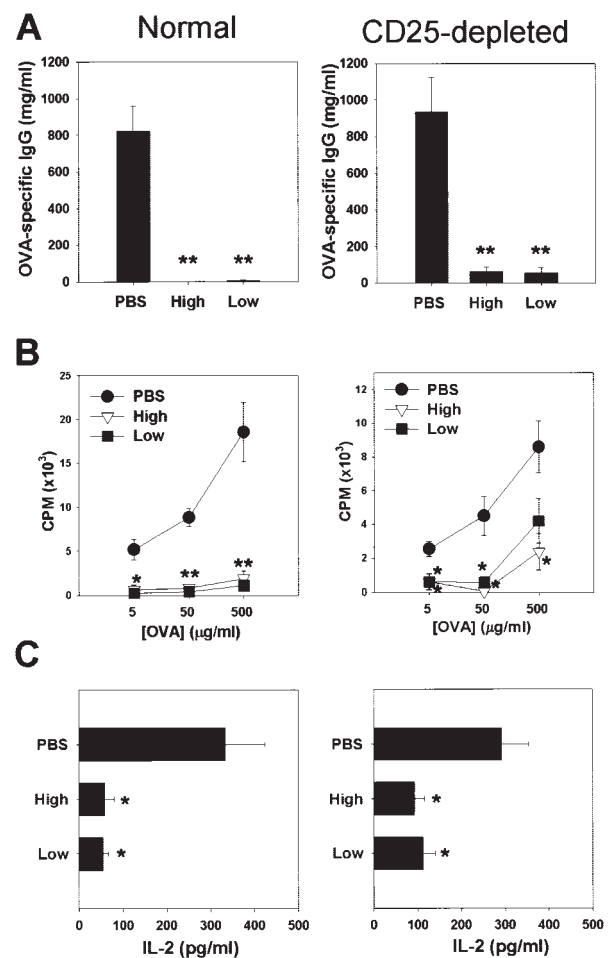


Fig. 3. Depletion of CD4⁺CD25⁺ T cells does not affect the maintenance of the established oral tolerance in vivo. Groups of BALB/c mice were fed a single 20-mg dose (high-dose regimen) or three consecutive daily 1 mg doses (low-dose regimen) of OVA or PBS as a control. A 400-μg anti-CD25 mAb or rat IgG was injected i.p. at 7 days after the last oral administration. After 2 weeks, all mice were primed twice with OVA in CFA or IFA. Ten days after the last priming, sera and lymphoid cells from spleen were obtained, and the concentration of OVA-specific IgG (A) and OVA-specific proliferation (B) and the content of IL-2 in culture supernatant (C) were analyzed as described in Materials and Methods. *, $P < 0.05$, and **, $P < 0.01$, in comparison with the PBS group.

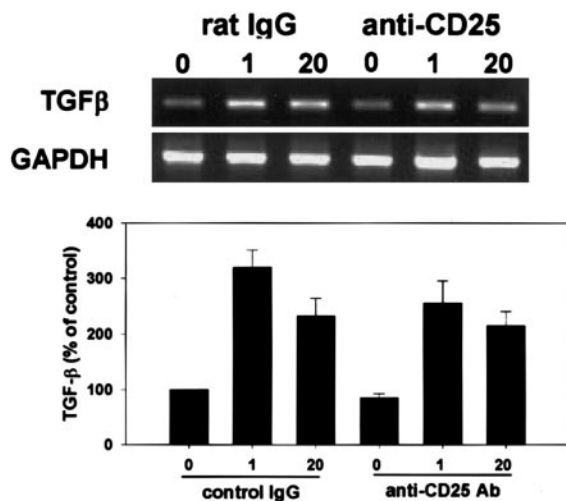


Fig. 4. Expression of TGF- β mRNA in the PP of OVA TCR-transgenic mice after oral administration of OVA. D011 mice received anti-CD25 antibodies (Ab) or rat IgG. Two weeks later, the mice were given 1 mg and 20 mg oral OVA or PBS alone. Total RNA was isolated from PP cells taken 6 h after feeding, and RT-PCR, using mouse-specific TGF- β 1 and GAPDH primers, was performed. The amounts of TGF- β product were calculated and are expressed relative to GAPDH expression and as a percent of control group values. Data are the mean $\pm$ SE of two separate experiments.

Depletion of CD25⁺ cells together with TGF- β neutralization reverses the induction of oral tolerance in vivo

As depicted in Figure 4, the increase of TGF- β mRNA in PP by oral OVA was also observed in CD25-depleted D011 mice, suggesting that this cytokine plays a role in regulating oral tolerance in the absence of CD25⁺ cells. Therefore, we next sought to determine whether the neutralization of TGF- β in CD25-depleted mice affected the induction of oral tolerance. To this aim, groups of mice that had been depleted of CD25⁺ cells by an i.p. injection of anti-CD25 mAb at day -14 were given a high dose (20 mg once) or low dose (1 mg for 3 days) of oral OVA from day 0. Then mice received anti-TGF- β mAb i.p. at days 0–2. After 2 weeks, all mice were immunized with OVA in adjuvant.

It is interesting that those mice given anti-CD25 mAb, anti-TGF- β mAb, and a high dose of oral OVA produced substantial amounts of OVA-specific antibody (Fig. 5C, left), amounts comparable with those seen in PBS-fed, control mice. We observed greater amounts of OVA-specific IgG in mice given a low dose of oral OVA than in the PBS control ($P < 0.05$). Regardless of dose, oral OVA also lost its ability to suppress OVA-specific proliferation in mice given anti-CD25 mAb and anti-TGF- β mAb (Fig. 5C, middle). When the concentration of OVA was low, OVA-specific proliferation was still less pronounced in the high-dose than in the PBS-fed group;

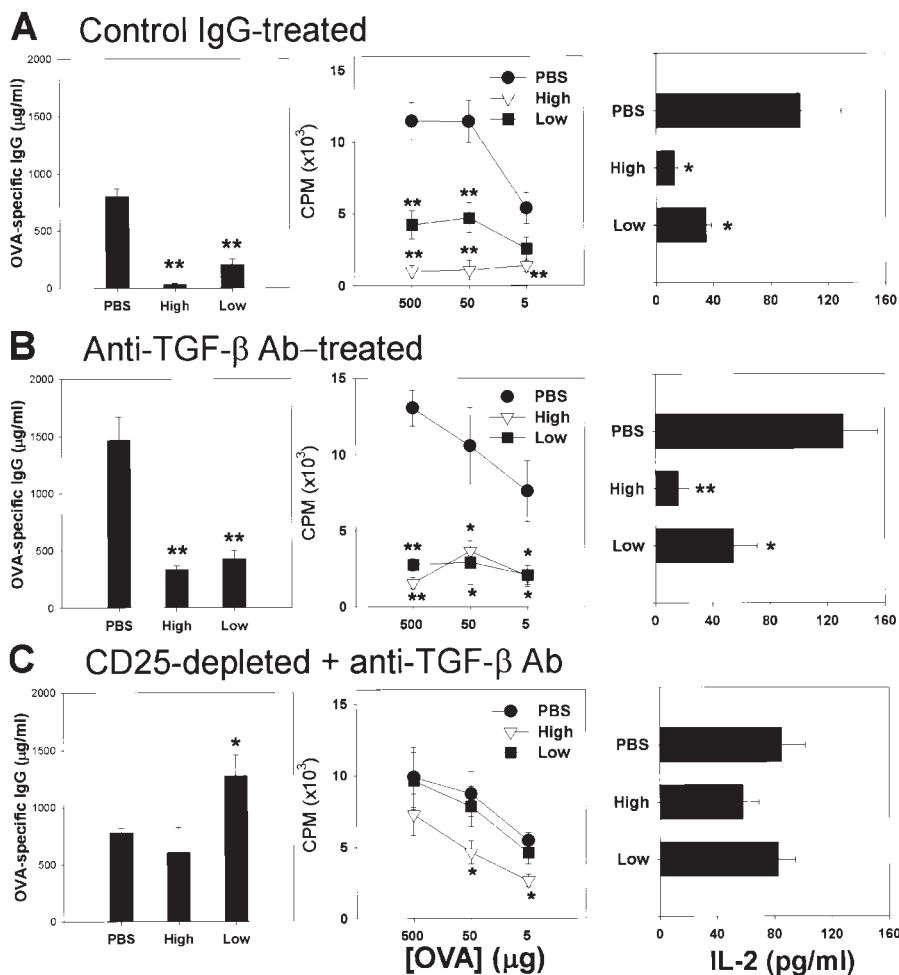


Fig. 5. Working in tandem with CD25 depletion, anti-TGF- β mAb blocks the induction of oral tolerance in vivo. Groups of BALB/c mice were given anti-CD25 mAb at day -14 (C) and were fed a high dose (20 mg once) or low doses (1 mg for 3 consecutive days) of OVA from day 0. Anti-TGF- β mAb was given i.p. at days 0–2 (B and C). Seven days later, the mice were primed and boosted with OVA at 2-week intervals. Ten days later, sera and lymphoid cells from spleen were obtained, and the concentration of OVA-specific IgG (left panels) and OVA-specific proliferation (middle panels) and the content of IL-2 in culture supernatant (right panels) were analyzed. *, $P < 0.05$, and **, $P < 0.01$, in comparison with the PBS group.

however, it was not completely suppressed, as it was in the control, IgG-treated, high-dose group (Fig. 5, middle). We observed similar results in IL-2 production (Fig. 5C, right). The decreased production of IL-2 by oral OVA was largely restored by treating mice with anti-CD25 mAb together with anti-TGF- β mAb in the high- and low-dose groups. As the PBS control group, which also received anti-CD25 and anti-TGF- β antibodies, showed normal immune responses, we concluded that the reversal of tolerance in OVA-fed mice was not the lingering effect of these antibodies on the immunization with OVA.

These results demonstrate that CD4⁺CD25⁺ T cell depletion, together with TGF- β neutralization, blocked the induction of oral tolerance regardless of the dose of oral OVA, although oral tolerance was not completely reversed by the administration of a high dose of oral OVA.

The limited expansion of OVA-specific T cells against OVA-priming in OVA-fed mice is restored in mice depleted of CD25⁺ cell and received anti-TGF- β antibody

The reversal of oral tolerance by CD25 depletion plus TGF- β neutralization implicates that OVA-specific T cells are not anergic and respond normally toward the followed priming with the antigen. Thus, we finally asked what happened to antigen-specific T cells following the antibody treatment. To explore this, we used an adoptive transfer study. Groups of mice were i.v.-injected with DO11 T cells at day 0 and then received 20 mg oral OVA or PBS at day 1. Some groups of mice had previously received anti-CD25 mAb at day -14 and/or were injected with anti-TGF- β antibodies for 3 consecutive days from day 1. These mice were primed with OVA in IFA and then lymphoid cells from MLN, and spleen were analyzed for the frequency of DO11 T cells by a flow cytometer.

As depicted in **Figure 6**, priming recipient with OVA increased the population of DO11 T cells in MLN of the PBS-fed mice in the percentage (Fig. 6A) and the absolute number (Fig. 6B). However, when the recipient mice were given oral OVA, the priming did not increase the population of DO11 T cells. This result indicates that DO11 T cells failed to expand against OVA challenge in mice given oral OVA. We observed similar results in mice treated with anti-TGF- β an-

tibodies. A weak but significant increase in the DO11 T cell was observed in mice depleted of CD25; however, it is far less profound than that of PBS-fed, primed mice (Fig. 6).

It is interesting that we observed a significant increase of the DO11 T cell in mice treated with anti-CD25 and anti-TGF- β antibodies (Fig. 6). The absolute number of DO11 T cells in this group was comparable with that of PBS-fed, primed mice, indicating that OVA-specific CD4⁺ T cells did not become anergic by oral OVA and respond normally against the followed priming with the same antigen in this group. We observed a similar pattern of results when we analyzed lymphoid cells from spleen (data not shown).

Collectively, these results demonstrate that although depletion of CD25 or neutralization of TGF- β alone failed to overcome anergy induction by oral administration of antigens in antigen-specific T cells, a combination of CD25 depletion and TGF- β neutralization allowed antigen-specific T cells to overcome anergy induction and respond to the subsequent challenge with the same antigen.

DISCUSSION

A recent study has demonstrated that immunosuppressive, antigen-specific CD4⁺CD25⁺ T cells can be generated in the periphery by administration of oral or i.v. antigen [10]. Another study showed that transfer of OVA-specific CD4⁺CD25⁺ T cells activated by oral OVA suppressed OVA-specific DTH responses in recipient mice [11]. Hence, it would seem reasonable to surmise that CD4⁺CD25⁺ T cells might be involved in the peripheral tolerance induced by exogenous antigen. Thus, a goal of the present study has been to delineate the possible role of these cells in peripheral tolerance induced by exogenous antigens using oral tolerance models. As the specific mechanism of oral tolerance seemed to vary with the dose of oral antigen, the oral tolerance models we established varied in their doses of oral antigen.

To examine the role of Tr cells in the induction of oral tolerance, we depleted mice of CD25-positive cells by an i.p. injection of anti-CD25 mAb and then administered to them a high or low dose of oral OVA. CD4⁺CD25⁺ T cells may play a role in low-dose oral tolerance, as the suppression of OVA-

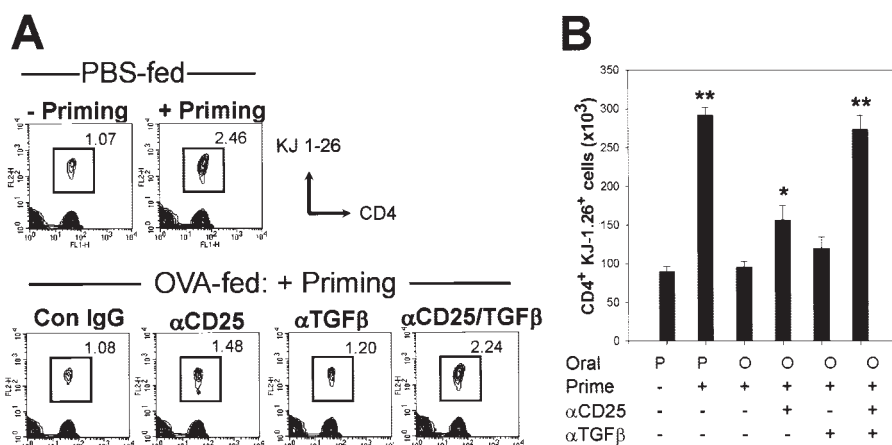


Fig. 6. CD25 depletion plus anti-TGF- β mAb enhance the dividing of OVA-specific T cells in OVA-fed mice against the subsequent challenge with the antigen. (A and B) KJ1.26⁺ cells from DO11.10 mice were i.v.-transferred into BALB/c mice (day 0). The recipient BALB/c mice were given anti-CD25 mAb (day -14) or anti-TGF- β mAb (days 1-3). The mice were given 20 mg oral OVA (day 1) and were then primed with OVA in IFA (day 14). Five days later, lymphoid cells from MLN were analyzed by flow cytometer. The inserted number indicates mean percentage of DO11 T cells (A). Absolute number of DO11 T cell was calculated and expressed as mean + SE. *, $P < 0.01$, and **, $P < 0.001$, in comparison with OVA-fed, control IgG-treated group (Con IgG). (A) Data represent one from four mice per group.

specific IgG production induced by a low dose of oral OVA was profoundly reversed in mice depleted of CD25-positive cells. However, the CD25-depleted group of mice given a high dose of oral OVA showed similar suppression to that seen in normal mice. Indeed, Thorstenson and Khoruts [10] found that a low dose of i.v. antigens was more effective in generating CD4⁺CD25⁺ T cells than a high dose. However, Dubois et al. [23] have recently reported that CD4⁺CD25⁺ T cells are required for the suppression of hapten-specific CD8 T cell responses (contact hypersensitivity) by oral administration of hapten using an in vivo depletion study. Such a finding does not necessarily contradict our own, as we speculate that hapten and protein antigens may have different requirements for the induction of oral tolerance. It is also possible that CD4⁺CD25⁺ T cells contribute to T cell tolerance in distinct ways for CD8 and CD4 T cells. Finally, as the researchers in the hapten study injected CD25-depleting antibodies during the sensitizing phase for hypersensitivity as well as the inductive phase for oral tolerance, the residual anti-CD25 antibody might not only have depleted Tr cells but also may have affected the T cell activation or dendritic cell function in vivo by blocking CD25 (IL-2 receptor α chain).

In this study, we have also examined the role of CD4⁺CD25⁺ Tr cells in the maintenance of oral tolerance by depleting CD25-positive cells in mice previously tolerized by oral OVA. We observed that the established OVA-specific tolerance was maintained regardless of CD25 depletion, indicating that CD4⁺CD25⁺ Tr cells are not required for the maintenance of oral tolerance. Two recent studies demonstrated that CD25⁺ Tr cells induce anergy and transfer suppressive activity to conventional CD4⁺ T cells in a process termed "infectious tolerance" [15, 16]. As hyporesponsiveness was induced in the CD25-depleted mice as well as in the normal mice, we conclude that infectious tolerance induced by CD4⁺CD25⁺ T cells is likely not the main mechanism for peripheral tolerance in our model.

TGF- β is a cytokine whose immunosuppressive traits have been well characterized. Nakamura et al. [17] showed that the suppressive properties of CD4⁺CD25⁺ Tr cells were abrogated if the TGF- β found on their surface was blocked [17]; another study, however, reported opposite results [18]. It has also been reported that oral antigens have generated TGF- β -secreting T cells, capable of suppressing antigen-specific responses [20, 27]. However, as oral tolerance could be induced in TGF- β knockout mice regardless of antigen dose, multiple mechanisms of tolerance must be presumed to coexist [22]. In addition, Tsitoura et al. [28] showed that neutralization of the immunosuppressive cytokines IL-10 and TGF- β did not abrogate the induction of mucosal tolerance. We showed an increase of TGF- β mRNA by oral administration of OVA in DO11 mice treated with control antibodies and anti-CD25 antibodies. According to our own observations, however, administration of a neutralizing antibody to TGF- β during the inductive phase did not reverse the induction of oral tolerance, regardless of the dose of oral OVA, although the suppression of antibody production was slightly weaker in anti-TGF- β -treated groups than that of control, antibody-treated groups. Thus, neutralization of TGF- β alone failed to block the induction of oral tolerance. However, we showed that CD4⁺CD25⁺ T cell

depletion together with TGF- β neutralization blocked the induction of oral tolerance by high and low doses of oral OVA. Using an adoptive transfer model, we observed that even after oral administration of OVA, DO11 T cells expanded normally against the subsequent priming with OVA in CD25-depleted and TGF- β -neutralized mice, and each treatment alone did not. Thus, the mechanism of action of Tr depletion plus TGF- β neutralization is that this regime rescues antigen-specific T cells from anergy and allows the cells to respond normally to their relevant antigen. Based on our observations, we propose that CD4⁺CD25⁺ Tr cells and TGF- β are involved in the induction of tolerance and play mutually compensatory roles in vivo. When an unharmed antigen is administered orally into a CD4⁺CD25⁺ Tr cell-free environment, the immune system may use a TGF- β -dependent, immunomodulatory pathway to induce tolerance by regulating the expansion of antigen-specific T cells and vice versa. Thus, our study may help explain the discrepant results regarding the role of TGF- β in mucosal tolerance. However, it is not clear whether the depletion of Tr cells plus neutralization of TGF- β would affect the maintenance of oral tolerance in the present study.

Under normal physiological conditions, the frequency of antigen-specific CD4⁺CD25⁺ T cells is probably low, and thus, it seems unlikely that these cells are solely responsible for the induction of antigen-specific tolerance in the periphery. However, in the present study, we could not rule out the possibility that Tr cells from subsets other than CD4⁺CD25⁺ are involved in peripheral tolerance to exogenous antigens. Unger et al. [29] demonstrated the immunosuppressive capacities of proliferating OVA-specific CD4⁺CD25⁺ T cells from intranasally tolerized mice. Moreover, Hauet-Broere et al. [30] recently showed that administration of oral antigen induced functional CD4⁺CD25⁺ as well as CD4⁺CD25⁺ Tr cells in gut-associated lymphoid tissue. We suggest that TGF- β plays a role in the induction of CD4⁺CD25⁺ Tr cells. As high doses of oral antigen were used in the above study, the induced cells may not be of the Th3 type, which is known to be induced by multiple low doses of oral antigen. An interesting subject for future research would be the comparison of the three types of regulatory cells (CD4⁺CD25⁺ Tr, CD4⁺CD25⁺ Tr, and Th3) with the role of TGF- β in inducing each.

Recently, Chen et al. [31] demonstrated that TGF- β induced the expression of FoxP3 mRNA and converted naïve CD4⁺CD25⁺ T cells to immunosuppressive CD4⁺CD25⁺ T cells. In our study, mice were depleted of CD25⁺ cells and given TGF- β -neutralizing antibody. In these mice, oral OVA may have failed to induce the conversion of antigen-specific CD4⁺CD25⁺ T cells into CD4⁺CD25⁺ Tr cells, and this failure may represent one mechanism by which the induction of tolerance is blocked in these mice.

Our study is novel in demonstrating that CD4⁺CD25⁺ T cells and TGF- β may play mutually complementary roles in the induction of peripheral tolerance by oral antigen. Elucidating the factors that influence peripheral tolerance to exogenous antigens is likely to provide important insights into the regulation of physiological immunity as well as to facilitate its application to immune disorders.

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