

# Suppression of serum IgE response and systemic anaphylaxis in a food allergy model by orally administered high-dose TGF- $\beta$

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## Abstract

Some epidemiological or association studies suggest that transforming growth factor- $\beta$  (TGF- $\beta$ ) in breast milk may be a decisive factor in diminishing the risk of allergic diseases during infancy. The observations have prompted us to investigate whether TGF- $\beta$ , when taken orally, can affect allergic immune responses. Repeated high-dose ovalbumin peptide (OVA) feeding was previously reported to induce OVA-specific IgE production and an anaphylactic reaction after intravenous challenge of OVA in OVA-TCR transgenic mice, which might represent a model for food allergy. By using this model, we showed here that oral administration of high-dose TGF- $\beta$  simultaneously with OVA feeding significantly inhibited the OVA-specific IgE elevation and anaphylactic reaction in OVA-TCR transgenic DO11.10 mice. These effects were associated with suppression of OVA-specific IL-4 production and GATA-3 expression and with up-regulation of IFN- $\gamma$  production and T-bet expression by splenocytes. Intra-peritoneal injection of anti-TGF- $\beta$ -neutralizing antibody abolished the inhibitory effects of orally administered TGF- $\beta$  on the serum IgE response and anaphylactic reaction after OVA feeding in DO11.10 mice. Interestingly, oral administration of high-dose TGF- $\beta$  suppressed activation-induced T cell death induced by OVA feeding in DO11.10 mice. We thus conclude that TGF- $\beta$ , when taken orally at high dose, has the capacity to modulate a food allergy-related reaction, at least in part, through its systemic activity.

## Introduction

Human milk is rich in nutrients, hormones and cytokines, which influence the growth, development and immune status of the newborn infant (1–3). These factors in human milk have been suggested to contribute to the reduced susceptibility of the breast-fed infants to infection and the development of allergic disease when compared with formula-fed infants (1–8), although the precise reasons remain unclear.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a multifunctional cytokine that regulates cell growth, differentiation and survival and is also involved in the regulation of the immune responses (9, 10). Human milk contains abundant TGF- $\beta$  although there is considerable variability in the concentration of TGF- $\beta$  in human milk ranging from ~200 to 3500 pg ml<sup>-1</sup> (11, 12). Previous studies have suggested that TGF- $\beta$  in human milk is associated with prevention of the development of allergic

disease during infancy (13, 14). For instance, Oddy *et al.* reported in their multivariate analyses that the risk of wheezing at 1 year of age was significantly decreased with increasing TGF- $\beta$ 1 dose received from breast-feeding (14). Although some immunoregulatory activities of TGF- $\beta$  such as induction of oral tolerance may explain such an anti-allergic property of TGF- $\beta$  in human milk (9), the mechanisms underlying those observations remain obscure. More importantly, although there is numerous evidence that TGF- $\beta$ , when applied systemically, affects several immune responses (9), it is not clear whether TGF- $\beta$ , when taken orally, can modulate systemic immune responses or not.

The primary aim of this study is therefore to investigate whether orally administered TGF- $\beta$  can affect systemic immune responses and prevent the development of allergic

## 2 Oral TGF- $\beta$ suppresses food allergy

disease. Shida *et al.* previously showed that oral administration of high-dose ovalbumin peptide (OVA) to OVA-specific TCR transgenic mice led to an increase in the levels of OVA-specific IgE in the sera and the subsequent intravenous challenge of OVA-fed transgenic mice with OVA resulted in anaphylactic reaction, which might be a useful tool for studies of the cellular and molecular mechanisms of the T cell and IgE responses to orally ingested antigen (15). By using this model, we tested whether oral administration of TGF- $\beta$  affected the food allergy-related immune reactions.

### Methods

#### Reagents

Recombinant human latent TGF- $\beta$ 1 was purchased from R&D Inc. (Minneapolis, MN, USA). The latent form of TGF- $\beta$ 1 was activated via acidification in 4 mM HCl containing 0.1% BSA, and, then, dissolved in saline before use. Chicken OVA was purchased from Sigma Chemical Co. (St Louis, MO, USA). Anti-TGF- $\beta$  mouse mAb that neutralized human/mouse TGF- $\beta$ 1, - $\beta$ 2 and - $\beta$ 3 activity (clone 1D11) was purchased from R&D Inc.

#### Mice

OVA-specific TCR transgenic mice on the BALB/c background, clone DO11.10, which recognizes the 323–339 peptide fragment of OVA, were originally obtained from K. Murphy (16) and housed under specific pathogen-free conditions in our facility. These mice were kept in conventional surroundings. Animal experiments were approved by the Institutional Review Board of Juntendo University and University of Yamanashi.

#### Study design

The DO11.10 mice (6–10 weeks old) were fed with 100 mg OVA and 5  $\mu$ g TGF- $\beta$ 1 or with 100 mg OVA plus control vehicle by gavaging every other day for a total of six times (day 1, 3, 5, 7, 9, 11). For some experiments, anti-TGF- $\beta$ -neutralizing antibody or isotype-matched control mouse IgG was intraperitoneally injected (100  $\mu$ g per mouse) on the same days as OVA and/or TGF- $\beta$  gavaging for a total of six times. To investigate the effects of systemic administration of TGF- $\beta$ 1 on immune responses induced by OVA feeding in DO11.10 mice, TGF- $\beta$ 1 or control PBS was subcutaneously injected (5  $\mu$ g per mouse) on the same days as OVA gavaging for a total of six times.

#### Flow cytometric analysis

Spleen cells ( $1 \times 10^6$ ) were stained with FITC-conjugated anti-CD4 mAb, PE-conjugated anti-clonotype-specific mAb KJ1-26 (these were purchased from BD Pharmingen, San Diego, CA, USA), Annexin V-FITC (BioVision, Research Products, Mountain View, CA, USA) or 7-aminoactinomycin D (7-AAD) (Sigma Chemical Co.) for 30 min. After washing with PBS, the cells were analyzed on FACSCalibur (BD, San Jose, CA, USA), and the data were analyzed by using WinMDI program (Scripps Research Institute, La Jolla, CA, USA).

#### Reverse transcription-PCR

Spleen cells were prepared into single-cell suspensions and CD4<sup>+</sup> T cells were isolated using T cell subset columns (R&D Inc.) according to the suggested protocol. Total RNA was then extracted by using the Isogen solution (Nippon Gene, Toyama, Japan) as recommended by the manufacture's instructions. Complementary DNA (cDNA) was synthesized from 2  $\mu$ g of total RNA using the first strand cDNA synthesis kit (Ready To Go; Amersham Biosciences Corp., Piscataway, NJ, USA). PCR amplification (Fas ligand and Fas; 94°C for 45 s, 55°C for 45 s and 72°C for 45 s; 35 cycles, T-bet and GATA-3; 94°C for 45 s, 60°C for 45 s and 72°C for 45 s; 30 cycles,  $\beta$ -actin; 94°C for 0.5 min, 56°C for 0.5 min and 72°C for 1 min; 35 cycles) was performed in a DNA engine cycler (MJ Research, Inc., Waltham, MA, USA). The PCR products were separated by 2.0% agarose gel electrophoresis and stained with 0.5  $\mu$ g ml<sup>-1</sup> ethidium bromide. The primer pairs (Fas ligand and Fas) used were purchased from R&D Inc. Mouse T-bet, GATA-3 and  $\beta$ -actin primers are as follows—T-bet: forward, 5'-AACCAG-TATCCTGTTCCCAGC-3', reverse, 5'-TGTCGCCACTGGAAG-GATAG-3'; GATA-3: forward, 5'-CTCCTTTTGTCTCTCTTTTC-3', reverse, 5'-AAGAGATGAGGACTGGAGTG-3' and  $\beta$ -actin: forward, 5'-TGGAATCCTGTGGCATCCATGAAAC-3', reverse, 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'.

#### Determination of antigen-specific IgE antibody in serum

Two days after the last feeding (day 13), the titer of OVA-specific IgE antibody in mouse serum was assessed by a 24-h passive cutaneous anaphylaxis reaction as described by Ovary (17).

#### Determination of antigen-specific IgG1 and IgG2a antibody in serum

The amount of OVA-specific IgG1 or IgG2a in mouse serum was measured by ELISA as described elsewhere (18). In brief, ELISA plates were coated with OVA (250  $\mu$ g ml<sup>-1</sup>), washed three times with PBS containing 0.05% Tween20 (PBST) and blocked with blocking buffer (PBS containing 2% BSA; Sigma Chemical Co.). Serum samples were added to the wells after 1 : 1000 or 1 : 3000 dilution in blocking buffer. As a control, serial dilutions of pooled serum from OVA-sensitized wild-type BALB/c mice were analyzed in each plate. After 1 h incubation, wells were washed with PBST, added either biotinylated anti-mouse IgG1 or IgG2a (Pharmingen) at 2  $\mu$ g ml<sup>-1</sup> in blocking buffer and incubated for 1 h. After washing, wells were incubated with 100  $\mu$ l of ExtrAvidin alkaline phosphatase (1 : 2000 dilution, Sigma Chemical Co.) for 45 min, washed with PBST and the reaction was developed with p-Nitrophenyl Phosphate Liquid Substrate System (pNPP) (Sigma Chemical Co.).

#### Induction of an anaphylactic reaction

Anaphylactic reaction was induced as previously described (15). Briefly, 1 mg of OVA dissolved in 0.1 ml PBS was injected into the tail vein of mice fed with OVA plus TGF- $\beta$ 1 or OVA plus control vehicle. The mice were observed for a 30-min period after OVA injection. The anaphylactic reaction was evaluated and rated according to four scores as follows: –, no reaction observed; +, the mice are slow and mobile if provoked; ++, the

mice are stationary even if provoked and +++, the mice lie down and have severe anaphylactic shock.

#### Cytokine ELISA

Spleen cells ( $5 \times 10^6$  cells) isolated from mice fed with OVA plus TGF- $\beta$ 1 or OVA plus control vehicle were re-stimulated with OVA ( $100 \mu\text{g ml}^{-1}$ ). Seventy-two hours after the culture, supernatants were collected for measurement of cytokines. The amount of IL-4, IL-12 and IFN- $\gamma$  in the culture supernatant of spleen cells was determined using murine IL-4, IL-12 and IFN- $\gamma$  ELISA kits (R&D Inc.).

#### Serum IgE levels

Blood was collected from DO11.10 mice at indicated times and serum samples were obtained by centrifugation and stored at  $-80^\circ\text{C}$  until use. Total IgE levels were measured by a sandwich murine IgE ELISA kit (Yamasa Co. Ltd, Chiba, Japan).

#### Data analysis

Data are summarized as mean  $\pm$  SD. An unpaired Student's *t*-test was used for the statistical analysis of the results.  $P < 0.05$  was considered to be significant.

### Results

#### *Repeated high-dose OVA feeding induced serum IgE elevation and an anaphylactic reaction following intravenous challenge with OVA in DO11.10 mice*

To determine whether orally administered TGF- $\beta$  could affect allergic immune responses, we examined the effect of orally administered TGF- $\beta$  on antigen-specific immune responses to orally ingested antigen in a mouse model of food allergy reported by Shida *et al.* (15). They showed that oral administration of high-dose OVA to OVA-specific TCR transgenic mice led to an increase in the levels of antigen-specific IgE in the sera and subsequent intravenous challenge of OVA-fed transgenic mice with OVA resulted in anaphylactic reaction.

According to their protocol with some modification, OVA-specific TCR transgenic mice DO11.10 were fed with 100 mg OVA and 5  $\mu\text{g}$  of TGF- $\beta$ 1 or with 100 mg OVA plus control vehicle every other day for a total of six times (day 1, 3, 5, 7, 9, 11). Two days after the last feeding (day 13), the mice were analyzed for antibody responses and anaphylactic reaction. This protocol induced total and OVA-specific serum IgE elevation and an anaphylactic reaction following intravenous OVA challenge in DO11.10 mice (Fig. 2 and Table 1, see below).

#### *Oral administration of TGF- $\beta$ inhibited activation-induced cell death of OVA-specific T cells induced by OVA feeding in DO11.10 mice*

Interestingly, spleens obtained from mice fed with OVA plus TGF- $\beta$ 1 according to the above protocol were relatively enlarged when compared with those obtained from mice fed with OVA plus control vehicle (Fig. 1A and spleen weight; OVA plus control vehicle:  $114 \pm 11$  mg versus OVA plus TGF- $\beta$ 1:

$144 \pm 13$  mg,  $n = 5$ ,  $P < 0.05$ ), suggesting that orally administered TGF- $\beta$ 1 affected systemic immune responses induced by OVA feeding in DO11.10 mice.

To determine the effect of oral administration of TGF- $\beta$ 1 on antigen-specific T cell responses, we examined the percentage of OVA-specific TCR-bearing CD4 T cells in splenic mononuclear cells by using KJ1-26 antibody recognizing the TCR (16). As shown in Fig. 1(B), the percentage of CD4<sup>+</sup>KJ1-26<sup>+</sup> T cells was significantly decreased in mice fed with OVA plus control vehicle when compared with non-treated control mice as previously described (19) (control mice:  $17.8 \pm 1.5\%$  versus OVA plus control vehicle:  $9.4 \pm 1.2\%$ ,  $n = 5$ ,  $P < 0.05$ ). Oral administration of TGF- $\beta$ 1 together with OVA restored the reduced percentage of CD4<sup>+</sup>KJ1-26<sup>+</sup> T cells (OVA plus control vehicle:  $9.4 \pm 1.2\%$  versus OVA plus TGF- $\beta$ 1:  $15.0 \pm 1.6\%$ ,  $n = 5$ ,  $P < 0.05$ ; Fig. 1B). The absolute number of CD4<sup>+</sup>KJ1-26<sup>+</sup> T cells was also significantly decreased in mice fed with OVA plus control vehicle when compared with non-treated control mice and it was comparable between non-treated control mice and OVA plus TGF- $\beta$ -fed mice (data not shown).

Because activation-induced T cell apoptosis via Fas/Fas ligand system was involved in the depletion of antigen-specific T cells by repeated high-dose OVA feeding in DO11.10 mice (19), we performed FACS analysis for splenic mononuclear cells 48 h after the first feeding (day 3) with an early apoptosis marker, Annexin V. As shown in Fig. 1(C), the percentage of Annexin V<sup>+</sup>7-AAD<sup>−</sup> cells (7-AAD was used to exclude non-specifically stained dead cells) in KJ1-26<sup>+</sup> T cells was increased in mice fed with OVA plus control vehicle when compared with non-treated control mice, which was also abrogated by oral administration of OVA plus TGF- $\beta$ 1. In addition, reverse transcription (RT)-PCR analysis showed that expression of Fas ligand, but not Fas, was decreased in CD4<sup>+</sup> splenic T cells obtained from mice fed with OVA plus TGF- $\beta$ 1 when compared with those obtained from mice fed with OVA plus control vehicle (Fig. 1D). Oral administration of TGF- $\beta$ 1 alone to DO11.10 mice did not affect their spleen size or other parameters examined here (data not shown). These results indicated that oral administration of TGF- $\beta$  inhibited activation-induced cell death (AICD) of OVA-specific T cells induced by OVA feeding in DO11.10 mice. The reduction of Fas ligand expression may explain, in part, the reduction of OVA-induced AICD in DO11.10 mice fed with OVA plus TGF- $\beta$ .

#### *Oral administration of TGF- $\beta$ suppressed food allergy-related immune responses induced by OVA feeding in DO11.10 mice*

We then examined the effects of orally administered TGF- $\beta$  on food allergy-related immune responses in this model. Total serum IgE elevation was completely suppressed in mice fed with OVA plus TGF- $\beta$ 1 when compared with mice fed with OVA plus control vehicle 2 and 9 days after the last feeding (Fig. 2A). The levels of OVA-specific IgE and IgG1 in the sera were also decreased, whereas the levels of OVA-specific IgG2a were increased in mice fed with OVA plus TGF- $\beta$ 1 when compared with mice fed with OVA plus control vehicle 2 days after the last feeding (Fig. 2B).

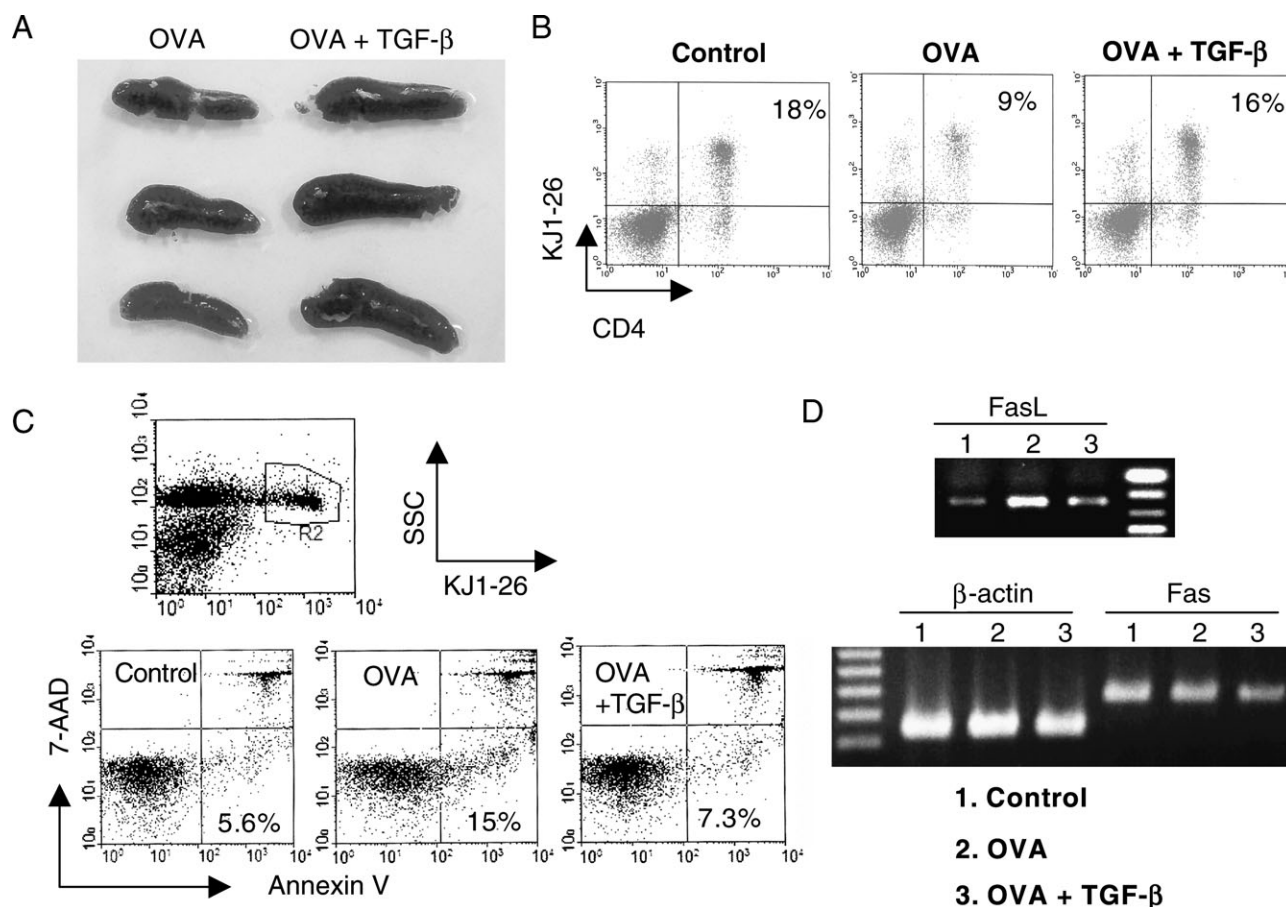


**Fig. 1.** Suppression of splenic T cell depletion after OVA feeding in DO11.10 mice by oral administration of TGF- $\beta$ . (A–B) DO11.10 mice were fed with 100 mg OVA plus 5  $\mu$ g TGF- $\beta$ 1 or 100 mg OVA plus control vehicle every other day for a total of six times. Mice were killed 2 days after the last feeding, and spleens were removed. (A) A representative picture showing relatively enlarged spleens removed from mice fed with OVA plus TGF- $\beta$ 1 (right) when compared with those from OVA plus control vehicle (left). (B) A representative FACS analysis showing the percentage of CD4 $^{+}$  KJ1-26 $^{+}$  T cells in splenic mononuclear cells. Spleen cells were stained with FITC-anti-CD4 mAb and PE-KJ1-26 mAb. (C–D) DO11.10 mice were fed with 100 mg OVA plus 5  $\mu$ g TGF- $\beta$ 1 or with 100 mg OVA plus control vehicle and were killed 2 days after the feeding (day 3) and spleen cells were removed. (C) A representative FACS analysis showing the percentage of Annexin V $^{+}$ 7-AAD $^{-}$  T cells in KJ1-26 $^{+}$  T cells. Spleen cells were stained with FITC-Annexin V, PE-KJ1-26 mAb and 7-AAD (FL3), then gated on KJ1-26 $^{+}$  cells (upper panel, R2), followed by two-color analysis. (D) RT-PCR analysis for Fas ligand/Fas expression. CD4 $^{+}$  T cells were prepared from the spleen, followed by RNA extraction. RT-PCR was then performed with specific primers for Fas ligand (FasL), Fas and  $\beta$ -actin. Similar results were obtained for at least three independent experiments. Control: non-treated control DO11.10 mice; OVA: DO11.10 mice fed with OVA; OVA + TGF- $\beta$ : DO11.10 mice fed with OVA plus TGF- $\beta$ 1.

In addition, OVA-induced IL-4 production by splenocytes was decreased, whereas OVA-induced IFN- $\gamma$  or IL-12 productions were increased in mice fed with OVA plus TGF- $\beta$ 1 when compared with mice fed with OVA plus control vehicle (Fig. 2C). Furthermore, RT-PCR analysis showed that expression of T-bet, a marker of T $_{H}$ 1-type immune responses (20), was increased, whereas expression of GATA-3, a marker of T $_{H}$ 2-type immune responses (20), was decreased in splenic CD4 T cells from mice fed with OVA plus TGF- $\beta$ 1 when compared with mice fed with OVA plus control vehicle (Fig. 3).

Importantly, mice fed with OVA plus TGF- $\beta$ 1 showed no or only a weak anaphylactic reaction although mice fed with OVA plus control vehicle showed an anaphylactic reaction after intravenous challenge of OVA as described previously (15) (Table 1). These results indicated that oral administration of TGF- $\beta$  suppressed serum IgE response and anaphylactic reaction associated with OVA feeding in DO11.10 mice.

#### *Anti-TGF- $\beta$ antibody abrogated the inhibitory effects of orally administered TGF- $\beta$ on serum IgE response and anaphylactic reaction associated with OVA feeding in DO11.10 mice*

To investigate the mechanisms underlying the above observations, we asked whether neutralization of systemic TGF- $\beta$  activity by anti-TGF- $\beta$ -neutralizing antibody affected the inhibitory effects of orally administered TGF- $\beta$  on serum IgE response and anaphylactic reaction associated with OVA feeding in DO11.10 mice.

As shown in Fig. 4 and Table 1, intra-peritoneal injection of anti-TGF- $\beta$  antibody abrogated the suppression of the total and OVA-specific IgE production and anaphylactic reaction by OVA plus TGF- $\beta$  feeding in DO11.10 mice. OVA-specific IgG2a production in mice fed with OVA plus TGF- $\beta$  was reduced to the background levels by the treatment with

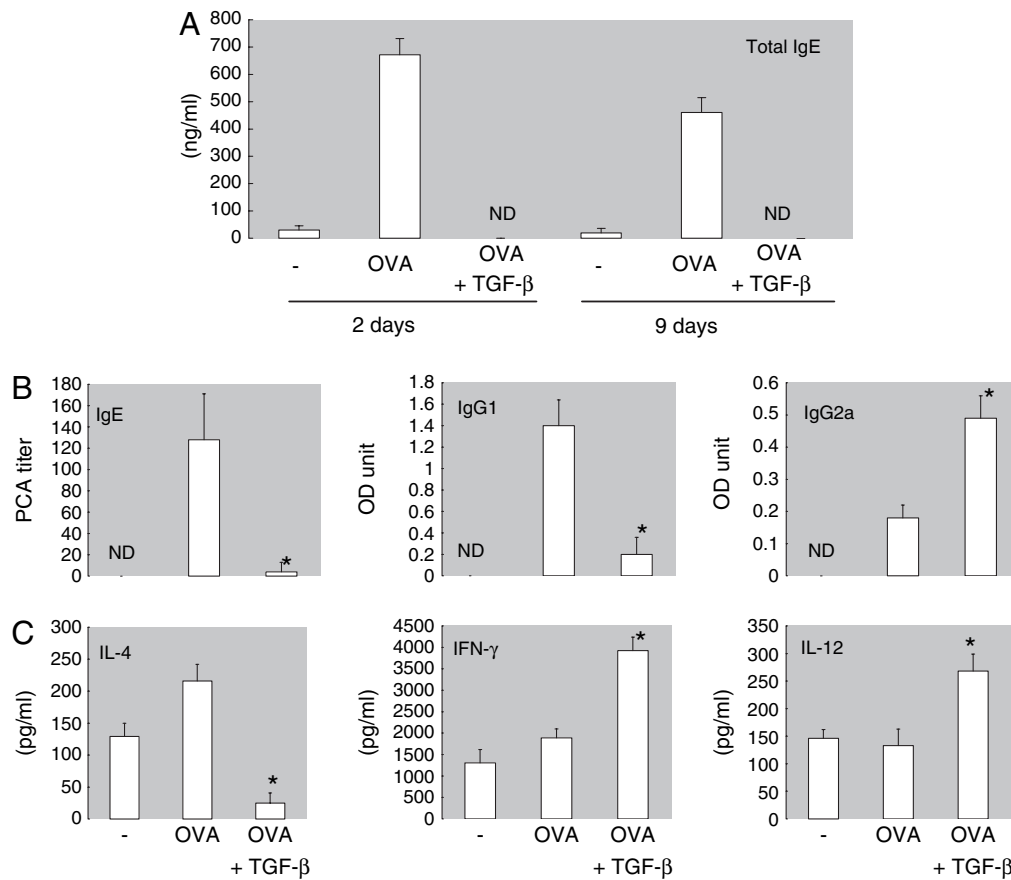


**Fig. 2.** Inhibition of Th2-type immune responses after OVA feeding in DO11.10 mice by oral administration of TGF- $\beta$ . DO11.10 mice were fed with 100 mg OVA plus 5  $\mu$ g TGF- $\beta$ 1 or OVA plus control vehicle every other day for a total of six times. Mice were sacrificed 2 days (or also 9 days for total serum IgE evaluation) after the last feeding and (A) total serum IgE levels were assessed by ELISA. (B) The titer of anti-OVA IgE, IgG1 or IgG2a antibody in mouse sera was assessed by a 24-h passive cutaneous anaphylaxis reaction or by ELISA. (C) Spleen cells were obtained from the mice 2 days after the third feeding (day 7), re-stimulated with OVA (100  $\mu$ g ml<sup>-1</sup>) for 72 h and the amount of IL-4, IFN- $\gamma$  or IL-12 in the culture supernatant was determined by ELISA. Values represent the mean  $\pm$  SD of five mice per group. \* $P$  < 0.05 compared with corresponding control. OVA: DO11.10 mice fed with OVA; OVA + TGF- $\beta$ : DO11.10 mice fed with OVA plus TGF- $\beta$ 1; ND: not detected.

**Table 1.** Suppression of an anaphylactic reaction by orally administered TGF- $\beta$

| Mice                                            | No. of mice showing anaphylactic reaction |   |    |     |
|-------------------------------------------------|-------------------------------------------|---|----|-----|
|                                                 | –                                         | + | ++ | +++ |
| Control                                         | 4                                         | 0 | 0  | 0   |
| OVA                                             | 0                                         | 1 | 4  | 1   |
| OVA + TGF- $\beta$ 1                            | 4                                         | 2 | 0  | 0   |
| TGF- $\beta$ 1                                  | 4                                         | 0 | 0  | 0   |
| OVA + TGF- $\beta$ 1 + $\alpha$ TGF- $\beta$ Ab | 0                                         | 2 | 4  | 0   |
| OVA + TGF- $\beta$ 1 + control Ab               | 3                                         | 3 | 0  | 0   |

OVA-specific TCR transgenic mice DO11.10 were fed with 100 mg OVA plus 5  $\mu$ g of TGF- $\beta$ 1 or with 100 mg OVA plus control vehicle every other day for a total of six times. Two days after the last feeding, the mice were challenged intravenously with OVA, and the anaphylactic reaction observed was evaluated. The extent of anaphylactic reaction was rated according to four scores, as described in Methods. Control: non-treated DO11.10 mice; OVA: DO11.10 mice fed with OVA; OVA + TGF- $\beta$ : DO11.10 mice fed with OVA plus TGF- $\beta$ 1; TGF- $\beta$ : DO11.10 mice fed with TGF- $\beta$ 1; OVA + TGF- $\beta$ 1 +  $\alpha$ TGF- $\beta$  Ab (or control Ab): DO11.10 mice fed with OVA plus TGF- $\beta$ 1 injected intra-peritoneally with anti-TGF- $\beta$  antibody (or control antibody).

anti-TGF- $\beta$  antibody (Fig. 4B). Thus, the inhibitory effects of orally administered TGF- $\beta$  on serum IgE response and anaphylactic reaction might be attributed, in part, to its systemic activity possibly appeared after absorption of orally administered TGF- $\beta$  from the mouse intestine.

#### Systemic administration of TGF- $\beta$ suppressed both serum IgE and IgG2a responses induced by OVA feeding in DO11.10 mice

Based on the findings that anti-TGF- $\beta$  antibody abrogated the inhibitory effects of orally administered TGF- $\beta$  on serum IgE response and an anaphylactic reaction, we asked whether systemic administration of TGF- $\beta$  could also show similar effects to orally administered TGF- $\beta$ . As shown in Fig. 5, subcutaneous injection of TGF- $\beta$ 1 simultaneously with OVA feeding suppressed not only OVA-specific IgE elevation but also OVA-specific IgG2a elevation in DO11.10 mice. Total IgE elevation was also suppressed by subcutaneous injection of TGF- $\beta$ 1, which was associated with the inhibition of anaphylactic reaction to intravenous challenge with OVA (data not shown). Furthermore, when purified mouse CD4 T cells were

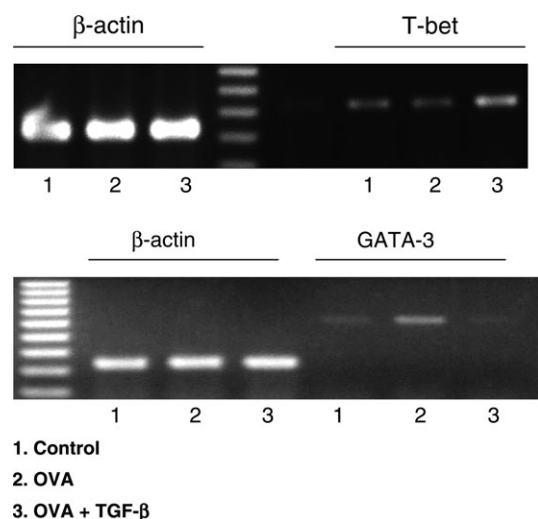


**Fig. 3.** Inhibition of GATA-3 expression in splenic CD4<sup>+</sup> T cells after OVA feeding in DO11.10 mice by oral administration of TGF- $\beta$ . DO11.10 mice were fed with 100 mg OVA plus 5  $\mu$ g TGF- $\beta$ 1 or OVA plus control vehicle every other day for a total of six times. Mice were sacrificed 2 days after the last feeding and CD4<sup>+</sup> T cells were prepared from the spleen, followed by RNA extraction. RT-PCR analysis was then performed with specific primers for mouse T-bet, GATA-3 or  $\beta$ -actin. Similar results were obtained for at least three independent experiments. Control: non-treated DO1.10 mice; OVA: DO11.10 mice fed with OVA; OVA + TGF- $\beta$ : DO11.10 mice fed with OVA plus TGF- $\beta$ 1.

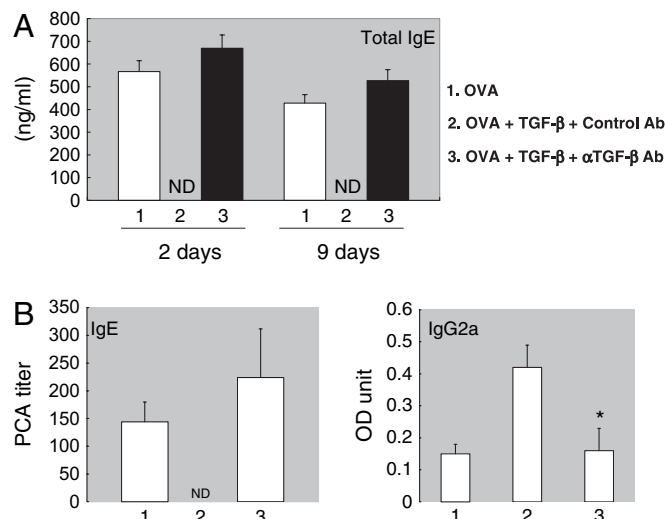


**Fig. 4.** Abrogation of the inhibitory effects of orally administered TGF- $\beta$  on serum IgE response after OVA feeding in DO11.10 mice by anti-TGF- $\beta$  antibody. DO11.10 mice were fed with 100 mg OVA plus 5  $\mu$ g TGF- $\beta$ 1 or OVA plus control vehicle every other day for a total of six times. Anti-TGF- $\beta$ -neutralizing antibody ( $\alpha$ TGF- $\beta$  Ab) (100  $\mu$ g per mouse) or isotype-matched control mouse IgG (control Ab) was injected intra-peritoneally on the same day as OVA and/or TGF- $\beta$ 1 feeding for a total of six times. Mice were sacrificed 2 days (or also 9 days for total serum IgE evaluation) after the last feeding and (A) total serum IgE levels were assessed by ELISA. (B) The titer of anti-OVA IgE or IgG2a antibody in mouse sera was assessed by a 24-h passive cutaneous anaphylaxis reaction or by ELISA. Values represent the mean  $\pm$  SD of five mice per group. \* $P$  < 0.05 compared with corresponding control. OVA: DO11.10 mice fed with OVA; OVA + TGF- $\beta$ : DO11.10 mice fed with OVA plus TGF- $\beta$ 1; ND: not detected.

cultured in T<sub>H</sub>1-skewed or T<sub>H</sub>2-skewed condition in the presence of TGF- $\beta$  *in vitro*, expression of T-bet or GATA-3, respectively, was inhibited as described before (21, data not shown). Thus, it appeared that systemic administration of TGF- $\beta$  suppressed both T<sub>H</sub>1- and T<sub>H</sub>2-type immune responses, whereas oral administration of TGF- $\beta$  suppressed T<sub>H</sub>1-, and augmented T<sub>H</sub>2-type immune responses induced by OVA feeding in DO11.10 mice.

## Discussion

Some epidemiological or association studies showing that TGF- $\beta$  in breast milk may reduce the risk of allergic disease during infancy have led us to investigate whether TGF- $\beta$ , when taken orally, can affect allergic immune responses. The results clearly demonstrated that TGF- $\beta$ , when taken orally as well as when applied systemically, had the capacity to modulate a food allergy-related immune response.

Interestingly, the effects of orally administered TGF- $\beta$  on a food allergy-related immune response were somewhat different from those of systemically applied TGF- $\beta$ . Orally

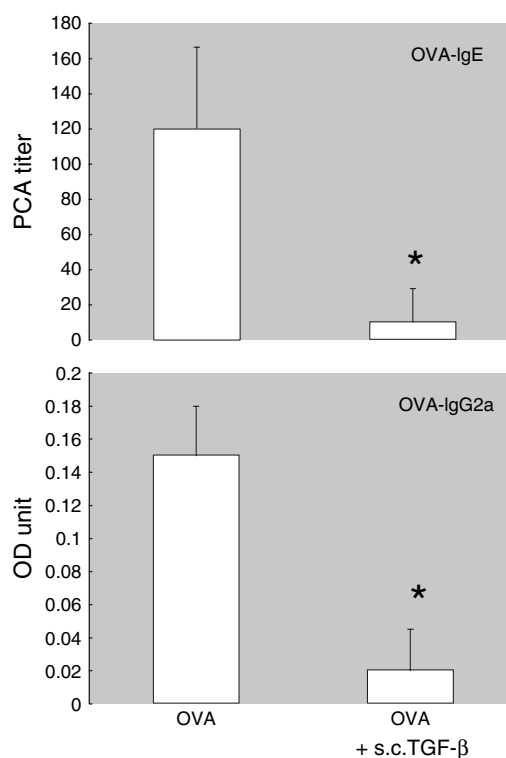


**Fig. 5.** Inhibition of OVA-specific IgE and IgG2a responses after OVA feeding in DO11.10 mice by subcutaneous (s.c.) injection of TGF- $\beta$ . DO11.10 mice were fed with 100 mg OVA or PBS every other day for a total of six times. TGF- $\beta$ 1 (5  $\mu$ g per mouse) or control vehicle was injected s.c. on the same days as OVA feeding for a total of six times. Mice were sacrificed 2 days after the last feeding and the titer of anti-OVA IgE or IgG2a antibody in mouse sera was assessed by a 24-h passive cutaneous anaphylaxis reaction or by ELISA. Values represent the mean  $\pm$  SD of five mice per group. \* $P$  < 0.05 compared with corresponding control. OVA: DO11.10 mice fed with OVA and injected s.c. with control vehicle; OVA + s.c. TGF- $\beta$ : DO11.10 mice fed with OVA and injected s.c. with TGF- $\beta$ 1.

administered TGF- $\beta$  suppressed T<sub>H</sub>2-type immune responses with significant T<sub>H</sub>1 skewing, whereas systemically administered TGF- $\beta$  suppressed both T<sub>H</sub>1- and T<sub>H</sub>2-type immune responses induced by high-dose OVA feeding in DO11.10 mice (Figs 2 and 5). Previous *in vitro* studies suggested that TGF- $\beta$  inhibited both T<sub>H</sub>1- and T<sub>H</sub>2-type T cell development associated with suppression of T-bet and GATA-3 (21), which was also confirmed by our hands (data not shown). The outcome of TGF- $\beta$  effects on T cells was shown to strongly depend on the cellular context such as stage of differentiation and cytokine milieu (22). It is thus possible that administration of TGF- $\beta$  through oral route stimulates many types of cells which produce various cytokines in response to TGF- $\beta$ , e.g. intestinal epithelial cells (23), and effects of TGF- $\beta$  on T cells are affected through the cytokine milieu. Clarification of mechanisms underlying the specific enhancing effects of orally administered TGF- $\beta$  on T<sub>H</sub>1-type immune responses is an important and interesting issue in future studies.

Because TGF- $\beta$  is secreted as a latent form and then activated by extremes of pH, heat, proteases and thrombospondin-1 extracellularly (24), the latent form of TGF- $\beta$  present in human milk is thought to be activated by acidification at low pH of gastric juice in the stomach and maintain its biological activity when milk is taken orally (3).

Consistent with the above-mentioned notion, Letterio *et al.* previously showed that orally administered TGF- $\beta$  was absorbed from the intestine and appeared in systemic circulation in mice (25). We also found in this study that neutralization of systemic TGF- $\beta$  activity by intra-peritoneal administration of anti-TGF- $\beta$  antibody abrogated the effects of orally administered TGF- $\beta$  on serum IgE response and anaphylactic reaction (Fig. 4). Taken together, systemic activity possibly appeared after absorption of orally administered TGF- $\beta$  from the mouse intestine might explain, at least in part, the effects of orally administered TGF- $\beta$  observed in the current study.

Our preliminary study showed that, when mice were fed with relatively low-dose TGF- $\beta$ 1 (5–10 ng per mouse), the effects on serum IgE response and anaphylactic reaction were marginal in contrast to the dose (5  $\mu$ g per mouse) used in current study (data not shown). Although TGF- $\beta$  may be relatively resistant to degradation by acidification of gastric juice or proteolytic enzyme in the intestine when compared with other cytokines, it should undergo such degradation to some extent. Therefore, we speculate that a small portion of high-dose TGF- $\beta$  escaped from the degradation might be absorbed from the mouse intestine, enter the circulation keeping its activity and affect systemic immune responses.

TGF- $\beta$  has been shown to suppress antigen-induced T cell death, at least in part, through regulation of Fas ligand expression (26), which was consistent with the current results (Fig. 1). For example, Chen *et al.* reported that activation-induced T cell death induced by anti-CD3 mAb was strikingly enhanced in TGF- $\beta$ 1 null mice with increase of Fas/Fas ligand expression (27). Our results also suggest that orally administered TGF- $\beta$  as well as endogenous TGF- $\beta$  can regulate T cell survival via regulation of Fas/Fas ligand system.

The relationship between inhibition of AICD and modulation of T<sub>H</sub>1/T<sub>H</sub>2-type immune responses by orally administered TGF- $\beta$  remains unclear. Naive, T<sub>H</sub>1-type and T<sub>H</sub>2-type T cells

have been suggested to have different susceptibility to AICD although it appears to be still controversial (28, 29). We speculate that orally administered TGF- $\beta$  might preferentially inhibit AICD of T<sub>H</sub>1-differentiating, but not T<sub>H</sub>2-differentiating, T cells, resulting in enhanced T<sub>H</sub>1-type responses as suggested by the literature (22).

Interestingly, we did not find any significant effects of orally administered TGF- $\beta$  on other body systems (e.g. the respiratory system) than the immune system in mice in our experimental protocol (data not shown). This may be due to the short half-life of active TGF- $\beta$  in circulation (1–2 min) (30) or different sensitivity to TGF- $\beta$  in different organs. In fact, we failed to detect clear differences in serum concentrations of TGF- $\beta$ 1 between non-treated mice and TGF- $\beta$ -fed mice at least up to 4 h after TGF- $\beta$ 1 feeding, which might be due to rapid clearance of the cytokine *in vivo* (data not shown). In anyway, unfavorable consequences that might occur by more long-term or high-dose oral administration of TGF- $\beta$ , such as fibrosis, must be investigated carefully in future studies.

In summary, we showed that orally administered TGF- $\beta$  could modulate a food allergy-related immune response. Thus, TGF- $\beta$ , when taken orally at high dose, appears to have the capacity to affect systemic immune responses. The results may explain, at least in part, the previous observations that TGF- $\beta$  in human milk is associated with prevention of the development of allergic disease during infancy (13, 14). However, physiological relevance of our study remains unclear because we cannot say that the total amount of orally administered TGF- $\beta$ 1 used in the current study (30  $\mu$ g per mouse) corresponds to the total amount of TGF- $\beta$ 1 that breast-feeding infants receive due to the difficulty of dosage comparison between mice and humans. Thus, a more direct approach (e.g. an experiment to test if neutralizing antibodies against TGF- $\beta$  suppress the ability of breast milk to reduce allergic responses.) is still necessary to clarify the role of TGF- $\beta$  in human milk.

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## Abbreviations

|              |                                     |
|--------------|-------------------------------------|
| 7-AAD        | 7-aminoactinomycin D                |
| AICD         | activation-induced cell death       |
| cDNA         | complementary DNA                   |
| OVA          | ovalbumin peptide                   |
| PBST         | PBS containing 0.05% Tween20        |
| RT           | reverse transcription               |
| TGF- $\beta$ | transforming growth factor- $\beta$ |

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## 8 Oral TGF- $\beta$ suppresses food allergy

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