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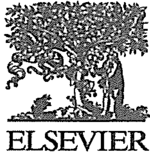

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T-cell receptor antagonist modifies cytokine secretion profile of naive CD4$^+$ T cells and their differentiation into type-1 and type-2 helper T cells

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Abstract

A T-cell receptor (TCR) antagonist is an analog of a peptide ligand for TCR that inhibits T-cell responses to the original peptide. We investigated the effects of a TCR antagonist on cytokine secretion of naive CD4$^+$ T cells and their differentiation into type-1 and type-2 helper T cells (Th1 and Th2) induced by stimulation with varying doses of an antigenic peptide. In the presence of a TCR antagonist peptide, proliferation of naive CD4$^+$ T cells and antigen dose-dependent secretion of interferon-γ, a typical Th1-type cytokine, by these cells was down-regulated. With respect to the secretion of interleukin-4 (IL-4), a typical Th2-type cytokine, the TCR antagonist raised the concentration of the antigenic peptide required to elicit maximal IL-4 production and, surprisingly, significantly increased the maximum level of IL-4 secretion. Similar effects induced by the TCR antagonist were observed on the Th1/Th2 differentiation of naive CD4$^+$ T cells. These results clearly indicate that, for naive CD4$^+$ T cells, a TCR antagonist has the potential to change the balance of Th1/Th2 cytokine secretion and even enhance Th2 responses.
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Keywords: TCR antagonist; Naive CD4$^+$ T cell; Th1/Th2 cytokine response

1. Introduction

The T-cell receptor (TCR) recognizes an antigen as a short peptide bound to a major histocompatibility complex (MHC) molecule on the surface of antigen presenting cells (APCs). Interaction between the TCR and MHC/peptide complex leads to activation of T cells, which is strictly regulated by environmental factors, including the structure and dose of TCR ligands. It has been well documented that changes in the structure and dose of an antigenic peptide modifies T-cell responses, not only in quantity, but also in quality, i.e. partial elicitation of effector functions [1–5] or alteration of cytokine responses [6,7]. In naive CD4$^+$ T cells from TCR-transgenic mice, differences in the structure or dose of an antigenic peptide profoundly affect polarized differentiation into two types of helper T cells, interferon-γ (IFN-γ)-predominant type-1 and interleukin (IL)-4-predominant type-2 helper T cells (Th1 and Th2) [8–11]. Furthermore, we have previously shown that changes in the structure or dose of an antigenic peptide altered the Th1/Th2-type cytokine secretion profile of naive CD4$^+$ T cells even in their primary responses [12,13].

Certain analogs of an antigenic peptide containing single amino acid substitutions in the TCR contact residues have been shown to fail to stimulate T-cell responses on their own, but are able to diminish, or even inhibit, T-cell activation induced by the original peptide. These peptides are known as TCR antagonists [14]. The inhibitory function of TCR antagonist has been shown not only in vitro but also in vivo [15–17]. Previous studies on TCR antagonists have mainly used T-cell clones [18,19] and, therefore, the effects of TCR antagonists on the primary responses of naive CD4$^+$ T cells and on their functional differentiation are yet to be elucidated.

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In the present study, we utilized naive $CD4^+$ T cells derived from ovalbumin (OVA)-specific TCR-transgenic mice (OVA23-3) and examined the effects of a TCR antagonist on distinct Thl/Th2 responses elicited by varying dose of a specific antigenic peptide. Our results demonstrate that a TCR antagonist not only inhibits T-cell responses but also modifies the cytokine secretion profile and Thl/Th2 differentiation of naive $CD4^+$ T cells.

2. Materials and methods

2.1. Mice

Six-week-old female BALB/c mice were purchased from Japan CLEA (Tokyo, Japan). OVA323-339-specific TCR Tg mice (OVA23-3) with the BALB/c genetic background [20] were generously provided by Prof. S. Habu (Tokai University, Isehara, Japan) and maintained in our animal facility. All studies were performed according to the Guidelines for Animal Experiments of the Faculty of Agriculture, The University of Tokyo.

2.2. Peptides

OVA323-339 (ISQAVHAAHAEINEAGR) and a series of single amino acid-substituted analogs of OVA323-339 were purchased from Mimotopes Pty., Ltd. (Clayton, Victoria, Australia).

2.3. Preparation of naive $CD4^+$ T cells and APC

Naive $CD4^+$ T cells were prepared as described previously [13]. T cell-depleted splenocytes, used as APCs, were prepared from splenocytes of BALB/c mice by incubation with anti-mouse Thy 1.2 anti-serum (Cederlane, Ontario, Canada), followed by incubation with low-toxicity rabbit complement (Cederlane), and were treated with 50 mg/ml mitomycin C (Sigma, St. Louis, MO, USA). Isolated naive $CD4^+$ T cells and APCs were routinely >96% $CD4^+CD62L^{high}$ and <5% $Thy1.2^+$, respectively.

2.4. T-cell proliferation assay and TCR-antagonist assay

Naive $CD4^+$ T cells (1×10^5 cells/well) in a total volume of 200 μl were cultured with APCs (3×10^5 cells/well) for 96 h in the presence of each peptide in 96-well flat-bottom plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA). Proliferation was assessed by measuring the incorporation of [^{3}H] thymidine (37 kBq/well) during the final 24 h of culture. To screen TCR-antagonist peptides, naive $CD4^+$ T cells (1×10^5 cells/well) were cultured with APCs (3×10^5 cells/well) for 96 h in the presence of OVA323-339 plus each analog peptide. For the prepulse TCR-antagonist assay, naive $CD4^+$ T cells (1×10^5 cells/well) were cultured in the presence of each analog peptide and APCs (3×10^5 cells/well) preincubated with OVA323-339 at 0.2 μM for 2 h at 37 °C.

2.5. Cytokine secretion assay

Naive $CD4^+$ T cells (1×10^5 cells/well) were cultured with APCs (3×10^5 cells/well) in the presence of peptides in 96-well flat-bottom plates in a total volume of 200 μl. The culture supernatants were recovered after 24 h for assaying IL-2 and after 72 h for assaying IL-4 and IFN-γ.

To examine the effect of TCR-antagonists on Thl/Th2 differentiation, primary cultures were set up in 24-well flat-bottomed plates with naive $CD4^+$ T cells (1×10^6 cells/well) and APCs (1×10^6 cells/well) plus various concentrations of OVA323-339 and 50 μM A326V in a total volume of 2 ml. After 7 days of priming, viable cells were recovered and counted. T cells (1×10^5 cells/well) were re-plated in 200 μl volume in 96-well plates with fresh APCs (3×10^5 per well) and 5 μM of OVA323-339. Supernatants were collected for cytokine analyses 48 h later for assaying IL-4, IL-5, and IFN-γ. The cytokine concentration was determined by means of two-site enzyme-linked immunosorbent assay (ELISA) as described previously [12].

2.6. Intracellular cytokine staining

Naive $CD4^+$ T cells (1×10^6 per well) and APCs (1×10^6 per well) were cultured with OVA323-339 at 0.05, 0.5, or 5 μM in the presence or absence of 50 μM A326V in 24-well plates in a total volume of 2 ml for 7 days. Viable cells (1×10^6 per well) were stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 6 h in the presence of monensin in 24-well plates. Cells were stained with anti-CD4 labeled with cy-chrome (BD PharMingen, San Diego, CA, USA). Cells were then fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and made permeable by washing twice with 0.5% saponin in PBS. Cells were incubated with anti-IFN-γ labeled with fluorescein isothiocyanate (BD PharMingen) and anti-IL-4 labeled with phycoerythrin (BD PharMingen). Their staining profiles were analyzed by BD LSR and CellQuest Software (BD Biosciences, Mountain View, CA, USA).

2.7. Quantitative RT-PCR

Naive $CD4^+$ T cells (1×10^6 per well) in a total volume of 2 ml were cultured with peptides in the presence of APC (3×10^6 per well) in 24-well plates. Cells were harvested 24 h after stimulation. Preparation of total RNA and reverse transcription was performed as described previously [13]. The LightCycler PCR and real-time detection system (Roche Diagnostics GmbH, Mannheim, Germany) was used for the measurement of relevant mRNAs for GATA-3 and GAPDH as described previously [13]. The level of GAPDH mRNA was used to normalize the amounts of assayable RNA in each

sample. The data are shown as relative expression index, compared to the lowest amount of mRNA detected.

3. Results

3.1. Screening of TCR-antagonist peptides for naive CD4+ T cells

In our previous study, we identified the essential residues in OVA323-339 that interact with the TCR of $CD4^+$ T cells derived from OVA23-3 mice. We also showed that substitutions of ^{326}Ala can modify the primary activation profile of naive $CD4^+$ T cells including proliferation and cytokine secretion [12]. Therefore, a series of analogs of OVA323-339 with all 19 possible substitutions for ^{326}Ala was prepared to identify TCR-antagonist peptides.

First, the degree of proliferation of naive $CD4^+$ T cells induced by stimulation with each analog was determined (Fig. 1A). Analogs with a substitution of Asp, Glu, Phe, Lys, Leu, Met, or Val for ^{326}Ala failed to induce T-cell proliferation. Next, we tested the ability of each analog to act as a TCR antagonist for the $CD4^+$ T cells stimulated with OVA323-339 (Fig. 1B). Analog peptides, A326D, A326F, and A326V effectively inhibited $CD4^+$ T-cell proliferation. Furthermore, a prepulse assay used to confirm TCR-antagonist activity demonstrated that A326V efficiently inhibited the proliferation of $CD4^+$ T cells stimulated with APC preincubated with OVA323-339 in a dose-dependent manner (Fig. 1C). These results show that A326V is able to act as a potent TCR antagonist for $CD4^+$ T cells derived from OVA23-3 mice. We then examined the effect of A326V on the proliferative response of $CD4^+$ T cells stimulated with various doses of OVA323-339. A326V, at 50 μM, down-regulated the proliferation of $CD4^+$ T cells stimulated with OVA323-339 at less than 5 μM.

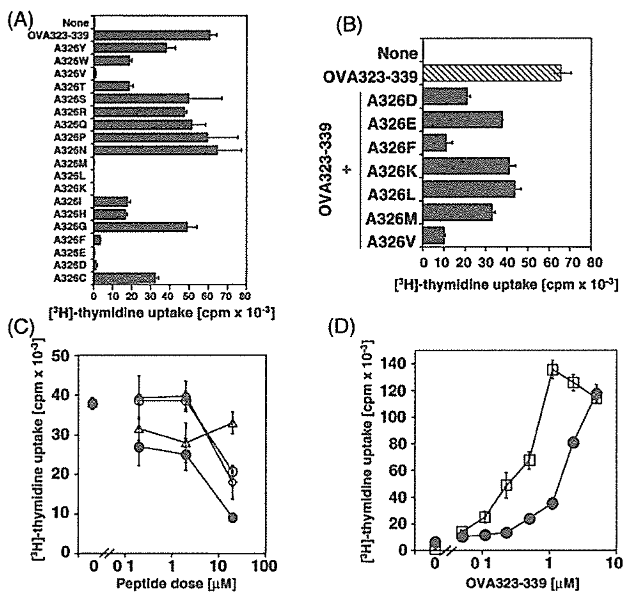

Fig. 1. Identification of OVA323-339 analogs with TCR-antagonist activity for naive $CD4^+$ T cells of OVA23-3 transgenic mice. Proliferative response of $CD4^+$ T cells was determined by culturing $CD4^+$ T cells in the presence of APCs and peptides for 96 h. (A) Proliferation of $CD4^+$ T cells stimulated with OVA323-339 or its analogs at 5 μM. (B) Proliferation of $CD4^+$ T cells stimulated with OVA323-339 at 0.02 μM in the absence or presence of each analog peptide at 20 μM. (C) A prepulse assay to determine TCR-antagonist activity of each analog peptide; proliferation of $CD4^+$ T cells stimulated with indicated concentrations of A326D (open circle), A326F (open diamond), A326V (closed circle), or A326L (open triangle) in the presence of APCs preincubated with OVA323-339 at 0.2 μM for 2 h at 37 °C. (D) Proliferation of $CD4^+$ T cells stimulated with various concentrations of OVA323-339 in the absence (open square) or presence (closed circle) of A326V at 50 μM. The data are shown as the average from triplicate cultures ± S.D. The results shown are representative of more than three independent experiments.

3.2. A326V did not induce cytokine secretion by itself but altered the cytokine secretion profile of naive CD4+ T cells and the Th1/Th2 differentiation profile induced by varying doses of OVA323-339

As we reported previously [13], OVA323-339 induced a distinct cytokine secretion profile in naive $CD4^+$ T cells from OVA23-3 mice, depending on the dose of the peptide (Fig. 2B–D, open square). Of all the doses tested, maximal IL-4 secretion was induced by stimulation at 0.1 μM and decreasing IL-4 secretion was observed by stimulation at higher concentrations. Secretion of IL-2 and IFN-γ was increased in a dose-dependent fashion. In contrast, A326V alone did not elicit secretion of IL-2, IFN-γ, or IL-4 at any of the tested doses (data not shown).

We examined the effects of A326V on cytokine secretion from naive $CD4^+$ T cells induced by OVA323-339. OVA323-339 at 0.5 μM, in the absence of A326V, induced IFN-γ secretion but not IL-4 secretion, a typical Th1-type cytokine response (Fig. 2A). Addition of A326V to the culture inhibited IFN-γ secretion completely and induced IL-4 secretion, resulting in a typical Th2 profile (Fig. 2A). These results clearly indicate that, whereas a TCR antagonist inhibits the proliferation of naive $CD4^+$ T cells in response to antigenic peptide stimulation, it has the potential to completely change the Th1/Th2 cytokine balance of these cells.

Next, the influence of A326V on the dose-dependent cytokine pattern induced by varying doses of OVA323-339 was analyzed. In the presence of A326V, secretion of IL-2 and IFN-γ was down-regulated at every dose of OVA323-339 tested (Fig. 2B and C). A326V appears to raise the concentration of OVA323-339 required to elicit secretion of the same levels of IL-2 and IFN-γ as those induced in the absence of the TCR antagonist. Likewise, in the case of IL-4, the dose of OVA323-339 eliciting secretion of the highest amount of IL-4 was shifted from 0.1 to 0.5 μM in the presence of A326V (Fig. 2D). Interestingly, the maximum amount of IL-4 secreted was increased considerably in the

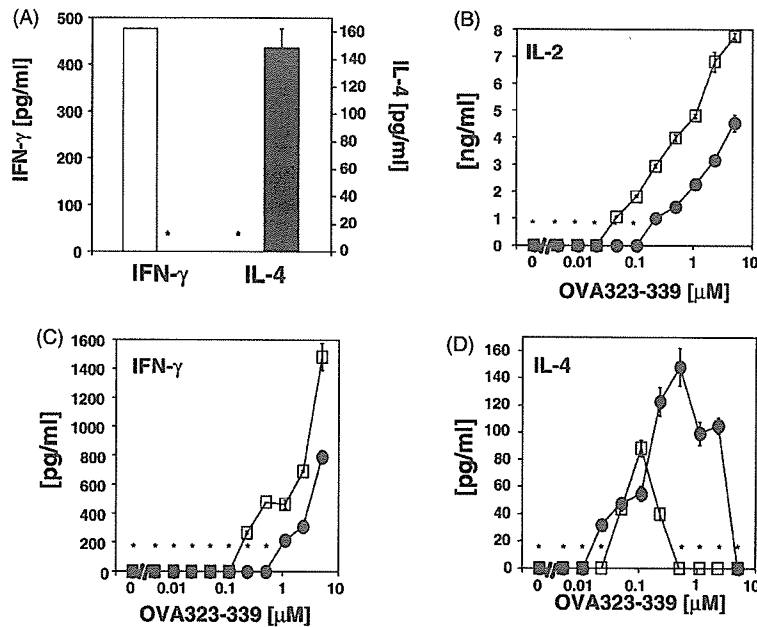

Fig. 2. A326V modified cytokine secretion profile of naive CD4+ T cells induced by various concentrations of OVA323-339. (A) Naive CD4+ T cells and APCs were cultured with 0.5 μM OVA323-339 in the absence (open bar) or presence (closed bar) of A326V at 50 μM. (B–D) Naive CD4+ T cells were stimulated with various concentrations of OVA323-339 in the absence (open square) or presence (closed circle) of A326V at 50 μM. The levels of IL-2 (B), IFN-γ (A and C), and IL-4 (A and D) in each culture supernatant were determined by means of ELISA. Detection limits were 750 pg/ml for IL-2, 188 pg/ml for IFN-γ, and 31 pg/ml for IL-4. *Not detected. The data are shown as the average from triplicate cultures ± S.D. The results shown are representative of more than three independent experiments.

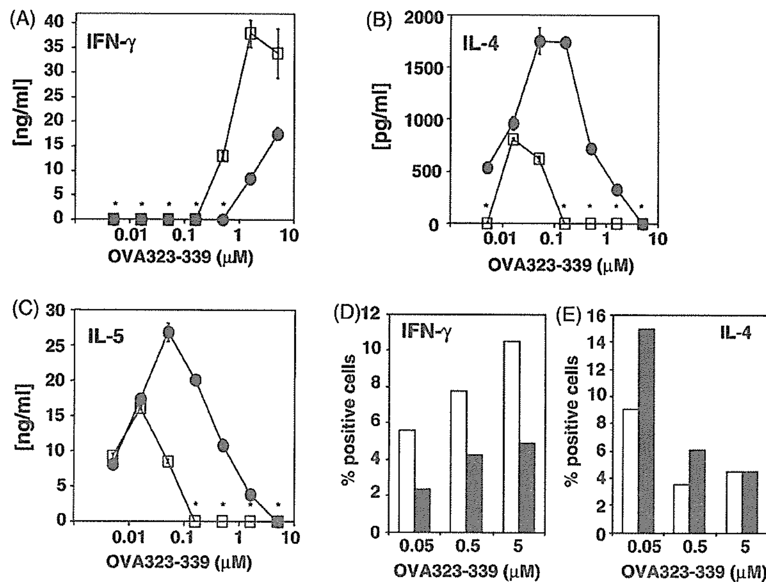

Fig. 3. A326V modified Th1/Th2 differentiation of naive CD4+ T cells. (A–C) Naive CD4+ T cells were primed with various concentrations of OVA323-339 plus APCs in the absence (open square) or presence (closed circle) of A326V at 50 μM. After 7 days of priming, viable cells were recovered and re-stimulated for 48 h with 5 μM OVA323-339 plus APCs. Supernatants were analyzed for levels of IFN-γ (A), IL-4 (B), and IL-5 (C) by means of ELISA. No cytokine was detected when cultured in the absence of OVA323-339. Detection limits were 11.3 ng/ml for IFN-γ, 225 pg/ml for IL-4, and 3.8 ng/ml for IL-5. *Not detected. The data are shown as the average from triplicate cultures ± S.D. The results shown are representative of more than three independent experiments. (D and E) Naive CD4+ T cells were primed with OVA323-339 at 0.05, 0.5, or 5 μM in the absence (open bars) or presence (closed bars) of A326V at 50 μM. After 7 days, viable cells were stimulated with PMA plus ionomycin in the presence of monensin for 6 h. Cells were fixed, permeabilized, and stained for intracellular IL-4 and IFN-γ. The staining profile was analyzed by FACS and percentages of IL-4+ (D) and IFN-γ+ (E) CD4+ T cells were shown. The results shown are representative of two independent experiments.

presence of A326V. These results show that the TCR antagonist induced not only a shift in the antigen-dose-dependent profile of Th1/Th2 cytokine secretion by naive CD4$^+$ T cells, but also an increase of Th2-type cytokine responses.

Fig. 3 shows the secondary cytokine responses of differentiated T cells generated by priming with OVA323-339 plus A326V for 1 week. In the presence of A326V at 50 μM together with the indicated concentrations of OVA323-339 in 1-week primary cultures of naive CD4$^+$ T cells, IFN-γ secretion induced by subsequent stimulation with OVA323-339 alone was decreased (Fig. 3A). As for Th2-type cytokines, IL-4 and IL-5, the dose of OVA323-339 eliciting the highest levels of cytokine secretion was shifted from 0.015 to 0.05 μM in the presence of A326V and the maximum levels of the cytokines secreted were significantly increased (Fig. 3B and C). We further investigated Th1/Th2 differentiation at the single cell level, by staining intracellular IL-4 and IFN-γ production. The presence of A326V in the primary culture decreased the development of IFN-γ-producing cells (Fig. 3D) but enhanced the differentiation of IL-4-producing cells (Fig. 3E). These results show that, as expected from the primary response of naive CD4$^+$ T cells, the TCR antagonist could also affect the Th1/Th2 differentiation profile.

3.3. A326V modified expression of GATA-3 mRNA

GATA-3 is an important transcription factor in Th2 cells. We have previously demonstrated that the expression of GATA-3 mRNA was altered depending on the dose of OVA323-339 and corresponded to that of IL-4 mRNA [13]. Fig. 4 shows that A326V altered the profile of GATA-3 mRNA expression in naive CD4$^+$ T cells stimulated with varying doses of OVA323-339 in a similar manner to that seen with IL-4 secretion by naive CD4$^+$ T cells. These results suggest that A326V may modify Th2 response by altering the expression of GATA-3.

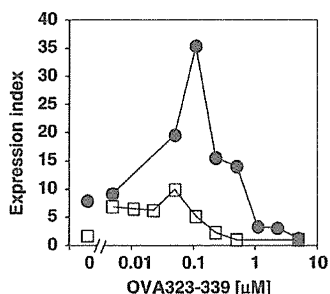

Fig. 4. A326V modified expression of GATA-3 mRNA by naive CD4$^+$ T cells. Naive CD4$^+$ T cells were cultured with 0.005–5 μM OVA323-339 plus APCs in the absence (open square) or presence (closed circle) of A326V at 50 μM. Cells were harvested 24 h following stimulation, and total RNA was prepared. Expression of the GATA-3 mRNA was analyzed by quantitative real-time RT-PCR, and values were normalized to the level of GAPDH mRNA. The results shown are representative of three independent experiments.

4. Discussion

In the present study, we demonstrate that a TCR antagonist has the potential to change the balance of Th1/Th2 cytokine secretion of naive CD4$^+$ T cells in response to the original antigenic peptide. Furthermore, the TCR-antagonist peptide, which inhibited the proliferation of naive CD4$^+$ T cells, significantly enhanced secretion of IL-4 by naive CD4$^+$ T cells and their Th2 differentiation induced in the presence of a specific dose of the original peptide. These results suggest that, for naive CD4$^+$ T cells, a TCR antagonist does not simply inhibit all of the T-cell responses as observed in assays using T-cell clones [18,19], but rather modifies the TCR-mediated signals so as to enhance at least one aspect of the T-cell responses.

To date, two potential mechanisms of TCR antagonism have been proposed. One hypothesis is that TCR antagonists directly interfere with the generation of a TCR-mediated signal by the original antigenic peptide by effectively preventing TCR oligomerization and functional TCR triggering [21]. Another possibility is generation of a negative signaling pathway by the antagonist that suppresses the activation pathway induced by the original antigenic peptide [22,23]. The latter hypothesis is supported by studies using T cells expressing two distinct TCRs having distinct antigenic specificity as a model system [24,25]. Using this model, it has been shown that a TCR antagonist for one TCR inhibited T-cell responses induced by signals mediated by the other TCR. However, opposing results have also been reported using similar experimental systems [26,27]. Thus, the mechanism of TCR antagonism is still controversial.

Our findings that a TCR antagonist increased the dose of the original antigenic peptide required to induce T-cell responses, equivalent to those induced in the absence of the antagonist (Fig. 2B–D), may be explained by the interference of generation of TCR-mediated signaling and/or the generation of negative signaling, leading to inhibition of all TCR-mediated signaling. In contrast, the notable increase in Th2 responses accompanied by the increase of GATA-3 mRNA expression in the presence of a TCR antagonist cannot be explained by these mechanisms, but seems to be explained by the generation of some negative or positive signaling, partially modifying TCR signaling. A recent study demonstrated that reduction of Erk activity led to a dramatic increase in IL-4 production of naive CD4$^+$ T cells and Th2 generation [28]. Thus, it is possible that TCR-mediated signaling induced by the TCR antagonist specifically inhibited activation of Erk.

Several physiological roles of endogenous TCR antagonists have been proposed. TCR antagonists have been reported to be involved in positive selection in the thymus [29–32]. Furthermore, several studies have demonstrated an important role for TCR antagonists in supporting survival and homeostatic proliferation of mature T cells in the periphery [33–35]. Our findings suggest that endogenous peptides functioning as TCR antagonists for CD4$^+$ T cells would af-

fect the Thl/Th2 response of these cells. Some self-peptides with TCR-antagonist activity for T cells specific for an allergen would have an influence on the onset and/or deterioration of the allergic reaction to the allergen by means of enhancement of Th2 responses.

In conclusion, we demonstrate here that a TCR antagonist for a naive $CD4^+$ T cell can affect its Thl/Th2 cytokine responses and functional differentiation and can enhance IL-4 secretion and Th2 differentiation. This indicates that, for naive $CD4^+$ T cells, TCR antagonists do not inhibit or down-regulate all responses to the original antigenic peptide and even enhance at least one aspect of the T-cell response. Analysis of the alteration of TCR-mediated signals in naive $CD4^+$ T cells induced in the presence of TCR antagonists would enhance our understanding of the mechanism of TCR antagonism.

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Ca^{2+} signaling down-regulates TGF-β1 gene expression in $CD4^+$ T cells

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Abstract

In the immune system, TGF-β1 exerts two major functions, anti-inflammatory and immuno-suppressive effects. This work aims to investigate the molecular mechanisms involved in the regulation of the TGF-β1 gene expression in $CD4^+$ T cells. The TGF-β1 gene expresses three transcripts of 2.5, 1.9, and 1.4 kb. The 1.9 kb mRNA which has the highest translation activity was the major transcript. The relationship between T cell receptor (TCR) stimulation and the expression of the gene was investigated. TCR stimulation with a low dose of antigen peptide enhanced the gene expression, whereas a higher dose suppressed the expression. TCR stimulation activates PKC/MAPK and Ca^{2+} signaling pathways. PMA increased the gene expression, whereas ionomycin decreased the gene expression, markedly. The results indicate that Ca^{2+} signaling down-regulates TGF-β1 gene expression. The molecular regulation of TGF-β1 gene expression is unique when comparing to other cytokine genes which are generally activated by Ca^{2+} signaling.
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Keywords: Anergic T cells; Ca^{2+} signaling; $CD4^+$ T cells; TGF-β1

Transforming growth factor-β1 (TGF-β1) is an immuno-inhibitory cytokine increasingly recognized as a key factor for immuno-regulation. In the immune system, TGF-β1 has been shown to have two major functions, anti-inflammatory and immuno-suppressive effects [1]. TGF-β1 producing T cells are limited to Th3 cells functioning in oral tolerance [2] and some regulatory T cells [3]. In addition, TGF-β1 has been implicated in participating in apoptosis [4,5]. Apoptotic T cells release both latent and active TGF-β1, so that tissues or cells near the apoptotic cells may be able to avert inflammation and tissue damage. It is thus of considerable interest to investigate the molecular mechanisms involved in TGF-β1 production from T cells for designing strategies to control unwanted immune responses such as autoimmunity, inflammation, and allergy.

The regulatory mechanisms governing the several steps from the transcription of the TGF-β1 gene to secretion of TGF-β1 from T cells are complicated and are poorly understood. Gene expression of T cell cytokines is, in general, induced by TCR-mediated signals together with signals dependent on co-stimulatory molecules [6] or exogenous cytokines [7]. In contrast, signals that induce the transcription of the TGF-β1 gene are not well documented. Stimulation through TCR activates protein kinase C (PKC), mitogen activated protein kinase (MAPK), and Ca^{2+} signaling pathways. Several reports suggest that activation of the PKC pathway enhances TGF-β1 secretion [8], however detailed molecular relationship between TCR signals and the expression of TGF-β1 mRNA is not well understood. Moreover, post-translational regulation involved in TGF-β1 production makes the mechanisms even more complicated. For example, some T cells may store TGF-β1 in the intracellular membrane-bound compartments (e.g.,

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mitochondria) and release the cytokine depending on changes in environments or outer stimuli [4].

The TGF-β1 gene lacking the TATA box is expressed in every leukocyte lineage. This nature of the gene makes the mode of the gene expression mechanism different from those of other cytokine genes such as IL-2, IL-4, IL-10, and IFN-γ. The reports using non-immuno-competent cells suggest that the TGF-β1 gene expresses three distinct transcripts of 2.5, 1.9, and 1.4 kb. The 2.5 kb mRNA that is the major transcript in many cells carries a highly GC rich 5′ untranslated region (UTR) of ~900 bases [9]. The UTR has been shown to be critical for translational regulation. The 1.4 kb transcript starts 25 bases upstream of the initiator codon AUG, whereas the 1.9 kb transcript has two start sites 366 and 401 bases upstream of the AUG codon [10]. The 2.5 and 1.4 kb transcripts appear to be poorly translated. In contrast, the 1.9 kb transcript is highly efficiently translated [10]. However, critical studies in terms of the relationship between immune response and TGF-β1 transcripts have not been reported.

In the present study, we focused on the relationship between TCR signals and the expression of TGF-β1 mRNA in $CD4^+$ T cells toward a better understanding of TGF-β1 expression in immune response. We present evidence that strong TCR stimulation down-regulates the expression of TGF-β1 mRNA and the activation of the Ca^{2+} signaling pathway causes a marked decrease in TGF-β1 gene expression.

Materials and methods

Mice. BALB/c mice were purchased from Japan CLEA (Tokyo, Japan). DO11.10 (DO) transgenic (Tg) mice carrying a TCR specific for ovalbumin peptide (OVAp) were kindly provided by M. Kubo (Research Institute for Biological Sciences, Tokyo University of Science, Chiba, Japan).

Cell culture, anergy induction, and cell stimulation. $CD4^+$ T cells were purified using CD4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) as previously described [11]. The purity of $CD4^+$ T cells was routinely estimated to be 94–98%. In some experiments, these $CD4^+$ T cells were differentiated into Th1 cells by a standard protocol [12]. Anergy was induced by treatment of Th1 cells (1×10^6 cells/ml) with 1 μM ionomycin for 16 h. For the experiments in Fig. 1, a Th2 clone (68-41) was used. For the experiments in Fig. 2, naïve $CD4^+$ T cells from DO Tg mice were stimulated with 0–5 μM OVAp. For the experiments in Fig. 3, a thymoma $CD4^+$ T cell line (EL-4) or naïve $CD4^+$ T cells were stimulated with ionomycin (50 nM) or phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) alone, or with both of them. For the experiments in Fig. 4, anergy induced Th1 cells were stimulated with anti-CD3 and anti-CD28 mAbs.

T cell proliferation assay. The T cell proliferation assay was performed in 96-well flat bottomed plates. $CD4^+$ T cells (5×10^4/well) in a total volume of 200 μl were stimulated with 0–5 μM OVAp in the presence of irradiated syngeneic spleen cells (2×10^5/well). Cells were cultured for 54 h. Proliferation was assessed by measuring the incorporation of [^{3}H]thymidine (1 μCi/well) added for the final 18 h of culture.

Real-time RT-PCR. Cells were harvested at indicated time points, and the total RNA was prepared using ISOGEN (Nippon Gene). The total RNA was treated with deoxyribonuclease I (Invitrogen), and cDNA was synthesized using oligo(dT) primers and Superscripts polymerase (Wako). The Light Cycler PCR and real-time detection

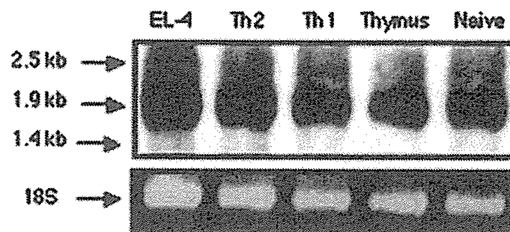

Fig. 1. Transcripts of the TGF-β1 gene. Total RNA was prepared from non-activated EL-4 cells, Th1 cells, the 68-41 Th2 clone, thymocytes, and naïve $CD4^+$T cells. The expression levels of TGF-β1 mRNA were determined by Northern blotting. The lower panel shows ethidium bromide (EtBr)-stained 18s rRNA as a control for equal loading.

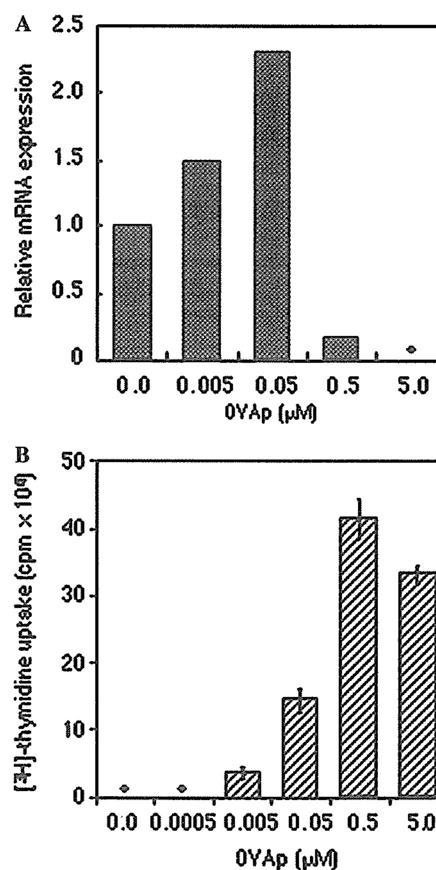

Fig. 2. Strong antigenic stimulation down-regulates TGF-β1 mRNA expression. (A) Naïve $CD4^+$ T cells derived from DO Tg mice were stimulated with varying dose of OVAp in the presence of antigen presenting cells (APCs) prepared from BALB/c mice spleen for 24 h. The expression levels of TGF-β1 mRNA were determined by real-time PCR. The results shown are representative of two independent experiments. (B) Naïve $CD4^+$ T cells derived from DO Tg mice were stimulated with varying dose of OVAp in the presence of APCs prepared as described above. Proliferative response was measured by [^{3}H]thymidine incorporation.

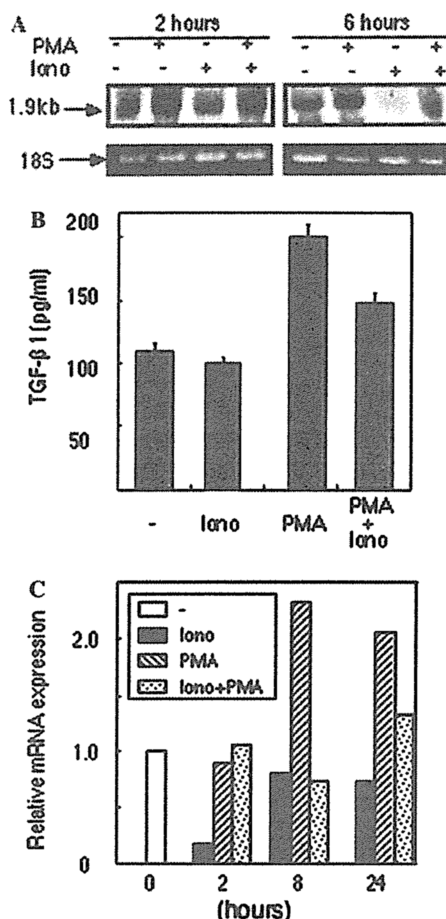

Fig. 3. Ca^{2+} signaling down-regulates TGF-β1 mRNA expression. (A) EL-4 cells were stimulated with ionomycin (50 nM) or PMA (100 ng/ml) alone, or with both of them, Cells were harvested after 2 and 6 h. The expression levels of TGF-β1 mRNA were determined by Northern blotting. The results shown are representative of three independent experiments. The lower panel shows EtBr-stained 18s rRNA as a control for equal loading. (B) EL-4 cells were stimulated as described above. Culture supernatants were recovered after 24 h and the concentration of TGF-β1 was measured. The data are shown as the average from triplicate cultures (±SD). (C) Naïve $CD4^+$ T cells were stimulated with ionomycin (200 nM) or PMA (50 ng/ml) alone, or with both of them. Cells were harvested after 2–24 h. Total RNA was prepared and the expression levels of TGF-β1 mRNA after 2, 8, and 24 h stimulation were determined by real-time PCR. The results shown are representative of three independent experiments.

system (Roche) was used for amplification and in-line quantification. The pairs of primers used were as follows: glyceraldehyde-3-phosphate dehydrogenenase (GAPDH) sense, 5′-TGAAACGGGAAGCTGG-3′; GAPDH antisense, 5′-TCCACCACCCTGTTTGGTGTA-3′; TGF-β1 sense, 5′-CCAAGGAGACGGAATACAGG-3′; TGF-β1 antisense, 5′-GTTCATGTCATGGATGGTGC-3′. The hybridization probe format was used to quantify the amplified fragment. Hybridization probes consisted of two different short oligonucleotides that hybridize close to each other in an internal sequence of the amplified fragments during the annealing phase of PCR cycles. One probe was labeled at the 5′-end with the Light Cycler Red 640 (LC640) fluorophore, and the other was labeled 3′-end with FITC. The pairs of hybridization probes used were as follows: GAPDH, 5′-CTGAGGACCAGGTTGT TGTCTCCTGCGA-FITC 3′ and 5′-Red 640-TTCAACAGCAACT CCCACTCTTCCACC-3′; TGF-β1, 5′-GTGACAGCAAAGATAA CAAACTCCAC-FITC 3′ and 5′-Red 640-TTCAACAGCAACTCCC ACTCTTCCACC-3′. Normalization was calculated by assessing the amounts of mRNA for GAPDH as an internal control for each sample.

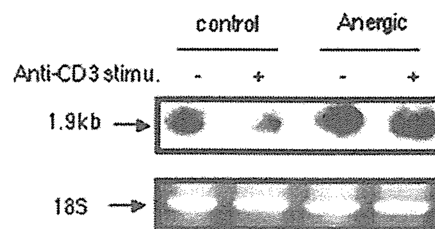

Fig. 4. Expression of TGF-β1 mRNA in anergic T cells after TCR stimulation. Th1 cells and anergy induced Th1 cells were stimulated with anti-CD3 and CD28 mAbs. Cells were harvested after 3 h to prepare the total RNA. The expression levels of TGF-β1 mRNA expression were determined by Northern blot analysis. The results shown are representative of two independent experiments. The lower panel shows EtBr-stained 18s rRNA as a control for equal loading.

Northern blot analysis. Cells were harvested at indicated time points, and the total RNA was prepared using ISOGEN (Nippon Gene). Equal amounts of RNA samples (3–5 μg) were fractionated on 1% agarose gels containing 2.4% formaldehyde and then transferred to positively charged nylon membranes. After fixation under calibrated ultraviolet irradiation, the membranes were hybridized with dioxigenin (DIG)-labeled riboprobes and visualized using alkaline phosphatase-labeled anti-DIG antibody following the manufacturer's instructions (Roche). For preparation of a TGF-β1 probe, an *Eco*RI fragment of mouse TGF-β1 cDNA was cloned into Bluescript (Statagene) as previously described. DIG-labeled riboprobe was synthesized using a DIG-RNA labeling kit (Roche), following the manufacturer's instructions.

Measurement of TGF-β1 concentration. EL-4 cells (1×10^6) in a total volume of 1 ml were stimulated with A23187 (100 nM) or PMA (50 ng) alone, or with both of them for 24 h. Concentration of TGF-β1 in culture supernatant was determined by ELISA using anti-TGF-β1 mAbs (A75-2.1) and biotin-A75-3.1 purchased from PharMingen (San Diego, CA).

Results

The 1.9 kb mRNA is the major transcript in T cells

Initially, we determined the molecular sizes of the TGF-β1 transcripts in non-activated T cells (EL-4, Th1 line, Th2 clone, thymocytes, and naïve $CD4^+$ T cells), since the gene transcription produces three different sizes of mRNA (1.4, 1.9, and 2.5 kb). The results suggest that the TGF-β1 gene is transcribed constitutively (Fig. 1). Interestingly, the expression levels of the 2.5 and 1.4 kb transcripts, in general, were marginal in these T cells, although EL-4 cells expressed a relatively stronger band of 2.5 kb. The 1.9 mRNA that is supposed to have a most efficient translation activity was the major transcript. Based on these results, we concentrated on the analyses of the 1.9 kb transcript in the following experiments.

Strong antigenic stimulation down-regulates the expression of TGF-β1 mRNA

The structure of the TGF-β1 gene promoter has been extensively characterized [13], however little is known about the molecular mechanisms regulating the expression of the gene transcripts in $CD4^+$ T cells. We measured the expression levels of TGF-β1 mRNA of OVA specific naïve T cells prepared from DO11.10 (DO) transgenic Tg mice after stimulation with various doses of OVAp by real-time PCR analysis. The addition of a relatively low dose of OVAp (up to 0.05 μM) enhanced its transcription levels, whereas a higher dose (more than 0.05 μM) suppressed the expression dramatically (Fig. 2A). Proliferation of T cells required a higher dose of OVAp (more than 0.05 μM) (Fig. 2B). Thus, the expression of TGF-β1 mRNA was inversely correlated with proliferation. The results suggest that TCR stimulation strong enough for clonal expansion of T cells down-regulates the expression of the TGF-β1 gene.

Activation of Ca^{2+} signaling pathway down-regulates TGF-β1 mRNA expression

TCR stimulation activates PKC/MAPK and Ca^{2+} signaling pathways [14,15]. Addition of PMA which is a PKC activator and ionomycin (a calcium ionophore) is able to mimic TCR stimulation. Weak or partial TCR stimulation is often associated with impaired Ca^{2+} signaling. These results prompted us to speculate that TGF-β1 mRNA down-regulation is accompanied by full activation in the Ca^{2+} signaling pathway. To this end, initially we utilized EL-4 cells due to the following reasons. It is known that EL-4 cells produce TGF-β1 that can be detected by ELISA [16]. Then, we will be able to investigate the relationship between the transcription of the TGF-β1 gene and the released TGF-β1 protein from the T cells with relative ease, unlike primary T cells.

EL-4 cells constitutively expressed the TGF-β1 transcripts and the addition of PMA enhanced the transcription levels in the time course assay (Fig. 3A). In contrast, ionomycin treatment resulted in a marked decrease in TGF-β1 mRNA expression after 6 h. In addition, the combination of PMA and ionomycin caused a decrease in the transcription levels after 6 h, but to a lesser extent compared to ionomycin alone. Next, we assessed the concentration of released TGF-β1 after 24 h of stimulation by ELISA (Fig. 3B). PMA enhanced the amount of secreted TGF-β1, whereas the addition of ionomycin plus PMA induced a decrease in the concentration of TGF-β1. Stimulation with ionomycin alone caused a decrease in the TGF-β1 level, but not to a great extent, suggesting that intracellular TGF-β1 was released as described by Chen et al. [5]. In general, the ELISA results were not contradictory to the Northern blot results.

Then, we examined the effects of PMA and ionomycin on the expression of the TGF-β1 transcripts in primary T cells to confirm the EL-4 results (Fig. 3C). Naïve $CD4^+$ T cells were stimulated with PMA or ionomycin alone, or with both of them. The expression levels were assessed by a real-time PCR method. PMA increased the expression of TGF-β1 mRNA more than twofold after 8 h. Ionomycin caused a significant decrease in TGF-β1 mRNA expression in the 2 h assay, but recovered to its original level in 8 h. Addition of PMA and ionomycin caused a significant decrease compared to the addition of PMA alone after 8 and 24 h. Thus, the naïve $CD4^+$ T cell results were reminiscent of the EL-4 results.

Expression of TGF-β1 mRNA is not down-regulated in anergic T cells

Anergic T cells often demonstrate impaired Ca^{2+} signaling [17,18]. Thus, treatment of primary Th1 cells with ionomycin is a standard method to tolerize T cells. Rao et al. [18] reported that T cells anergized by this method demonstrate a strong impaired Ca^{2+} mobilization. The transcription of the TGF-β1 gene was examined in T cells anergized by pre-treatment of ionomycin after 24 h (Fig. 4). Proliferative response of these T cells diminished as described by Macian et al. [19] (data not shown). Stimulation of primary T cells with anti-CD3 mAb suppressed the expression of TGF-β1 mRNA. In contrast, TCR stimulation with anti-CD3 mAb did not diminish TGF-β1 mRNA expression in anergic T cells either. The amount of TGF-β1 produced from these primary T cells was below the detection level by ELISA.

Discussion

TGF-β1 is a pleiotropic growth factor exerting different functions on cell growth and differentiation depending on cell type [1]. In the immune system, TGF-β1 is one of the most important immuno-suppressive cytokines produced from T cells, however its behavior in several T cell responses is not well understood. Therefore, it is of interest to investigate the molecular mechanisms involved in TGF-β1 gene expression. In the current study, we carried out a series of experiments to clarify the relationship between TGF-β1 expression and T cell responses including Ca^{2+} metabolism, proliferation, and anergy. The TGF-β1 gene was constitutively transcribed from naïve T cells and the major transcript was 1.9 kb which has the most efficient translation activity. In our experiments, the expression levels of the 2.5 and 1.4 kb transcripts were marginal, however other reports on non-immunological cells demonstrate that the 2.5 kb mRNA is the major transcript. The reason for this discrepancy is not clear.

TGF-β1 mRNA expression was accelerated with an antigen dosage that was too low for T cell proliferation, whereas a higher antigen dosage inducing T cell proliferation caused a marked suppression in the gene expression. The gene expression profile contrasts with those of other cytokine genes involved in Thl/Th2 cells. In addition, we and other investigators have demonstrated that antigen peptides with weak binding affinity or altered peptide ligands (APLs) are able to induce the expression of the TGF-β1 gene in T cells [20–23]. The involvement of Ca^{2+} signaling in the regulation of TGF-β1 gene expression has been reported. Stimulation of T cells with peptides with weak binding affinity or APLs induces a decrease in proliferation and impaired Ca^{2+} signaling [20,24,25]. T cells generated in oral tolerance produce TGF-β1, and generally the proliferative response of these T cells is low [26]. Recently, we reported that the level of Ca^{2+} influx of T cells derived from orally tolerized mice was diminished [17], suggesting that the level of Ca^{2+} signaling is low in TGF-β1 producing T cells. These results suggest that the expression of the TGF-β1 gene is enhanced in T cells with the impaired Ca^{2+} signaling mechanism. Strong TCR stimulation with anti-CD3 mAb to in vitro anergized T cells did not cause a decrease in TGF-β1 gene expression. As described above, the anergic T cells carry an impaired Ca^{2+} mobilization mechanism. It is plausible that the regulatory mechanism of the TGF-β1 gene is not functioning in anergic T cells due to the impaired Ca^{2+} signaling.

The Ca^{2+} ionophore ionomycin decreased the expression of the TGF-β1 gene in EL-4 cells. However, the amount of TGF-β1 secreted from EL-4 cells did not show a concomitant reduction, suggesting that TGF-β1 stored inside the cell was released. T cells carry a certain amount of the cytokine in mitochondria and on the cell surface [4]. After some stimulation, TGF-β1 is released. It is also known that $CD4^+$ T cells release intracellular TGF-β1 by apoptotic signaling, so that apoptotic T cells could avoid an inflammatory response in the nearby environment [4,5]. Thus, it is difficult to judge the production levels of TGF-β1 from its gene transcription levels. Treatment of EL-4 cells with PMA and ionomycin induced a decrease in TGF-β1 concentration compared with PMA alone. The decreased amount of the released TGF-β1 protein reflected the decreased transcription level of the TGF-β1 gene. The TGF-β1 levels produced in T cells stimulated with PMA alone exceeded the production levels stimulated with PMA and ionomycin. The results suggest that the down-regulation of TGF-β1 transcription is one of the mechanisms involved in the observed decrease in TGF-β1 production.

What are the underlying biochemical reasons involved in the regulation of TGF-β1 gene expression by Ca^{2+} signaling? The transcription of the TGF-β1 gene is active constitutively in resting T cells. As well, it could be beneficial for T cells that a TCR signal inducing full activation of T cells elicits a decrease in TGF-β1 gene expression. The immuno-suppressive factor, TGF-β1, produced from T cells may quench the activation of the T cells. The production of the immuno-suppressive cytokine may also contribute to induction and maintenance of an anergic state. Our finding that a low dose stimulation of TCR by a specific antigen enhances the gene expression may support the results of the activation of TGF-β1 gene expression by partial or weak TCR stimulation.

PMA stimulation alone is able to accelerate TGF-β1 gene expression. This may suggest that the PKC signaling pathway regulates the gene expression positively. It is clear that impaired/weak Ca^{2+} signaling is associated with positive regulation of TGF-β1 gene expression. The possible involvement of Ca^{2+} signaling in the positive regulation of TGF-β1 gene expression requires further investigation. Mechanisms responsible for the down-regulation of the TGF-β1 gene are not well understood. One possibility is that the Ca^{2+} signaling pathway directly controls its promoter activity. This possibility is supported by the finding that several calcium responding elements exist in the promoter region of the TGF-β1 gene [13]. Another possibility is that the Ca^{2+} pathway controls the stability of the TGF-β1 mRNA. Previous reports on the effects of calcium metabolism on mRNA stability may support this hypothesis [27,28].

In summary, we have demonstrated that Ca^{2+} signaling down-regulates the expression of the TGF-β1 gene. TGF-β1 is one of the most important immuno-suppressive cytokines. Further elucidation of the molecular mechanisms involved in the positive and negative regulation of TGF-β1 gene expression will aid in the control of immune responses.

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Oral administration of food antigen induces T cell mediated intestinal inflammation: A model using TCR-transgenic mice

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Abstract. To investigate the mechanisms inducing food-sensitive intestinal inflammation, we focused on the OVA23-3 mouse, a transgenic mouse strain expressing a T cell receptor that recognizes ovalbumin (OVA). Mice administered an egg-white (EW) diet containing OVA showed a trend of loose feces and significant weight loss. Histology of the jejunum showed severe inflammation with villous atrophy. Thus, we studied the role of T cells and intestinal microflora in the development of the inflammation. Severe villous disruption was observed in sections of the jejunum from OVA23-3 mice and RAG-2 gene-deficient OVA23-3 mice fed with EW-diet. Further, a larger number of T cells was found in the lamina propria of the jejunum of EW-diet fed OVA23-3 mice, RAG-2 gene-deficient mice and germfree OVA23-3 mice compared with those of control-diet fed mice. However, severe inflammation was not detected in the jejunum of germfree OVA23-3 mice. $CD4^+$ T cells from the MLN of EW-diet fed OVA23-3 mice showed a Th2 cytokine secretion profile. These observations have thus clarified that antigen-specific Th2 cells play important roles in the development of intestinal inflammation. Although the presence of indigenous bacteria was not essential for the inflammation, T cells could mediate a more severe inflammatory response in their presence.

Keywords: Food allergy, oral administration of antigen, intestinal inflammation antigen-specific T cells, Th2 type $CD4^+$ T cells, probiotics, intestinal flora

1. Introduction

The mechanisms inducing food-sensitive intestinal inflammation remain to be clarified due to the lack of excellent animal models [2,3]. In an animal model of food allergy, the sole administration of antigens orally should induce 1) an increase of antigen specific IgE in the serum and 2) gastrointestinal inflammation and other symptoms. Previous studies in our laboratory showed that feeding egg-white (EW) diet to OVA23-3 mice, a transgenic mouse strain expressing an OVA-specific T cell receptor, elicited an OVA-specific serum IgE response [5]. Further, we found that OVA23-3 mice fed with EW-diet demonstrated intestinal inflammation accompanied with wasting diseases and diarrhea which are

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similar to clinical symptoms of food allergy patients (H, Nakajima-Adachi; submitted). Thus, this mouse could serve as a unique model to investigate the mechanisms of food-sensitive intestinal inflammation. In this study, we firstly analyzed the roles of T cells in this inflammation and secondly we investigated the effect of intestinal flora on intestinal inflammation.

2. Materials and methods

2.1. Mice

Conventional OVA23-3 TCR-transgenic mice (CV OVA23-3 mice), transgenic for OVA 323–339 specific and I-A^d restricted TCR-$\alpha\beta$, on a BALB/c genetic background were kindly provided by Professor Sonoko Habu of Tokai University and housed under Specific pathogen free (SPF) conditions. OVA23-3 mice were crossed to RAG-2 gene-deficient mice to produce RAG-2 gene-deficient (RAG-KO) OVA23-3 mice. Germfree (GF) OVA23-3 mice, which lack intestinal flora, were obtained from CV OVA23-3 mice by hysterectomy and foster nursed by lactating GF BALB/c mice in an isolator.

2.2. Administration of OVA

OVA23-3 mice were fed a pelleted diet containing egg-white protein at a concentration of 20% for 4 weeks. The EW diet contains approximately 10.4% OVA. A Casein (CN) diet was used as dietary control.

2.3. Histology and immunohistochemistry

Specimens were obtained from the jejunum, ileum and colon on week 4. Intestinal samples were fixed in 4% phosphate-buffered formaldehyde (pH7.2). Paraffin sections (thickness 3 μm) were stained with hematoxylin and eosin for identification of inflammatory cells, or with toluidine blue (pH4.1) for identification of mast cells. Intestinal specimens for immunohistochemistry were placed into molds and frozen in liquid nitrogen. Before staining, slides were fixed in acetone at $-20\,^{\circ}$C for 10 minutes. Blocked sections were incubated with biotinylated anti-mouse antibodies and then with streptavidin-HRP. DAB (Sigma) was used to stain $CD3^+$, $CD8^+$ cells.

2.4. Preparation of the mesenteric lymph nodes (MLN) cells and $CD4^+$ T cells.

$CD4^+$ T cells were purified from the MLN of transgenic mice by means of magnetic cell sorting (Miltenyi Biotech GmbH, Bergish Gladbach, Germany) with anti-CD4 beads.

2.5. Cell culture and cytokine assay with ELISA

Purified $CD4^+$ T cells (1×10^5 cells) were cultured with 0 or 1.0 mg/ml OVA in flat-bottom 96-well plates and 4×10^5 mitomycin C treated BALB/c splenocytes as antigen presenting cells (APCs) in RPMI 1640 media containing 10% heat-inactivated FCS (Sigma). For cytokine assays, supernatant samples were collected at 48 hours. IL-4 and IFN-γ cytokine production was measured by ELISA.

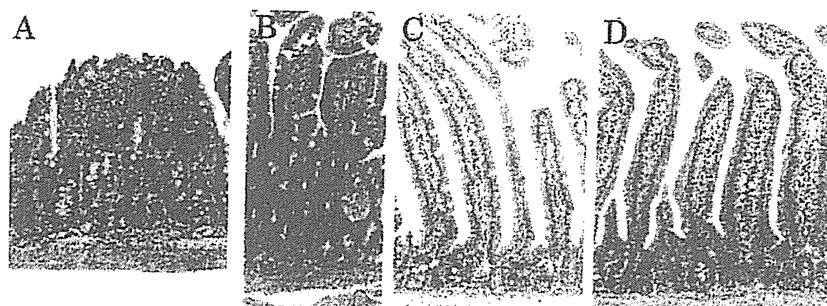

Fig. 1. Histology of jejunum from OVA23-3 mice fed with EW-diet. Samples were obtained on day 28 and stained with hematoxylin and eosin. A: OVA23-3 mice fed with EW-diet, B: RAG-KO OVA23-3 mice fed with EW-diet, C: GF OVA23-3 mice fed with EW-diet, D: OVA23-3 mice fed with CN (control) diet. (Original magnification: A, B, C and D 20×).

3. Results and discussion

3.1. Histologic analysis of the jejunum from OVA23-3 mice, RAG-KO OVA23-3 mice and GF OVA23-3 mice

Histologic features such as villous atrophy, crypt hyperplasia, goblet cell hyperplasia and infiltration of inflammatory cells, were detected in the jejunum of OVA23-3 mice fed with EW-diet (Fig. 1(A)). Mice fed with control (CN)-diet showed normal villous architecture (Fig. 1(D)). Severe inflammation was also detected in the jejunum of RAG-KO OVA23-3 mice fed with EW-diet (Fig. 1(B)). All T cells of RAG-KO OVA23-3 mice express the OVA-specific TCR, and these mice cannot produce antibodies due to lack of mature B cells. Thus, these observations suggest that OVA-specific T cells mediate intestinal inflammation, even in the absence of B cells. Severe inflammation was not detected in the duodenum and upper jejunum from GF OVA23-3 mice fed with EW-diet (Fig. 1(C)).

3.2. The number of CD3$^+$ T cells detected in the jejunum of OVA23-3 mice, RAG-2 gene KO OVA23-3 mice and GF OVA23-3 mice

The number of T cells detected in the jejunum of EW-diet fed OVA23-3 mice was significantly increased compared with CN-diet fed OVA23-3 mice (Table 1). Significant aggregates of T cells were also detected in EW-diet fed RAG-KO OVA23-3 mice and GF OVA23-3 mice. This immunohistochemical observation of the jejunum further supports the important role of antigen-specific T cells in inflammation. Indeed, a previous study of patients with food-sensitive enteropathy suggested that activated CD4$^+$ cells in the lamina propria may contribute to mucosal damage [4].

3.3. Cytokine production by CD4$^+$ T cells in the MLN

CD4$^+$ T cells from the MLN were cultured with OVA and cytokine secretion was examined by ELISA. High levels of IL-4 were secreted by CD4$^+$ T cells from OVA23-3 mice fed with EW-diet compared with mice fed with CN-diet (Table 2). CD4$^+$ T cells from mice fed with CN-diet did not secrete detectable levels of IL-4. Conversely, higher levels of IFN-γ were secreted by CD4$^+$ T cells from the MLN of CN-diet fed mice than EW-diet fed mice. We also found that a much higher level of IL-4 (524 pg/mL) was secreted by CD4$^+$ T cells from the MLN after 7 days administration of EW-diet compared with 28 days. These data showed that dysregulated Th2 cells play important roles in the pathological features of inflammation in the small intestine [1].