# Midazolam inhibits IgE production in mice via suppression of class switch recombination

(ミダゾラムはクラススイッチ組み換えの抑制を介してマウスの IgE 産生を抑制する)

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Abstract

Anaphylactic shock is characterized by increased capillary permeability and a decline in blood pressure due to excessive production of IgE. Midazolam (MDZ) is reported to have immunomodulatory properties. However, little is known about the effect of MDZ on the production of IgE antibody. We examined whether MDZ can suppress antigen-specific and total IgE production followed by IgE class switch recombination (CSR). MDZ was administered intraperitoneally to mice prior to ovalbumin (OVA) plus native cholera toxin (nCT) immunization. Serum OVA-specific and total IgE responses, and surface IgE-positive B cells were analyzed by ELISA and Flow cytometry. Furthermore, expression levels of CSR associated molecules such as germ-line transcript ε (εGLT), germ-circle transcript ε (εCT), AID, and Id2 in spleen were compared. Interferon-gamma (IFN-γ) and interleukin (IL)-4 mRNA and their protein levels were also examined in spleen and serum. MDZ significantly suppressed OVA-specific and total IgE levels in plasma and surface IgE-positive B cells in spleen. Moreover, MDZ-treated mice had significantly reduced levels of εGLT and εCT. Furthermore, although IFN-γ mRNA and protein levels were significantly elevated, IL-4

mRNA and protein levels were reduced in MDZ-treated mice. Therefore, MDZ may be an important modulator of allergic responses through its ability to downregulate IgE production.

## Introduction

Immunoglobulin E (IgE) plays a central role in the pathogenesis of many allergic diseases, such as allergic asthma, allergic rhinitis, and atopic dermatitis. Furthermore, immediate hypersensitivity reactions to anesthetic and associated agents used during the perioperative period have been reported with increasing frequency in most developed countries (1). In sensitized individuals, both total and allergen–specific IgE antibodies (Abs) are produced at high levels and bind to high-affinity FcεRs on mast cells and basophils surfaces, leading to the release of preformed and newly synthesized mediators that initiate immunologic cascades and inflammatory reactions. Therefore, inhibition of IgE production is an ideal strategy to ameliorate allergic disease. Until now, therapeutic means to suppress IgE production have not been clinically available, although neutralization of pre-existing IgE has been applied to patients with severe allergic asthma (2). Although IgE has a relatively short half-life in plasma, mechanisms that tightly control IgE class switch recombination (CSR) are thought to account for the low levels of IgE normally observed in plasma (3-5). CSR replaces the

heavy chain constant region Cμ gene with a targeted Cε gene by recombination of a switch region μ (Sμ) with a Sε region present in the respective targeted CH gene. Consequently, CSR allows the expression of an Ig that has the same antigen specificity with a secondary heavy chain isotype (IgG, IgA, or IgE) that exhibits different effector functions. Regulation of CSR in B cells is coordinated with the germ line transcription (GLT) of CH genes, as well as the induction of activation-induced cytidine deaminase (AID) expression (6).

Midazolam (MDZ), a benzodiazepine (BZD), is an intravenously administered anesthetic used for premedication, induction and maintenance of general anesthesia, as well as for sedation in the treatment of nervous dental patients. There are two types of BZD receptors, central type BZD receptors (CBRs) and peripheral type BZD receptors (PBRs) (7, 8). CBRs are expressed mainly in the central nervous system (7), while PBRs are detected in many peripheral tissues, cells and organs, such as the kidney, endocrine organs and monocytes (7, 8). PBRs are reportedly involved in regulation of cellular proliferation, immunomodulation, steroidogenesis, and oxidative processes, as well as programmed cell death (7, 8). MDZ has also been shown

to inhibit IL-6 mRNA expression in human peripheral blood mononuclear cells (9), and to suppress lipopolysachccaride (LPS)-induced nitric oxide and TNF-α release from rat microglia via PBRs (10). These results led us to speculate that MDZ might modulate immune system function. However, the exact mechanism of MDZ effects on IgE production remains to be fully elucidated.

Therefore, in this study, we examined the effect of i.p. administrated MDZ on total and antigen-specific IgE levels as well as IgE CSR in serum and splenocytes from ovalubmin (OVA) plus cholera toxin (CT)-immunized BALB/c mice. Furthermore, we investigated the immunomodulatory activities of MDZ on induced interferon-gamma (IFN-γ) and interleukin-4 (IL-4) production and their mRNA expressions in serum and splenocytes from OVA-immunized BALB/c mice.

## Materials and Methods

### *Mice*

Female BALB/c Cr Slc mice were purchased from Sankyo Laboratories (Tokyo, Japan) and were maintained under specific pathogen–free conditions at an experimental facility of the Nihon University School of Dentistry at Matsudo, Chiba, Japan. Mice were 8 to 12 weeks old when used for the experiments. All food and water were sterile. All animals were maintained and used in accordance with the guidelines for the care and use of laboratory animals of the Nihon University School of Dentistry at Matsudo.

### *Optimization of MDZ dose*

In humans, the loading dose of MDZ is 0.15 mg/kg, followed by a continuous infusion of 1 – 7 μg/kg/min. The maximum dose is 6 to 10 mg/kg (11, 12). Therefore, each mouse (five mice/group) was administered different intraperitoneal (i.p.) doses (1 mg, 3 mg, or 10 mg/kg) of MDZ in 100 μl phosphate-buffered saline (PBS) in order to establish the minimal effective dose for MDZ. Mice were monitored daily for staggering, a reduction in physical activity, sedation, and loss of the righting reflex.

*Immunization and sample collection*

BALB/c mice (five mice/group) were administered MDZ (3 mg/kg) or PBS alone via i.p. injection and then immunized intraperitoneally with 1 mg of ovalbumin (OVA, fraction V; Sigma-Aldrich, St. Louis, MO) plus 1 μg of native cholera toxin (nCT) in PBS on days 0, 7, and 14. On day 21, serum samples were collected and mononuclear cells were isolated from the spleens of both MDZ-treated and untreated mice that were immunized with OVA and nCT.

*OVA-specific and cytokine-specific ELISA*

The presence of OVA-specific IgE and total IgE Abs in serum was determined using an ELISA kit (Rebisu®, Shibayagi, Japan). Levels of cytokines in serum were measured using ELISA. A Quantikine™ mouse immunoassay kit (R&D Systems, Minneapolis, MN) was used to detect IFN-γ and IL-4 in the plasma of both MDZ-treated and control mice immunized with OVA plus nCT.

*Flow cytometry*

To characterize the frequency of surface-IgE–positive ($sIgE^+$) B cells, $2 \times 10^5$ mononuclear cells were incubated with phycoerythrin (PE)-labeled anti-IgE Ab and allophycocyanin-tagged B220 (BioLegend, San Diego, CA). Samples were then subjected to FACS analysis (BD Biosciences, San Jose, CA).

*Quantitative real time-PCR analysis*

Total RNA was purified from splenic cells using Trizol reagent (Invitrogen, Tokyo, Japan) following the manufacturer's protocol. Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) was used to generate cDNA from purified total RNA obtained from each mouse. Quantitative real-time RT-PCR analyses were performed using a Thermal Cycler Dice real-time PCR system (Takara, Shiga, Japan) in accordance with the manufacturer's protocol. The initial denaturation step was carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 25 sec, and 60°C for 35 sec. Each gene was tested in triplicate. Target RNA levels were

normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The levels of germ-line transcript ε (εGLT), germ-circle transcript ε (εCT), AID, IFN-γ, and GAPDH cDNA were determined by quantitative PCR using the following primer pairs:

εGLT: 5′-GCACAGGGGGCAGAAGAT-3′,

5′-CCAGGGTCATGGAAGCAGTG-3′

εCT: 5′-TTGGACTACTGGGGTCAAGG-3′,

5′-CAGTGCCTTTACAGGGCTTC-3′

AID: 5′-GGCTGAGGTTAGGGTTCCATCTCAG-3′,

5′-GAGGGAGTCAAGAAAGTCACGCTGGA-3′

Id2: 5′-ATCGTCTTGCCCAGGTGTCGTTCT-3′,

5′-AGCATCCCCCAGAACAAGAAGGTG-3′

IFN-γ: 5′-CGGCACAGTCATTGAAAGCCTA-3′,

5′-GTTGCTGATGGCCTGATTGTC-3′

IL-4: 5′-CATCGGCATTTTGAACGAGGTCA-3′,

5′-CTTATCGATGAATCAGGCATCG-3′

GAPDH: 5′-TGTGTCCGTCGTGGATCTGA-3′,

5′-TTGCTGTTGAAGTCGCAGGAG-3′

*Statistical analysis*

Student's *t*-test (two-tailed) was used for all statistical analyses and *P* values <0.05 were considered statistically significant.

Results

*MDZ inhibits Ag-specific IgE responses in serum*

We examined the optimal dose of i.p. administration of MDZ. Various doses (1 mg, 3 mg, and 10 mg/kg/mouse) of MDZ were administered i.p. to five mice per each group. Of the mice that were given a 3 mg/kg dose of MDZ, at least four out of five demonstrated evidence of anesthesia, such as staggering, a reduction in physical activity, sedation, and/or loss of the righting reflex (Table 1). As 3 mg/kg of MDZ appeared to be the optimal concentration to induce a hypnotic-anesthetic state, this concentration was used in all subsequent experiments. To evaluate Ag-specific IgE responses, mice were given MDZ i.p. 30 min before being immunized with OVA plus nCT (also via the i.p. route). Serum was collected from each mouse 7 days after the third i.p. injection, after which IgE levels were determined using ELISA. As shown in Figure 1, MDZ inhibited OVA-specific and total IgE production. This effect of MDZ on IgE production reflects a reduction in surface IgE expression on B cells, as confirmed by FACS analysis (Fig. 2).

*Suppression of IgE CSR in MDZ treated-mice*

We examined the effects of MDZ on CSR-mediated regulatory genes in the spleen cells of mice that had been immunized with OVA plus nCT. The levels of mRNA specific for εGLT, εCT, AID, and Id2 were determined using quantitative real-time PCR. Spleen cells from mice not treated with MDZ served as a positive control. The level of AID-specific mRNA was comparable in MDZ-treated and untreated mice (Fig. 3). In contrast, significant inhibition of εGLT (A) and εCT (B) mRNA production was observed in MDZ-treated mice compared with control mice. Interestingly, Id2, which suppresses IgE CSR, was increased in MDZ-treated mice. These data indicate that inhibition of IgE CSR by MDZ is caused by suppression of εGLT and εCT synthesis.

*Induction of IFN-γ but not IL-4 expression in spleen cells*

Since the number of surface $IgE^+$ B cells and the frequency of IgE CSR were lower in MDZ-treated mice than untreated controls, we speculated that some soluble factors might be involved in MDZ-mediated suppression of CSR.

We assessed the production of the cytokines IFN-γ and IL-4, which are known to regulate IgE CSR in activated B cells (13). After confirming that the level of OVA-specific IgE in serum was decreased in MDZ-treated mice on day 21 (7 days after the last immunization), spleen cells were collected and the levels of IFN-γ and IL-4 mRNA were determined using real time-PCR. Significant induction of IFN-γ–specific mRNA transcript production was detected in the spleen cells of MDZ-treated mice (Fig. 4). However, the level of IL-4–specific mRNA was significantly reduced in MDZ-treated compared to control mice. Moreover, altered levels of IFN-γ and IL-4 cytokines in serum correlated with mRNA transcript levels in MDZ-treated mice (Fig. 5).

## Discussion

In this study, we demonstrated that MDZ inhibits the production of OVA-specific IgE in serum as well as the production of surface $IgE^+$ B cells in spleen cells in concert with suppression of IgE CSR. Moreover, increased IFN-γ and decreased IL-4 mRNA and their similar protein levels were detected in serum and spleen cells from MDZ-treated mice. These data suggest that inhibition of Ag-specific IgE production by MDZ is associated with differential regulation of cytokines. Therefore we focused on MDZ-mediated immunomodulation of Th1/Th2 cell balance and suppression of IgE production. It now seems that Th1 and Th2 responses are strongly tied to IFN-γ and IL-4, respectively. We first examined the ability of MDZ to modulate the *in vivo* production of IFN-γ and IL-4 in spleen cells from BALB/c mice immunized with OVA plus nCT in order to elucidate the potential for MDZ to induce a Th1-skewed response. In this trial, MDZ administration effectively reduced the level of OVA-specific IgE in serum (Fig. 1). In parallel with suppression of IgE in serum, i.p. administration of MDZ resulted in increased IFN-γ production concomitant with suppression of

IL-4 production in serum (Fig. 5). The reduction in serum IgE could be explained by enhanced IFN-γ production and suppressed IL-4 production since IL-4 signaling is a prerequisite for IgE synthesis in B cells. It is well known that Th2 cytokines such as IL-4 and IL-5 are essential for IgE production and IgE-mediated allergic responses because of their function in recruiting B cells, mast cells, and eosinophils involved in allergic inflammatory reactions (14, 15). In contrast, the Th1 cytokine IFN-γ has inhibitory effects on both IgE production (13) and Th2 differentiation (16). The balance between Th1 and Th2 cytokines is therefore considered crucial for IgE production. In contrast, MDZ significantly increased total IgG producition (data not shown). Since IFN-γ-production was enhanced by MDZ, it is thought that IgG production was derived from MDZ. The results of *in vivo* cytokine production analyses indicate that i.p. administration of MDZ has an immunomodulatory effect that results in a Th1-skewed cytokine response. Therefore, it is possible that MDZ modulates the balance between Th1 and Th2 responses through induction of Th1 responses in systemic immunity, thereby leading to suppression of IgE production. This is the first demonstration that MDZ administered via an i.p. route can lower the level of

Ag-specific IgE in serum.

MDZ significantly inhibited CSR to IgE through inhibition of εGLT and εCT expression. It was shown that cytokines play important roles in the induction of GLT of distinct $C_H$ genes. For example, ε-GLT is induced by IL-4 and IL-13 (17). In contrast, IFN-γ and IL-21 inhibit the ε-GLT (18, 19). Furthermore, the expression of Id2 was increased by MDZ. Id2 suppresses IgE CSR by inhibiting the εGLT-inducing activities of E2A and Pax5 (20, 21). It has been shown that TGFβ inhibits IgE CSR through the induction of Id2 (5). Therefore, MDZ may increase Id2 expression via the secretion of TGFβ, since MDZ could enhance TGFβ production in serum (data not shown).

Naïve CT is a complex molecule with multiple antigenic and metabolic effects that binds to GM1 gangliosides on the surface of target cells eventually resulting in increased adenylate cyclase activity, thereby increasing the intracellular concentration of cAMP resulting in the secretion of $H_2O$, $Na^+$, $K^+$, and $HCO_3^-$ into the intestinal lumen in addition to its antigenic effects (22). As a mucosal adjuvant, CT given via oral routes induces Th2 cells that secrete high levels of IL-4 (23, 24). The IL-4 cytokine provides a helper signal for the induction of IgE Abs that may cause

anaphylactic reactions (25, 26). Indeed, oral and parenteral administration of CT has been reported to induce Th2 responses, such as IgE Ab production, in rodents and humans (27-29). CT induces maturation of human dendritic cells (DC) and licences them for Th2 priming (30). Since allergic sensitization occurs when antigen-presenting cells such as DC in the lymphatic system signal T cells which then interact with B cells to induce IgE production, MDZ may inhibit the adjuvant effect of CT on DC.

In summary, although the mechanism(s) by which MDZ alters the Th1/Th2 balance are yet to be elucidated at the cellular and molecular level, this is the first demonstration that i.p. administration of MDZ can lower serum IgE levels *in vivo*. These findings suggest that MDZ may be an important modulator of allergic responses through its ability to downregulate IgE production.

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| Midazolam | Stagger | Decrease in physical activity | Sedation | Loss of righting reflex |
|---|---|---|---|---|
| 1mg/kg | 2 / 5 | 3 / 5 | 1 / 5 | 5 / 5 |
| 3mg/kg | 4 / 5 | 5 / 5 | 4 / 5 | 5 / 5 |
| 10mg/kg | 5 / 5 | 5 / 5 | 5 / 5 | 5 / 5 |

Table. 1 Optimization of MDZ dose

Various doses (1 mg, 3 mg, and 10mg/kg/mouse) of MDZ were administered intraperitoneally to BALB /c mice (5 mice / group). Ten minutes later, behavioural effects by MDZ were examined.

Fig. 1

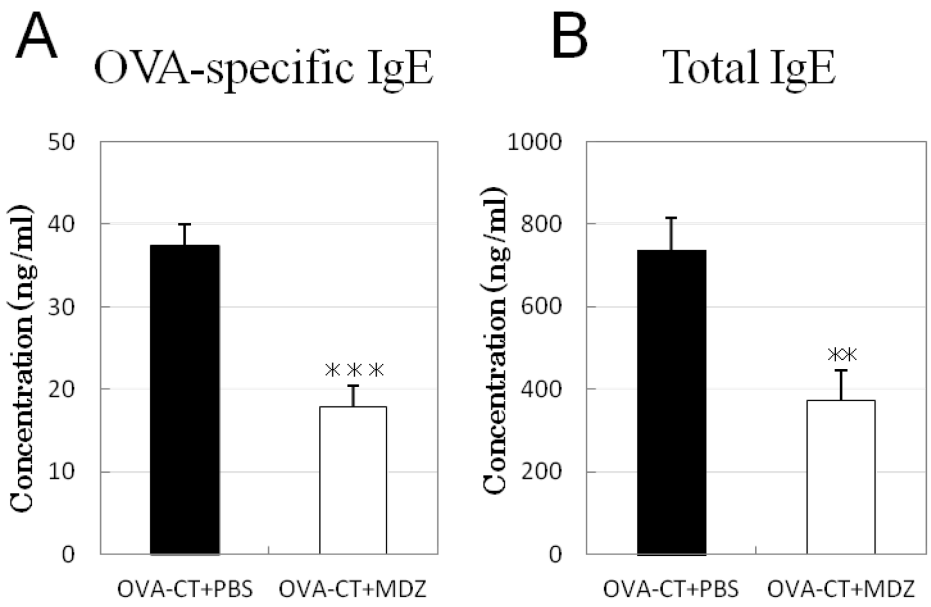
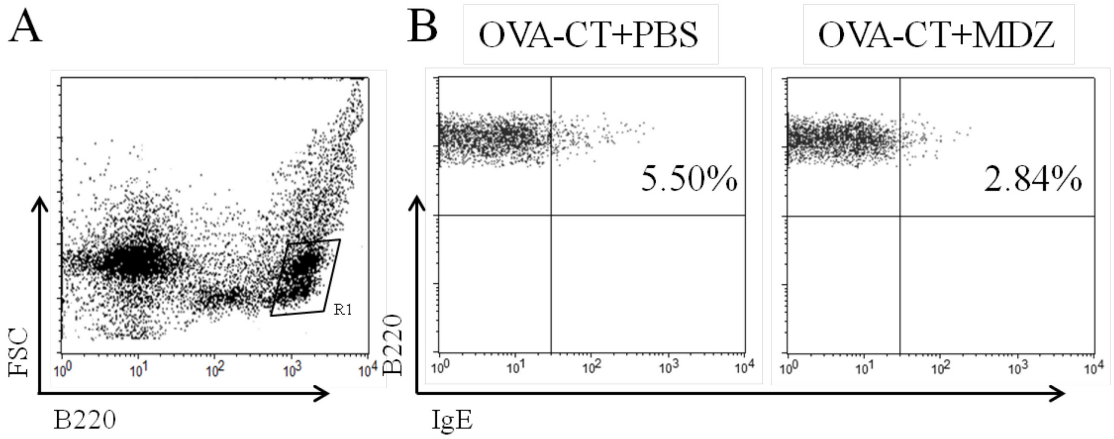



Figure 1. IgE response in serum
BALB/c mice were pre-administered MDZ (3 mg/kg) or PBS alone via i.p. injection and then immunized with OVA plus nCT on days 0, 7, and 14 via i.p. injection. Serum samples were collected on day 21, and the OVA-specific and total IgE responses were examined by ELISA. Results are expressed as the mean ± SD of five mice per group. $^{***}P<0.001$ compared with control (OVA plus nCT + PBS).

Fig. 2



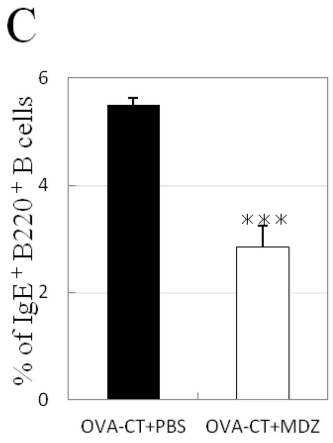




Figure 2. Frequency of surface IgE-positive B cells
BALB/c mice were pre-administered MDZ or PBS and then immunized with OVA plus nCT or PBS alone as described in the legend to Figure 1. Spleens were collected on day 21 and single cell suspensions were prepared. The cells were stained with Alex Flouor 647- conjugated anti-mouse B220 and PE- conjugated anti-mouse IgE, and analyzed by flow cytometry on a FSC/B220 plot (A) or IgE/B220 plot (B). The B cell population (Gate R1) was further gated for B220 binding and forward scatter (FSC) properties (A) by selecting those that were stained with anti-IgE Abs. The results are representative of five experiments. Panel C shows the summary results of five mice per group. Results are expressed as the mean ± SD $^{***}P<0.001$ compared with control (OVA plus nCT + PBS).

Fig. 3

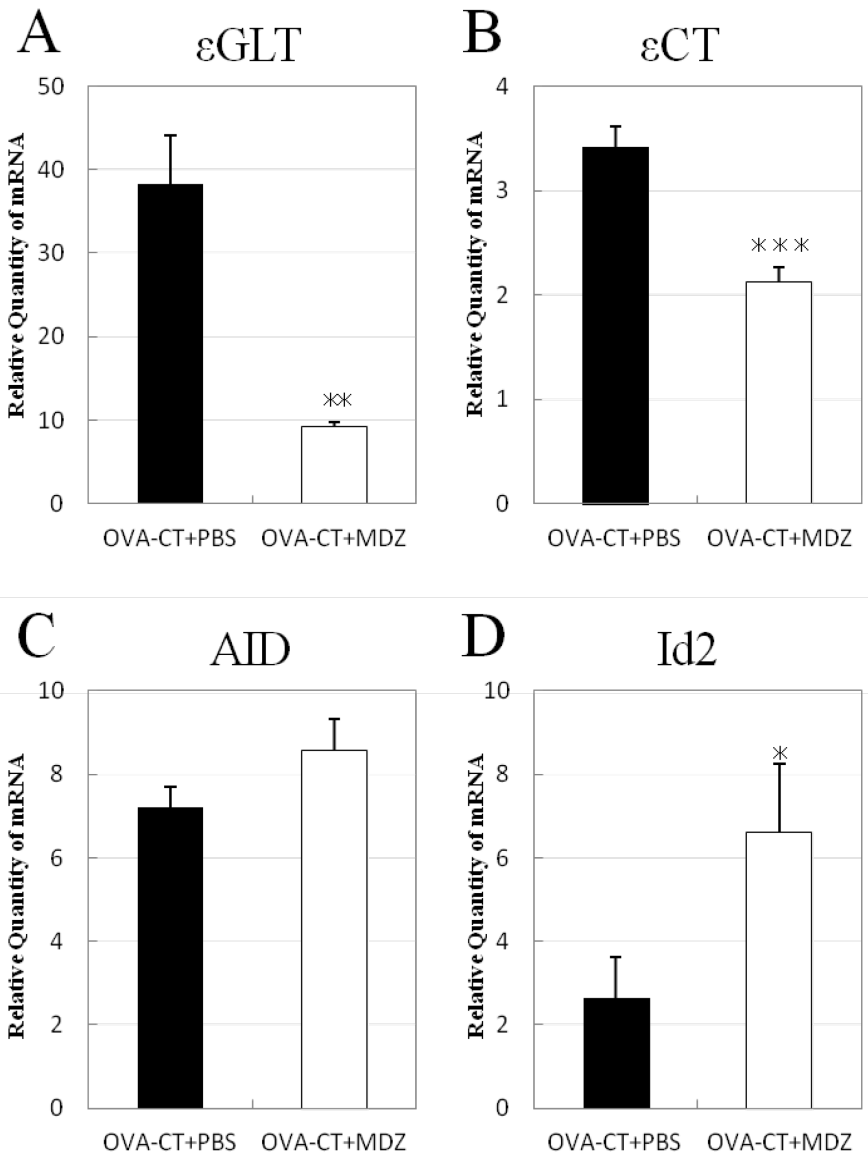



Figure 3. Expression of class switch recombination-associated molecules
BALB/c mice were pre-administered MDZ or PBS and then immunized with OVA plus nCT as described in the legend to Figure 1. Spleens were removed on day 21 and total RNA was isolated. The levels of εGLT (A), εCT (B), AID (C), and Id2 (D) mRNA transcripts were determined using quantitative RT-PCR. Relative mRNA levels were determined after normalization to the level of GAPDH mRNA. Values represent mean values ± SD (n=5). $^{*}P<0.05$ compared with control (OVA plus nCT + PBS). $^{**}P<0.005$ compared with control (OVA plus nCT + PBS). $^{***}P<0.001$ compared with control (OVA plus nCT + PBS).

Fig.4

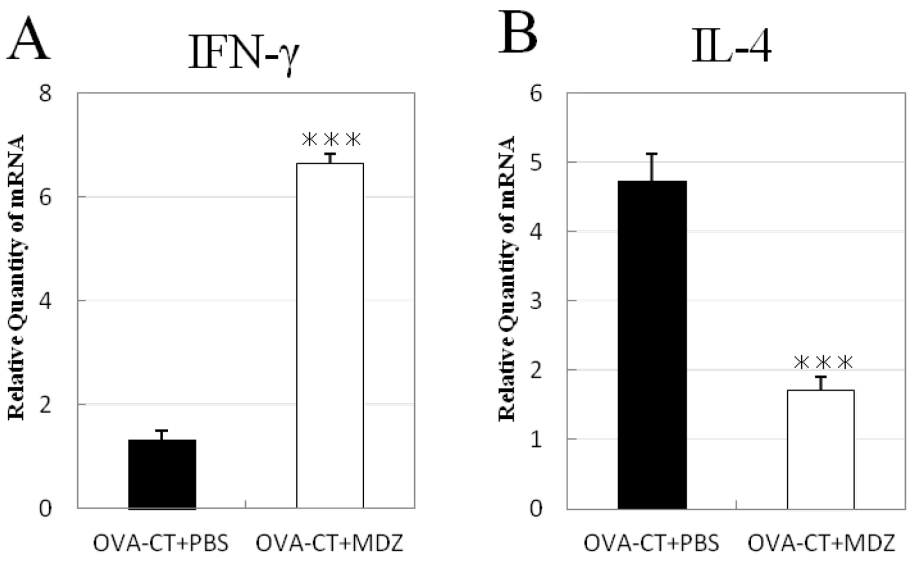



Figure 4. Expression of IFN-$\gamma$ and IL-4 mRNA
BALB/c mice were pre-administered MDZ or PBS and then immunized with OVA plus nCT as described in the legend to Figure 1. Spleens were removed on day 21 and total RNA was isolated. The levels of IFN-$\gamma$ (A) and IL-4 (B) mRNA transcripts were determined using quantitative RT-PCR. Relative mRNA levels were determined after normalization to the level of GAPDH mRNA using real-time RT-PCR. Values represent the mean values ± SD (n=5). $^{***}P<0.001$ compared with control (OVA plus nCT + PBS).

Fig.5

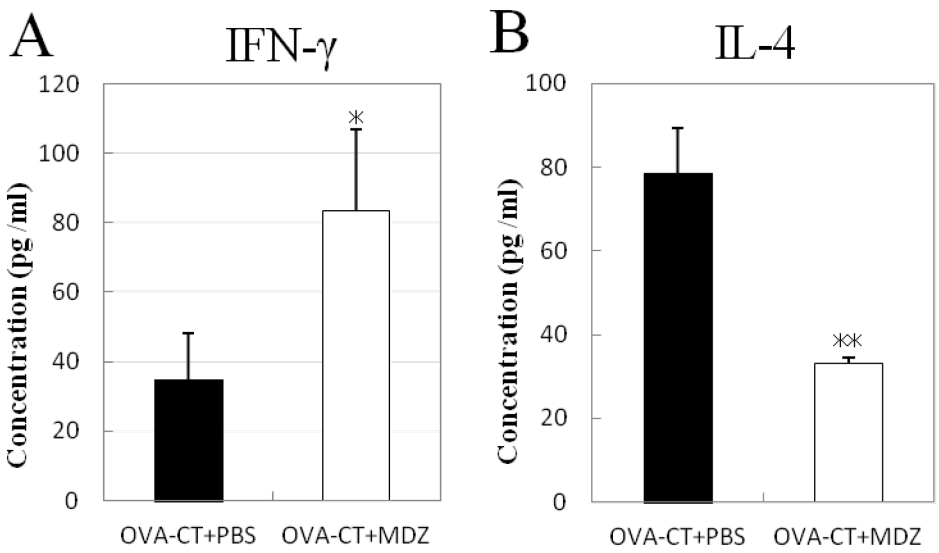



Figure 5. Serum levels of IFN-$\gamma$ and IL-4 cytokines
BALB/c mice were pre-administered MDZ or PBS and then immunized with OVA plus nCT as described in the legend to Figure 1. Plasma samples were collected on day 21 and the levels of IFN-$\gamma$ (A) and IL-4 (B) were determined using a Quantikine™ IFN-$\gamma$ and IL-4 immunoassay kit (R&D Systems, Minneapolis, MN). Values represent mean values ± SD (n = 5). $^{*}P<0.05$ compared with control (OVA plus nCT + PBS). $^{**}P<0.005$ compared with control (OVA plus nCT + PBS).