

S-adenosyl-L-homocysteine hydrolase inactivation curtails
ovalbumin-induced immune responses

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Running Title:

AdoHcy hydrolase inhibitor suppresses OVA specific immune responses

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DZ2002, methyl 4-(adenin-9-yl)-2-hydroxybutanoate; MDL 28,842,
(Z)-5'-fluoro-4',5'-didehydro-5'-deoxyadenosine; AdoHcy,
S-adenosyl-L-homocysteine; CFA, complete Freund's adjuvant; DNFB,
2,4-dinitrofluorobenzene; ELISA, enzyme-linked immunosorbent assay; IL,
interleukin; OVA, ovalbumin; PBS, phosphate-buffered saline; TMB,
3,3',5,5'-tetramethylbenzidine; Th1, type 1 helper T cell; Th2, type 2 helper T cell

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Abstract

The reversible S-adenosyl-L-homocysteine (AdoHcy) hydrolase inhibitor methyl 4-(adenin-9-yl)-2-hydroxybutanoate (DZ2002) suppresses macrophage activation and function. The effects of DZ2002 on T cell function, however, are still unclear. Here, we examined whether DZ2002 alters type 1 helper T cell (Th1) and/or type 2 helper T cell (Th2) immune responses, and whether these effects are associated with both the inhibition of AdoHcy hydrolase and intracellular elevation of endogenous AdoHcy. Male C57BL/6 mice immunized with ovalbumin (OVA) were treated with DZ2002 (1, 5, 25 mg/kg/day) after which lymphocyte proliferation, cytokine production and IgG responses to OVA were monitored. Administration of DZ2002 dose-dependently suppressed OVA-specific lymphocyte proliferation and anti-OVA IgG production compared to controls. IL-2 and IFN- γ , as well as anti-OVA IgG2a and IgG3, indicators of Th1 immune responses, were markedly decreased in mice treated with DZ2002, whereas IL-4 and anti-OVA IgG1, indicators of Th2 immune responses, were only mildly suppressed. AdoHcy hydrolase activity in spleens of DZ2002-treated mice was substantially blocked, and not surprisingly, AdoHcy levels were significantly elevated compared to controls. Finally, similar immunosuppressive effects were also observed in mice treated with AdoHcy. These data strongly indicate that DZ2002 suppresses antigen-induced specific immune responses, particularly Th1 responses, through inhibition of AdoHcy hydrolase and elevation of endogenous AdoHcy.

Introduction

S-adenosyl-L-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1) is a highly conserved ubiquitous enzyme of 432-amino acids that catalyzes the hydrolysis of AdoHcy to adenosine (Ado) and homocysteine (Hcy) in eukaryotic cells (Yuan et al., 1999). Conversely, inhibition of the AdoHcy hydrolase leads to the accumulation of intracellular levels of AdoHcy, a potent feedback inhibitor of all S-adenosyl-L-methionine-dependent cellular transmethylation, including methylations of proteins, lipids, and nucleic acids (Saso et al., 2001; Wu et al., 2005). As lymphocytes appear more dependent upon transmethylation reactions than most other cell types for their activation/function (German et al., 1983), AdoHcy hydrolase is thought to be an attractive target for the design of immunosuppressive and anti-inflammatory agents.

In recent years, several AdoHcy hydrolase inhibitors have been characterized. These compounds block T cell proliferation and IL-2 production (Wolos et al., 1993a; Wu et al., 2005), and exhibit both prophylactic and therapeutic effects in T cell mediated immune models, including 2,4-dinitrofluorobenzene (DNFB) induced delayed-type hypersensitivity reactions (Saso et al., 2001; Wu et al., 2005), allogeneic skin graft rejection, and collagen- (Wolos et al., 1993b, c) and peptidoglycan polysaccharide-induced arthritis (Saso et al., 2001). Although the precise mechanism(s) by which inhibition of AdoHcy hydrolase leads to the suppression of immune cell proliferation and activation are still unknown, three pathways have been

proposed: 1) decreases in transmethylation reactions due to the accumulation of AdoHcy, 2) depletion of intracellular levels of homocysteine, 3) and/or blockade of necessary signaling molecules such as phosphatidyl inositol kinase (Wolos et al., 1993a; Saso et al., 2001; Wu et al., 2005). Thus far, the direct linkage between these pathways and immunosuppression has not been examined.

To date, three types of AdoHcy hydrolase inhibitors have been described: the irreversible type I and type II inhibitors, and the reversible type III inhibitors (Yuan et al., 1999). These inhibitors either take advantage of AdoHcy hydrolase's 3'-oxidative activity (type I), thus becoming irreversibly trapped in the active site of the enzyme, its 5'-hydrolytic activity, which permanently alters the active site (type II), or by reversibly binding to the open form of the enzyme (type III). Due to the relatively long turn-over rate of AdoHcy hydrolase ($t_{1/2} \sim 24$ hrs), the irreversible inhibitors manifest significant toxicity, while type III inhibitors show greatly reduced toxicity yet still retain a similar ability to block the enzyme's activity (Yuan et al., 1999).

Recently, we reported that a potent ($K_i=17.9$ nM) type III inhibitor of AdoHcy hydrolase, DZ2002, obstructs both T cell and macrophage activation/function through downregulation of cytokine production and costimulatory molecule expression (Wu et al., 2005). Moreover, DZ2002 treatment reduced DNFB-induced delayed-type hypersensitivity responses, indicating a direct inhibitory effect on T cells, in particular, CD4⁺ T cells (Wu et al., 2005).

In the present study, we used DZ2002 to test the hypothesis that blockade of AdoHcy hydrolase leads to thymus-dependent antigen-specific immunosuppression in ovalbumin (OVA) immunized mice. We investigated the effects of DZ2002 on proliferative responses, cytokine levels, and antibody production following OVA immunization. In addition, we examined whether DZ2002 administration modulated Th1- versus Th2-type immune responses. Finally, to determine whether elevation of AdoHcy due to the inhibition of AdoHcy hydrolase by DZ2002 contributes to the immunosuppressive effects seen with DZ2002, we compared the effects of the methylation inhibitor AdoHcy (Boger et al., 2000a,b) with those of DZ2002 in OVA immunized mice. We found that indeed, DZ2002 blocked OVA-specific T cell proliferation as well as anti-OVA IgG2a and IgG3 production. In addition, cytokine responses to OVA were decreased in DZ2002-treated mice, and were associated with increased levels of intracellular AdoHcy.

Materials and Methods

Reagents

AdoHcy hydrolase inhibitor methyl 4-(adenin-9-yl)-2-hydroxybutanoate (DZ2002) was synthesized at Diazyme Laboratories (San Diego, CA, USA). Ovalbumin (OVA; grade V), dimethylsulfoxide (DMSO), acetonitrile, AdoHcy, Ado, Hcy, Ado deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride, and TMB were supplied by Sigma Chemical Co. (St. Louis, MO, USA). CFA was purchased from Difco Laboratory (Detroit, MI, USA). RPMI 1640 was bought from GIBCO BRL Life Technologies Inc (Gaithersburg, MD, USA), and fetal calf serum (FCS) was obtained from Hyclone Laboratories Inc. (Logan, Utah, USA). [³H] thymidine was provided by The Shanghai Institute of Applied Physics, Chinese Academy of Science (Shanghai, P.R. China). ELISA kits for IL-2, IFN- γ and IL-4 were purchased from BD PharMingen (San Diego, CA, U.S.A.). Rabbit anti-mouse IgG, IgG1, IgG2a, or IgG3 antibodies labeled with horseradish peroxidase were bought from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Animals

Male C57BL/6 mice, aged 6 to 8 weeks, were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. All animals were housed in specific pathogen-free conditions. Experiments were carried out according to the NIH Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica.

Experimental protocol

OVA (2 mg/ml) was diluted in phosphate-buffered saline (PBS) and emulsified in an equal volume of complete Freund's adjuvant (CFA). 100 µg of OVA in the CFA emulsion was injected s.c. into 2 different sites on the backs of shaved mice ($n=8$ mice/group). DZ2002 (1, 5, and 25 mg/kg/day) was dissolved in PBS containing 0.4% DMSO and administered i.p. following OVA immunization and continued throughout the study. Doses of DZ2002 were chosen on the basis of previous *in vivo* data (Wu et al., 2005) and our own preliminary experiments. As a control, an equal volume of PBS containing 0.4 % DMSO was injected daily into control mice i.p. ($n=8$ mice). As an additional control, the methylation inhibitor AdoHcy (0.2, 1, and 5 mg/kg/day) was dissolved in PBS containing 0.4 % DMSO and also administered i.p. into mice ($n=8$ mice) from days 0 to 21. Our own preliminary pharmacokinetic experiments determined that spleen levels of AdoHcy attained by direct injection (0.2 ~ 5 mg/kg AdoHcy) were 250 ~ 280 nmol/g spleen, corresponding to the concentrations that treatment with DZ2002 (1 ~ 25 mg/kg) achieved. The dosages of AdoHcy were selected based on previous reports that documented *in vivo* physiological effects (Gharib et al., 1985; Fonlupt et al., 1979a, b), and our own previously mentioned preliminary experiments.

Proliferation assay

Spleens and draining lymph nodes (LN) (axillary and inguinal) ($n=8$ mice/group) were removed and cell suspensions prepared as previously described with minor modifications (Feng et al., 2002; Wu et al., 2005). Briefly, erythrocytes were lysed

with tris-NH₄Cl. Remaining cells (5×10^5 cells/well) were then cultured in triplicate in 200 μ l RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2-mercaptoethanol in 96-well flat-bottom plates (Corning Incorporated, Corning, NY) in the presence of 100 μ g/ml OVA. After 60 hrs, 0.5 μ Ci/well [³H]-thymidine was added to each well for 12 hrs and, cells were harvested onto glass fiber filters, and [³H]-thymidine incorporation was measured by a Trilux Microbeta liquid scintillation counter (PerkinElmer Life Science, Boston, MA).

Cytokine measurement

Splenocytes and draining LN cells ($n=8$ mice/group) were prepared as described above. Cells (5×10^5 cells/well) were cultured in triplicate in 96-well flat-bottom plates (Corning) in the presence of 100 μ g/ml OVA. After 48 hrs, culture supernatants were harvested and stored at -20 °C. Concentrations of IL-2, IL-4 and IFN- γ were determined by ELISA as described by the manufacturer.

Determination of OVA-specific antibody

Serum samples ($n=6$ mice/group) were collected on day 21 following OVA-immunization. Levels of OVA-specific total IgG, IgG1, IgG2a, and IgG3 antibodies were determined by ELISA as previously described (Yoshino et al., 2003; Yoshino et al., 2004). Briefly, 96-well plates (Corning) were coated with OVA (100 μ g/mL) overnight. Wells were washed with PBS containing 0.05% Tween-20, then blocked with PBS containing 10% FCS. Next, serum samples were incubated for 30

min, plates were washed, and Ig subclasses were revealed using rabbit anti-mouse IgG, IgG1, IgG2a, or IgG3 conjugated to horseradish peroxidase and an appropriate substrate. Absorbance at 570 nm was read using a SpectraMax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Results are expressed as absorbance units at $OD_{570} \pm S.E.M$ (Yoshino et al., 2003).

Determination of AdoHcy Hydrolase Activity

Spleens from mice ($n=6$ mice/group) treated with increasing dosages of DZ2002 or vehicle were collected on day 21 after immunization with OVA. Tissue was homogenized in iced-cold saline and, after centrifugation at $3,000 \times g$ for 15 min at $4^{\circ}C$, the supernatant was collected and the protein concentration was determined by the Coomassie brilliant blue method (Fu et al., 2005). Supernatant was then used for the measurement of AdoHcy hydrolase activity.

Since AdoHcy hydrolase catalyzes the synthetic reaction from Ado and Hcy to AdoHcy as well as the hydrolytic reaction from AdoHcy to Ado and Hcy, AdoHcy hydrolase activity in spleens was determined in the synthetic direction by measuring the rate of AdoHcy formation from Ado and Hcy according to previously described methods (Yuan et al., 1994; Saso et al., 2001). Briefly, 10 μ l of the supernatant obtained from splenocytes was added to 20 μ l of 10 mM Ado, 20 μ l of 1 mM Ado deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride, and 50 μ l of 62.5 mM Hcy. This solution was then added to 400 μ l of 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA, and incubated at $37^{\circ}C$ for 10 min.

The reaction was terminated by the addition of 20 μ l of 5N HClO₄. After centrifugation, the supernatant was collected and analyzed for AdoHcy by HPLC (Agilent 1100; Palo Alto, CA, USA) using a C18 reversed phase column (Agilent Zorbax SB-C18, 5 μ m, 50 mm \times 2.1 mm; Palo Alto, CA, USA). The elution was carried out in two sequential linear gradients: 6 to 15 % A over 0 to 9 min, 15 to 50 % B over 9 to 15 min, where mobile phase A is acetonitrile and B is 50 mM sodium phosphate buffer, pH 3.2, containing 10 mM L-heptanesulfonic acid. The peak of AdoHcy was monitored at 258 nm and the concentration of AdoHcy was determined by comparison of the area under the curve with that of a known quantity of AdoHcy using a standard curve. One unit of enzyme activity was defined as the amount of enzyme that can synthesize 1 μ mol of AdoHcy/min/mg of protein.

Measurement of AdoHcy in Spleen

Spleens were quickly excised from mice ($n=6$ mice/group) which were treated with various dosages of DZ2002 or vehicle, and snap frozen in liquid nitrogen and stored at -80°C. Approximately 100 mg of spleen tissue was homogenized in four volumes of 0.4M HClO₄. After centrifugation at 10,000 \times g for 15 min at 4°C, the supernatant was filtered through a 0.2- μ m polypropylene syringe filter. A 5- μ l aliquot of the acid extract was applied directly onto the HPLC column as previously described (She et al., 1994; Wang et al., 2001). Results are expressed in nmol/gram wet tissue.

Statistical analysis

Results are expressed as mean \pm S.E.M. One-way analysis of variance followed by Dunnett's post test was used to determine differences between groups where appropriate. $P < 0.05$ was considered significant.

Results

DZ2002 reduces OVA-induced lymphocyte proliferation and cytokine production

Recently, we reported that the reversible AdoHcy hydrolase inhibitor DZ2002 had little effect on ConA stimulated T cells with regards to either proliferation or IL-2 production (Wu et al., 2005). Here, we examined the effects of increasing concentrations of DZ2002 on OVA-induced lymphocyte proliferation at 1, 5, and 25 mg/kg/day. Seven, 14, and 21 days following OVA immunization ($n=8$ mice/group), splenocytes and draining LN (axillary and inguinal) cells were assessed for their capacity to proliferate to OVA *ex vivo*. When splenocytes were stimulated with OVA at 100 $\mu\text{g/mL}$, responses of DZ2002-treated mice were dose-dependently decreased compared to splenocytes from vehicle-treated control mice. For example, on day 21, proliferative responses to OVA were suppressed by 44, 55 and 70 % at 1, 5, and 25 mg/kg DZ2002 respectively (1 mg/kg = $22,671 \pm 2,126$, 5 mg/kg = $18,178 \pm 1,783$, and 25 mg/kg = $12,171 \pm 1,836$ cpm versus vehicle-treated = $40,388 \pm 1,749$ cpm), (Figure 1A). Similarly, when LN cells from DZ2002- and vehicle-treated mice were stimulated *in vitro* with OVA, proliferative responses were blocked in a dose-dependent manner in DZ2002-treated mice (Figure 1B).

We then analyzed whether DZ2002 affected OVA specific cytokine production from DZ2002- or vehicle-treated mice ($n=8$ mice/group). We found that levels of IL-2, IL-4 and IFN- γ , produced by either splenocytes or LN cells, were decreased at all time points by prior treatment with DZ2002 (Figures 2A and 2B). Interestingly, DZ2002 administration appeared to reduce OVA-induced production of

Th1-associated cytokines such as IL-2 and IFN- γ , more than the Th2-associated cytokine, IL-4. As depicted in Figure 2A, on day 21, the highest dosage (25 mg/kg/day) of DZ2002 showed ~83 % inhibition of IL-2 (25 mg/kg = 20.9 ± 1.8 pg/ml versus vehicle-treated = 125.3 ± 4.7 pg/ml) and ~93% inhibition of IFN- γ production (25 mg/kg = 588.8 ± 34.3 pg/ml versus vehicle-treated = $8,782.7 \pm 37.4$ pg/ml) from splenocytes, whereas only ~32 % inhibition of IL-4 production was noted (25 mg/kg = 15.0 ± 0.6 pg/ml versus vehicle-treated = 22.2 ± 1.3 pg/ml). Similarly, the highest dosage (25 mg/kg/day) of DZ2002 showed ~86% inhibition of IL-2 (25 mg/kg = 33.2 ± 2.1 pg/ml versus vehicle-treated = 228.2 ± 26.1 pg/ml) and ~92% inhibition of IFN- γ production (25 mg/kg = 613.9 ± 22.7 pg/ml versus vehicle-treated = $7,499.1 \pm 147.7$ pg/ml) by LN cells, whereas IL-4 was inhibited by only ~18 % (25 mg/kg = 13.3 ± 0.4 pg/ml versus vehicle-treated = 16.3 ± 0.2 pg/ml) (Figure 2 B). We tried to confirm that Th1-type cytokines were preferentially reduced as compared to Th2 cytokines by quantitating a second Th2-associated cytokine, IL-5. Unfortunately, IL-5 levels were too low to detect in our assay (data not shown).

DZ2002 blocks anti-OVA IgG antibody production

To examine the effects of DZ2002 on *in vivo* OVA-specific antibody production, serum levels of anti-OVA IgG, IgG1, IgG2a, and IgG3 antibodies were determined on day 21 ($n=6$ mice/group). As depicted in Figure 3, serum levels of total anti-OVA IgG in DZ2002-treated animals were significantly decreased compared with serum samples from vehicle-treated mice (Figure 3). Treatment with 25 mg/kg/day of DZ2002 showed 52% (25 mg/kg = 0.125 ± 0.013 versus vehicle-treated = $0.262 \pm$

0.035 O.D. units) and 67% (25 mg/kg = 0.172 ± 0.040 versus vehicle-treated = 0.523 ± 0.089 O.D. units) decreases in anti-OVA-IgG2a and IgG3 production, respectively, while a 44% (25 mg/kg = 0.333 ± 0.082 versus vehicle-treated = 0.594 ± 0.021 O.D. units) reduction in anti-OVA IgG1 levels was noted.

DZ2002 inhibits AdoHcy hydrolase activity and leads to the accumulation of intracellular AdoHcy

To examine whether inhibition of AdoHcy hydrolase is directly associated with immunosuppression in OVA immunized mice, AdoHcy hydrolase activity was measured in spleens collected from DZ2002- and vehicle-treated mice ($n=6$ mice/group). We found that administration of DZ2002 reduced AdoHcy hydrolase activity (47.2, 48.6, and 51.5 % at dosages of 1, 5, 25 mg/kg/day, respectively; Figure 4A) in treated mice (1 mg/kg = 18.9 ± 1.1 , 5 mg/kg = 18.5 ± 0.6 , and 25 mg/kg = 17.4 ± 1.2 nmole SAH/min/mg protein versus vehicle-treated = 35.9 ± 1.8 nmole SAH/min/mg protein). These data therefore suggested that, following DZ2002 treatment, AdoHcy is likely accumulating intracellularly in tissues and should be detected. Indeed, inhibition of AdoHcy hydrolase by DZ2002 resulted in the accumulation of intracellular AdoHcy (Figure 4B). As predicted, AdoHcy levels in spleens of DZ2002-treated animals were modestly dose-dependently elevated compared with spleen tissue from controls (1 mg/kg = 253.0 ± 22.5 , 5 mg/kg = 308.0 ± 13.3 , and 25 mg/kg = 381 ± 19.6 nmol/g spleen versus vehicle-treated = 233.0 ± 18.9 nmol/g spleen).

AdoHcy also suppresses OVA-induced immune responses in mice

As DZ2002 potently blocks AdoHcy hydrolase activity and likely results in the accumulation of intracellular AdoHcy, the effects of increasing dosages of AdoHcy (0.2, 1, 5 mg/kg) treatment *in vivo* on OVA-induced immune responses were next investigated. Administration of 1 and 5, but not 0.2, mg/kg/day of AdoHcy resulted in significant decreases in proliferation of splenocytes (Figure 5A) and LN cells (Figure 5B) to OVA ($n=8$ mice/group). Similarly, most Th1-associated responses to OVA including IL-2 and IFN- γ secretion by both splenocytes (Figure 6A) and LN cells (Figure 6B), and anti-OVA IgG2a and IgG3 production (Figure 7) were decreased in mice given 0.2, 1, and 5 mg/kg/day of AdoHcy, respectively ($n=8$ or 6 mice/group). In contrast, Th2 responses to OVA, including IL-4 synthesis by lymphocytes (Figure 6A and 6B) and anti-OVA IgG1 production (Figure 7), were only partially inhibited in mice given the same dosage of AdoHcy. These immunosuppressive effects of AdoHcy were similar to those of DZ2002.

Discussion

The present study demonstrated that treatment of mice with DZ2002 not only decreased OVA-induced lymphocyte proliferation and anti-OVA IgG antibody production, but also suppressed Th1- and Th2-associated immune responses, in particular, Th1 responses. These effects were accompanied by inactivation of AdoHcy hydrolase activity, and by the accumulation of AdoHcy in the spleen. Moreover, similar to DZ2002 treatment of mice, treatment with AdoHcy also suppressed OVA-induced lymphocyte proliferation, anti-OVA IgG antibody production, and Th1- and Th2-type immune responses. This study provides evidence that DZ2002 may be an effective pharmaceutical agent in the prevention of antigen-specific immune responses, and likely exerts its immunosuppressive effects by increasing intracellular AdoHcy levels, thus blocking cellular transmethyations.

An increasing number of people throughout the world are affected by autoimmune diseases with very limited therapeutic options. These diseases are associated with humoral or cell-mediated immune responses against one or more of the body's own self-determinants (Lernmark et al., 2001). Blockade of immune responses to self-antigens that contribute to disease has remained an elusive and challenging problem. We and others, however, have recently established that AdoHcy hydrolase inhibitors are effective in the treatment of several immune-mediated disease animal models, including DNFB-induced delayed-type hypersensitivity reactions (Saso et al., 2001; Wu et al., 2005) and collagen-induced arthritis (Wolos et al., 1993c). This study demonstrated that like other AdoHcy

hydrolase inhibitors, DZ2002 effectively suppressed immune responses to OVA. Supporting our data, Wolos et al. previously reported that a different AdoHcy hydrolase inhibitor, MDL 28,842, also suppressed OVA-induced LN cell proliferation in mice (Wolos et al., 1993a). In combination with our data, these results indicated that AdoHcy hydrolase blockers possess inhibitory properties for antigen-specific immune responses that may play crucial roles in the pathogenesis of autoimmune diseases, and should therefore be further evaluated as possible pharmaceutical agents in the prevention and/or treatment of autoimmune diseases.

Nonetheless, it should be mentioned that previously we documented an inhibitory effect of DZ2002 on *in vitro* macrophage function, and on delayed-type hypersensitivity responses which, although primarily initiated by CD4⁺ T cells, also require macrophage recruitment (Wu et al., 2005). Based on the above data, and our experiments presented here, we cannot rule out that during the single immunization with OVA, the antigen presentation capacity of macrophages and dendritic cells was similarly altered. Logically, decreased antigen presentation cell (APC) activity would therefore lead to the development of fewer T cells, resulting in a smaller OVA-responding T cell compartment showing decreased overall proliferative capacity and cytokine production in response to OVA. Consequently, direct inhibition of T cell function and/or decreased APC activity are likely dual mechanism(s) responsible for the *in vivo* inhibitory effect of DZ2002, and are currently being further studied in autoimmune animal models.

It is long been recognized that antigen-specific IgG antibody production is T cell

dependent. Previous studies with the AdoHcy hydrolase inhibitor MDL 28,842 demonstrated decreased levels of OVA-specific IgG (Wolos et al., 1993a). Consistent with that report, the present study also demonstrates that DZ2002 suppresses anti-OVA IgG production. The reductions in anti-OVA antibody production by inhibition of the AdoHcy hydrolase is likely explained by blockade of antigen-specific lymphocyte activation. In fact, we recently demonstrated that DZ2002 suppressed immune responses to sheep red blood cells, indicating that DZ2002 also has an effect on antibody-secreting B cells (Wu et al., 2005). It is therefore likely that AdoHcy hydrolase inhibitors also suppress T and B cell activation/function.

Naive CD4⁺ T cells primarily differentiate into one of two effector phenotypes, Th1 or Th2 cells. Th1 cells primarily produce IL-2, IFN- γ , and TNF- α whereas Th2 cells primarily produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Seder et al., 1994; Theofilopoulos et al., 1999). Previously, Wolos et al. reported that MDL 28,842 reduced *in vitro* IL-2 production by T cells following mitogen-stimulation (Wolos et al., 1993a). In the present study, we demonstrated that DZ2002 treatment significantly decreased production of Th1-associated cytokines such as IL-2 and IFN- γ , but IL-4, a Th2-type cytokine, was also reduced in response to OVA. Interestingly, decreases in IL-2 and IFN- γ were more significant than reductions seen in IL-4. Moreover, DZ2002 administration significantly suppressed the production of Th1-associated IgG subclasses such as IgG2a and IgG3. Although a reduction in the Th2-associated subclass IgG1 was also noted in DZ2002-treated mice, decreases

in IgG2a and IgG3 appeared to be greater than those seen in IgG1. Since IgG2a, IgG3 and IgG1 production are dependent on Th1 and Th2 cells (Burstein et al., 1993; Isakson et al., 1982), respectively, these results suggest that DZ2002 may preferentially inhibit Th1 driven immune responses as compared to Th2 immune responses following OVA immunization. Previous reports have established that several factors, such as the strain of animal, dose of antigen, route of injection and usage of adjuvants may contribute to the preferential induction of Th1 or Th2 responses (Abbas et al., 1996). In our experiment, vehicle-treated C57BL/6 mice immunized subcutaneously with OVA mixed with complete Freund's adjuvant had less abundant IL-4 producing T cells, and enhanced IFN- γ production, that is, a relatively Th1 skewed response. That the Th1-associated responses appeared more sensitive to DZ2002 than Th2-type responses may be related to the factors mentioned above.

DZ2002, selected out of hundreds of analogues, is a very potent inhibitor of AdoHcy hydrolase with an optimal K_i of ~17.9 nM (Wu et al., 2005). The present study demonstrated that, *in vivo*, DZ2002 treatment substantially inhibited splenic AdoHcy hydrolase activity. As predicted, intracellular AdoHcy levels in spleens of DZ2002-treated animals were significantly elevated compared to controls.

Next, we investigated whether the methylation inhibitor AdoHcy affected antigen-specific immune responses in OVA-immunized mice. Previous reports have shown that AdoHcy can be found intracellularly in both the spleen and liver following i.p. administration (Gharib et al., 1985). We found that, indeed, similar to DZ2002,

in vivo treatment with AdoHcy suppressed OVA-specific immune responses. More recently, others demonstrated that a known methylase inhibitor, 5'-methylthioadenosine, decreased *IL-2*, *IL-4* and *IFN- γ* gene expression in T lymphocytes (Mowen et al., 2004; Richard et al., 2005). Together, these results provide evidence that the suppressive effect of DZ2002 on antigen-specific immune responses is likely related to the elevation of endogenous AdoHcy.

It is known that high levels of AdoHcy can block phosphatidylinositol kinase, an enzyme responsible for second messenger signaling in cellular activation (Pike and DeMeester, 1988). Since T cell activation via the T cell receptor utilizes the phosphatidylinositol-signaling pathway (Desai et al., 1990), inhibition of phosphatidylinositol kinase would likely inhibit T-cell activation. Alternatively, eukaryotic mRNA contains a transmethylation-dependent methylated 5'-cap structure that may prevent protein degradation by phosphatases and ribonucleases (Konarska et al., 1984). Methylation is also required by certain cell membrane signal transduction molecules (Mowen et al., 2004; Richard et al., 2005). In these proteins, methylation is required for the protein to translocate to its correct site in the cell membrane (Tan et al., 1991). Therefore, reductions in transmethylation by AdoHcy may lead to increased degradation and/or decreased translation of message, thus preventing the proper expression and function of receptors required for optimal lymphocyte activation and cytokine production. Any one or combination of these mechanism(s) could be responsible for the inhibitory effect seen with DZ2002 and certainly warrant further study.

In conclusion, the present study demonstrated that the AdoHcy hydrolase inhibitor DZ2002 suppressed antigen-specific immune responses, especially Th1-associated responses, and that the inhibitory effect of DZ2002 is likely related to the elevation of endogenous AdoHcy. Therefore, we believe that the data warrant further evaluation of DZ2002 as a potential therapeutic agent for immune-mediated diseases with a Th1-type bias, such as multiple sclerosis.

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Footnotes

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Legends for Figures

Figure 1

Administration of DZ2002 inhibits OVA-induced splenocyte (A) and LN cell (B) proliferation. OVA-immunized mice were treated with DZ2002 at 1, 5, and 25 mg/kg/day by i.p. injection. On the indicated day, splenocytes and draining LN cells were recovered and 5×10^5 cells were incubated with 100 $\mu\text{g/ml}$ OVA for 72 hrs. Cultures were pulsed with 0.5 $\mu\text{Ci/well}$ [^3H] thymidine for the final 12 hrs. Results are expressed as mean \pm S.E.M. of eight mice. * $P < 0.05$, ** $P < 0.01$ compared with vehicle control (Dunnett's test). Three independent experiments were performed with similar results.

Figure 2

Administration of DZ2002 inhibits OVA-induced splenocyte (A) and LN cell (B) cytokine production. OVA-immunized mice were treated with DZ2002 at 1, 5, and 25 mg/kg/day by i.p. injection. On the indicated days, splenocytes and draining LN cells were recovered and 5×10^5 cells were incubated for 48 hrs with 100 $\mu\text{g/ml}$ OVA. Culture supernatants were collected and analyzed by ELISA for levels of IL-2, IL-4 and IFN- γ . Results are expressed as mean \pm S.E.M. of eight mice. * $P < 0.05$, ** $P < 0.01$ compared with vehicle control (Dunnett's test). Three independent experiments were performed with similar results.

Figure 3

Administration of DZ2002 reduces production of anti-OVA IgG antibody and its subclasses. OVA-immunized mice were treated with DZ2002 at 1, 5, and 25 mg/kg/day by i.p. injection. On day 21 post-immunized anti-OVA IgG, IgG1, IgG2a, and IgG3 levels were determined by ELISA. Results are expressed as mean \pm S.E.M. of six mice. * P <0.05, ** P <0.01. compared with vehicle control (Dunnett's test). Three independent experiments were performed with similar results.

Figure 4

In vivo administration of DZ2002 inactivates AdoHcy Hydrolase (A) and leads to the intracellular accumulation of AdoHcy (B). OVA-immunized mice were treated with DZ2002 at 1, 5, and 25 mg/kg/day by i.p. injection. On day 21 post-immunized, spleens were harvested and the activity of AdoHcy hydrolase and levels of AdoHcy were determined by HPLC. One unit of enzyme activity was defined as the amount of enzyme that can synthesize 1 μ mol of AdoHcy/min/mg of protein. AdoHcy levels are expressed in nmol/gram wet tissue. Results are expressed as mean \pm S.E.M. of six mice. * P <0.05, ** P <0.01. compared with vehicle control (Dunnett's test). Three independent experiments were performed with similar results.

Figure 5

Administration of AdoHcy inhibits OVA-induced splenocyte (A) and LN cell (B) proliferation. OVA-immunized mice were treated with AdoHcy at 0.2, 1, and 5 mg/kg/day by i.p. injection. On day 21 post-immunized, splenocytes and draining LN cells were recovered and 5×10^5 cells were incubated with 100 μ g/ml OVA for 72 hrs. Cultures were pulsed with 0.5 μ Ci/well [3 H] thymidine for the final 12 hrs. Results are expressed as mean \pm S.E.M. of eight mice. $**P < 0.01$. compared with vehicle control (Dunnett's test). Two separate experiments were performed with similar results.

Figure 6

Administration of AdoHcy inhibits OVA-induced splenocyte (A) and LN cell (B) cytokine production. OVA-immunized mice were treated with AdoHcy at 0.2, 1, and 5 mg/kg/day by i.p. injection. On day 21 post-immunized, splenocytes and draining LN cells were recovered and 5×10^5 cells were incubated for 48 hrs with 100 μ g/ml OVA. Culture supernatants were collected and analyzed by ELISA for levels of IL-2, IL-4 and IFN- γ . Results are expressed as mean \pm S.E.M. of eight mice. $*P < 0.05$, $**P < 0.01$ compared with vehicle control (Dunnett's test). Two separate experiments were performed with similar results.

Figure 7

Administration of AdoHcy reduces production of anti-OVA IgG antibody and its subclasses. OVA-immunized mice were treated with AdoHcy at 0.2, 1, and 5 mg/kg/day by i.p. injection. On day 21 post-immunized, anti-OVA IgG, IgG1, IgG2a, and IgG3 levels were determined by ELISA. Results are expressed as mean \pm S.E.M. of six mice. * P <0.05, ** P <0.01 compared with vehicle control (Dunnett's test). Two separate experiments were performed with similar results.

Figure 1

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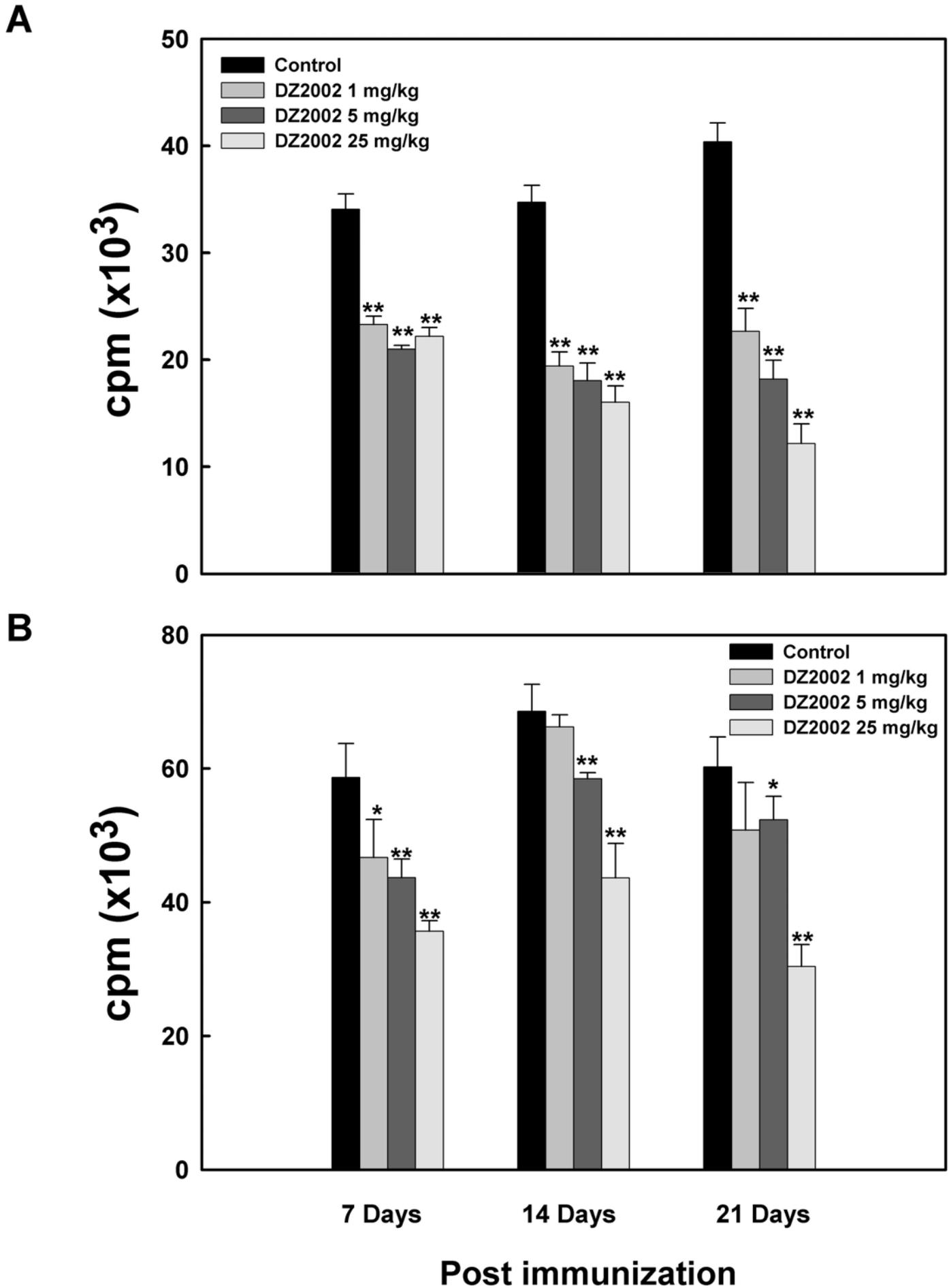


Figure 2

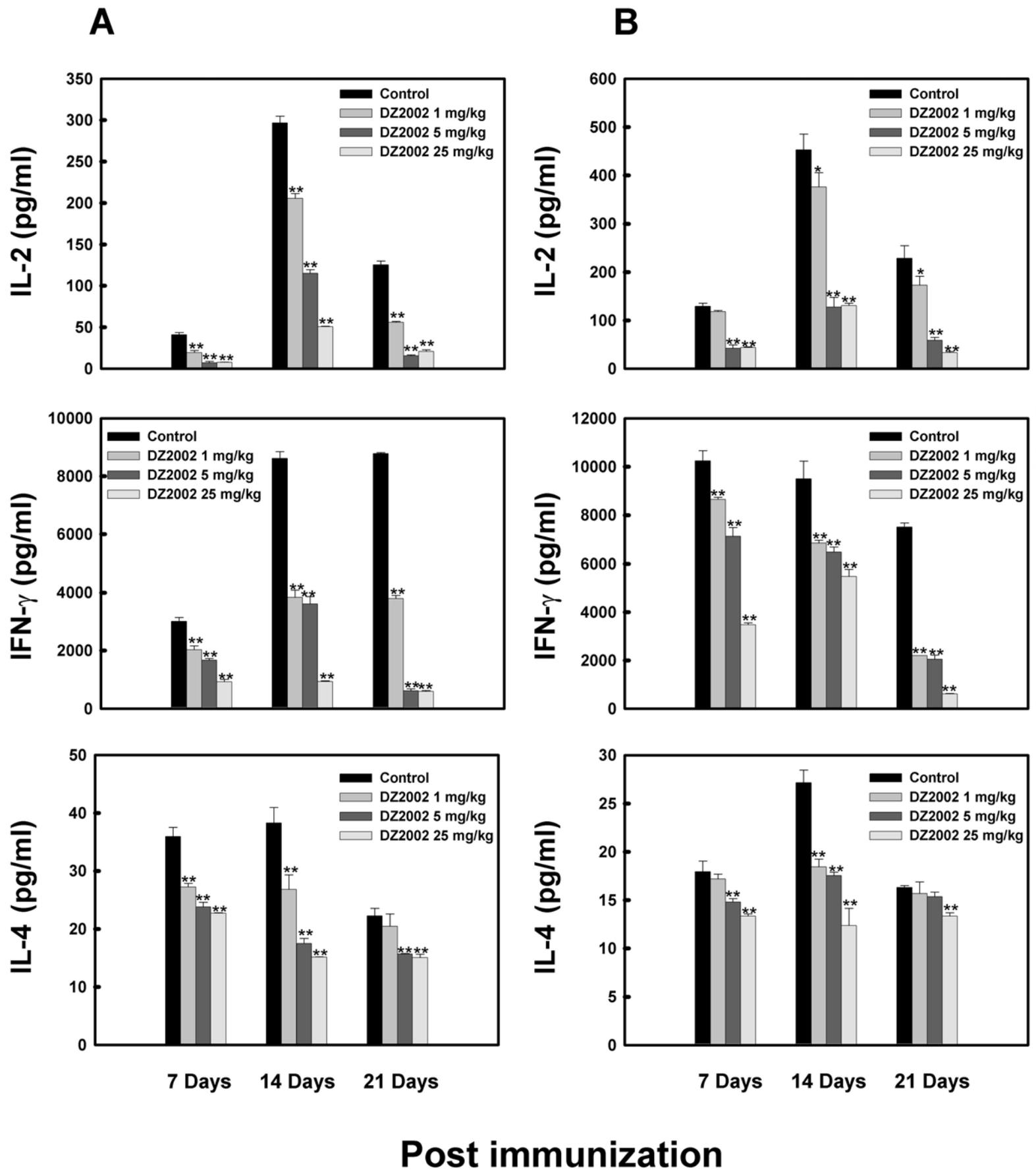
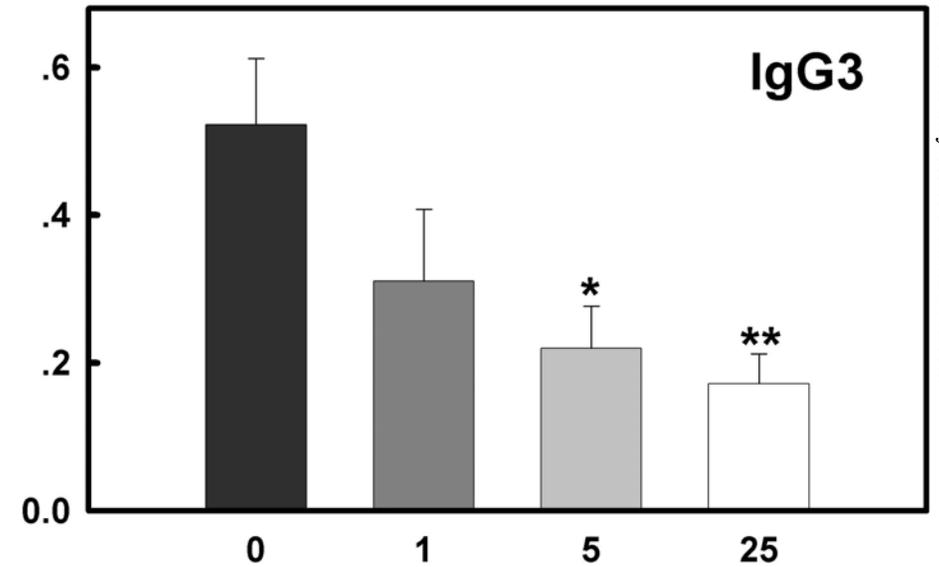
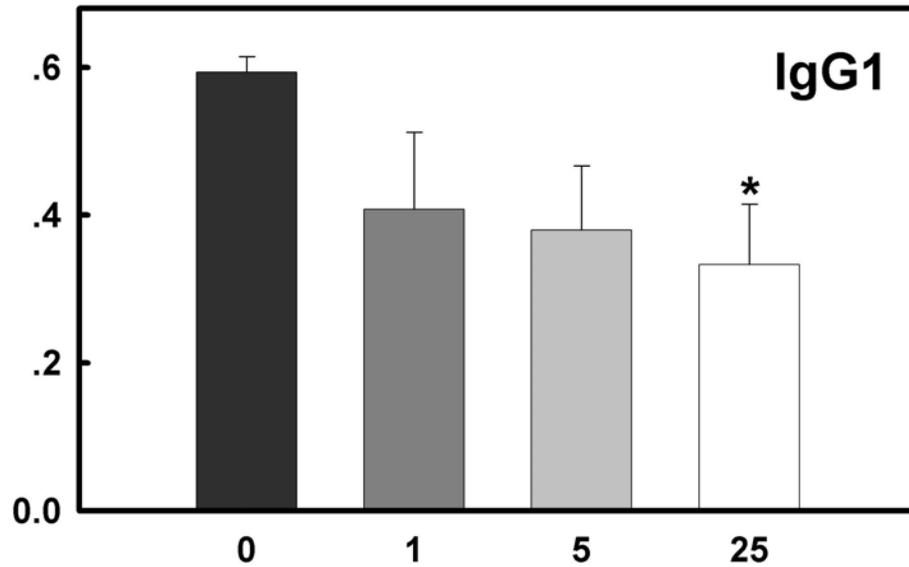
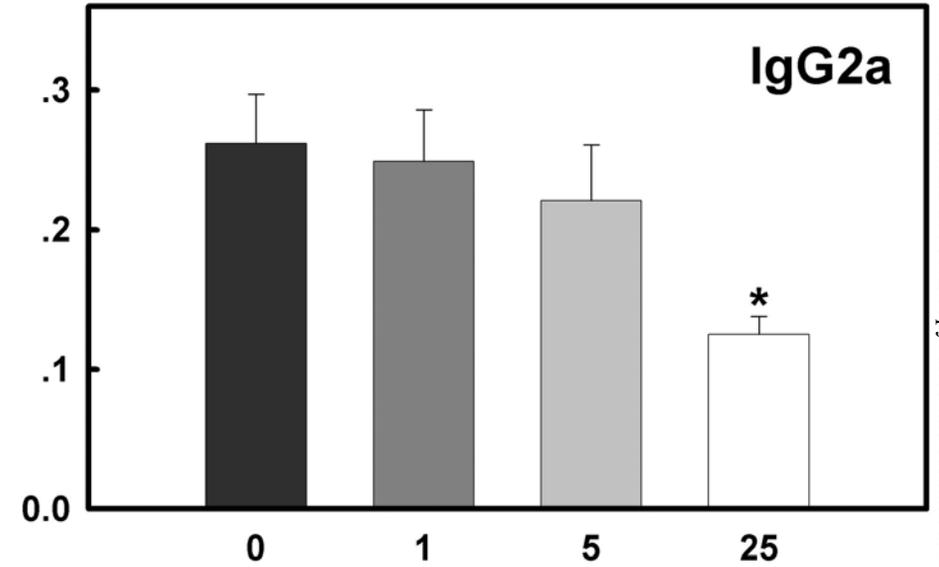
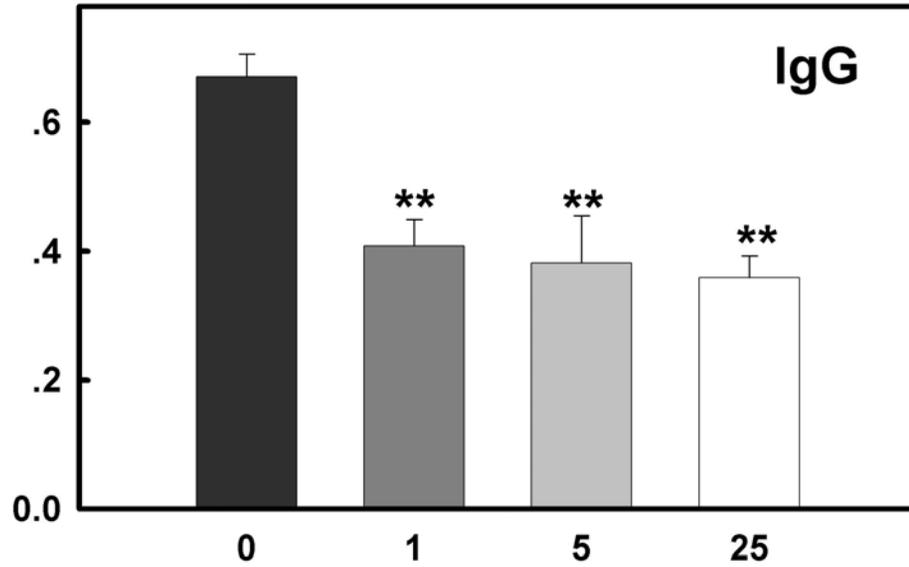


Figure 3

Optical Density (570 nm)



DZ2002 (mg/kg)

Figure 4

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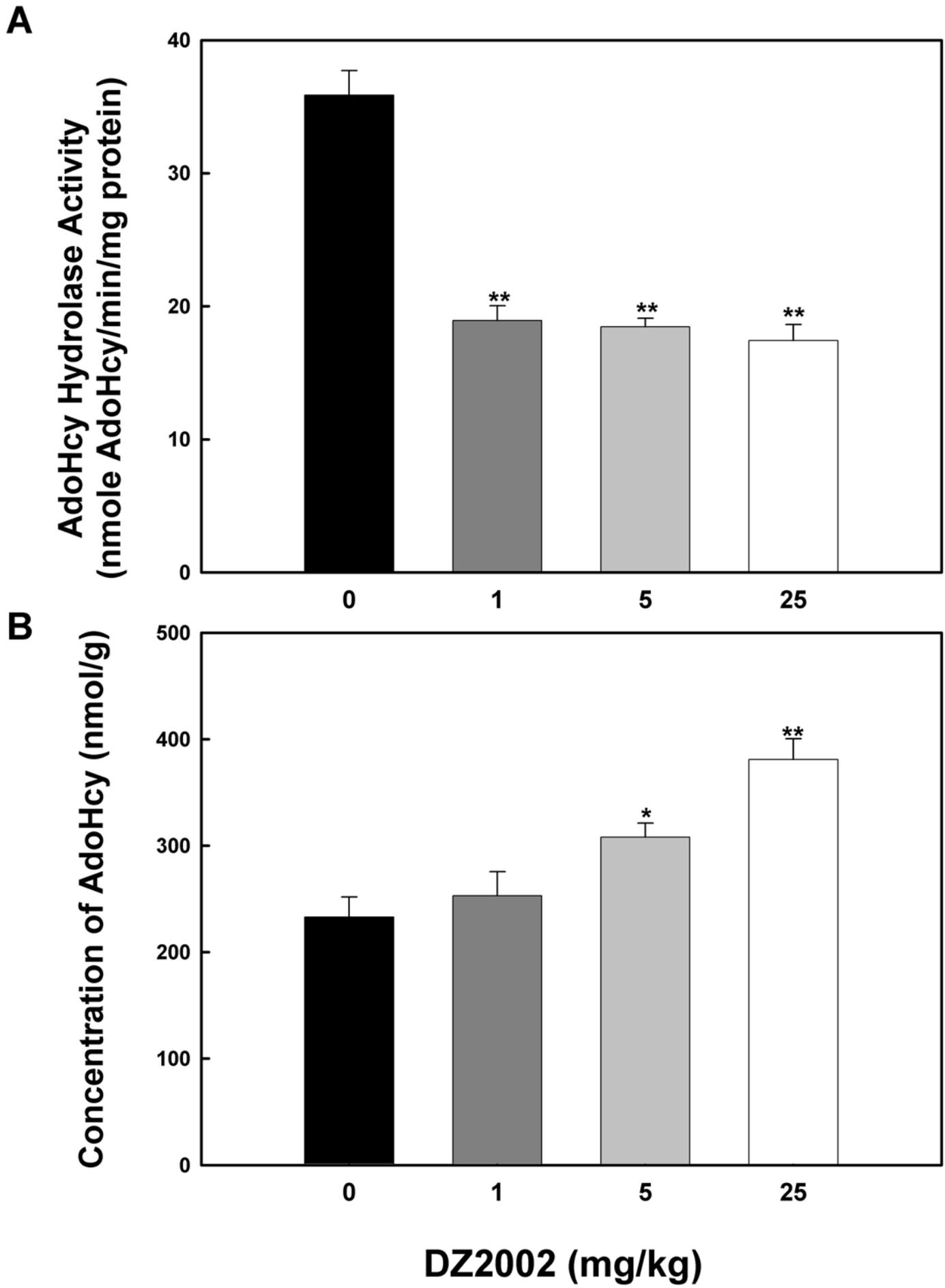


Figure 5

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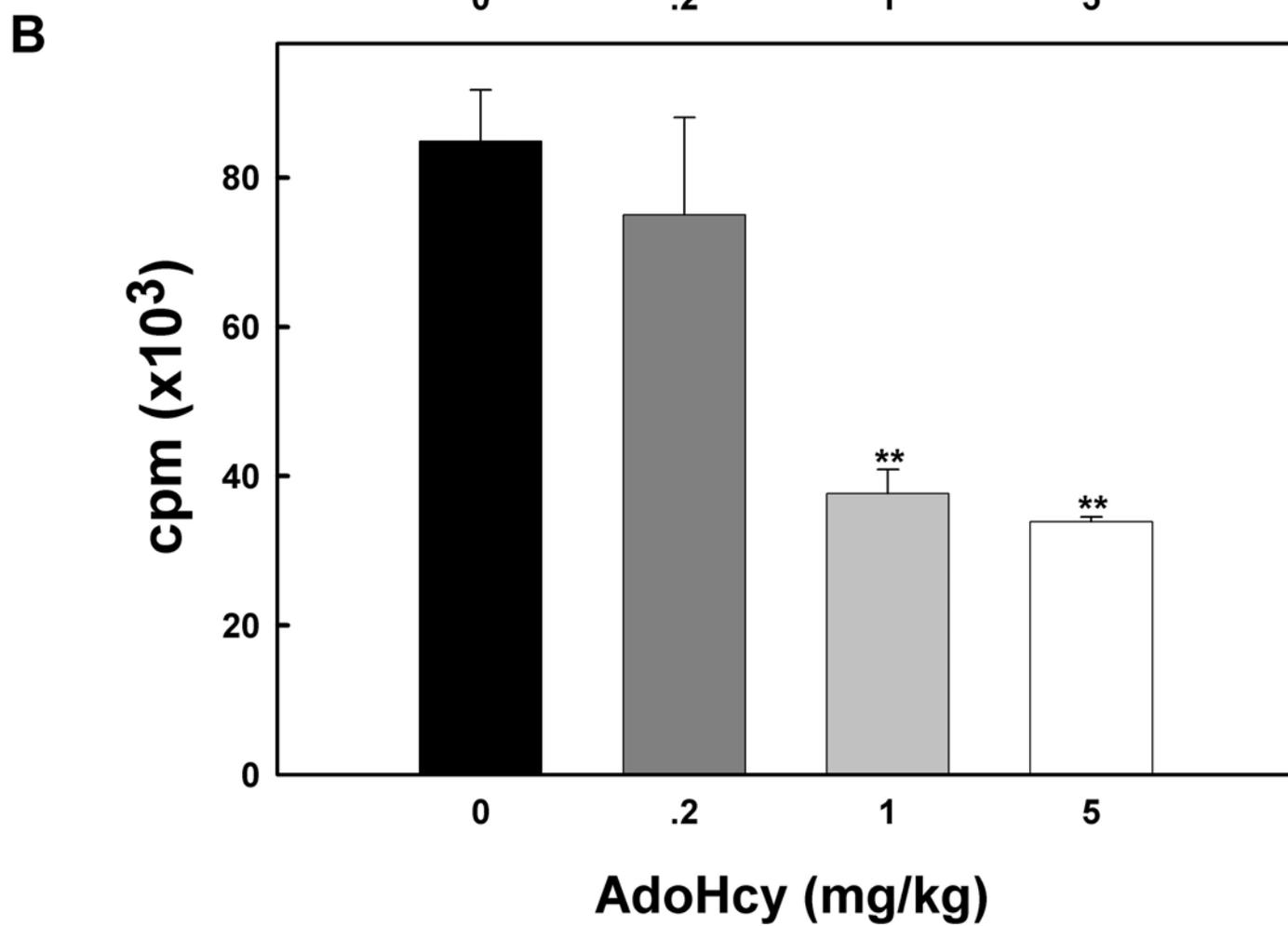
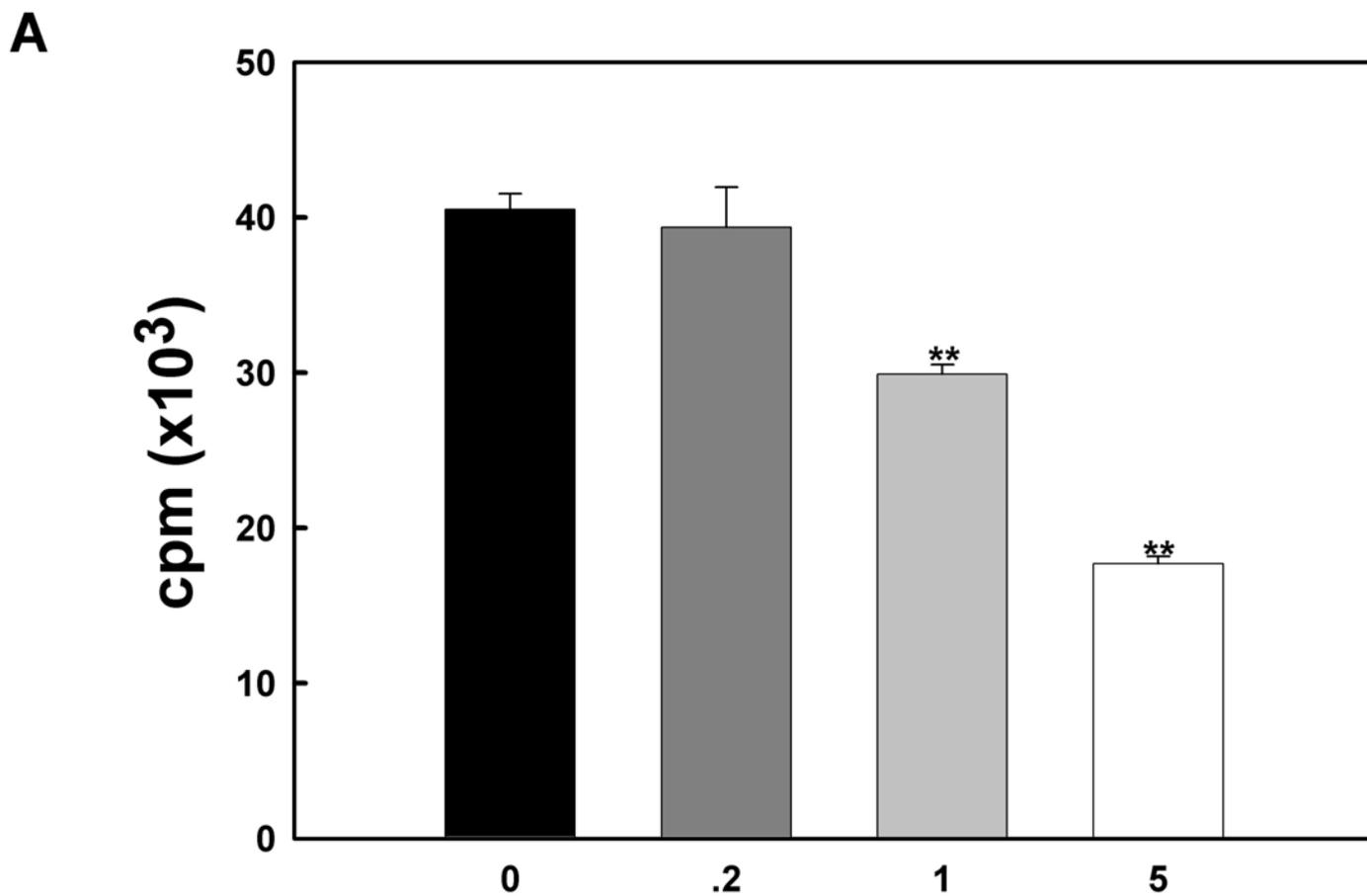


Figure 6

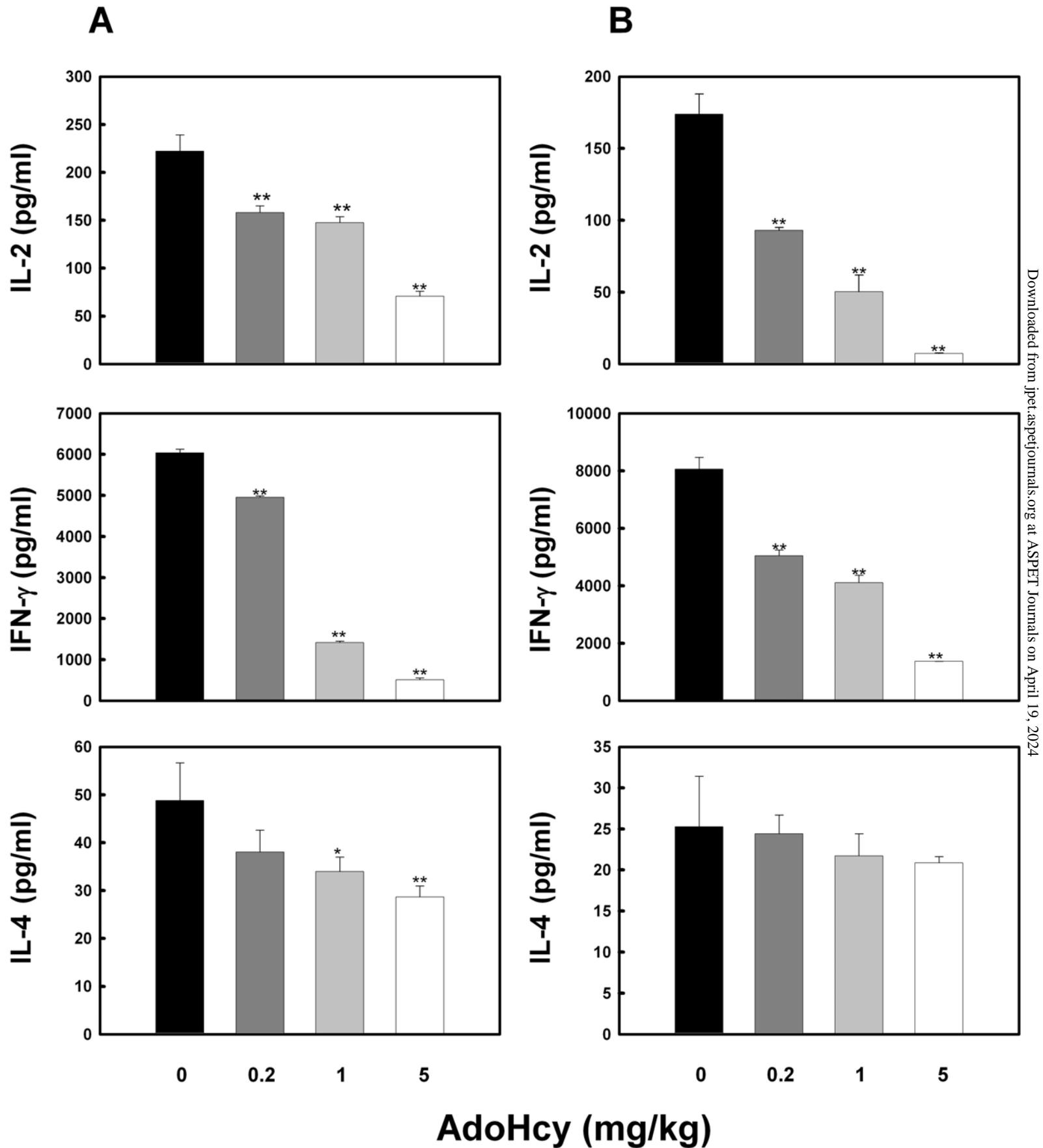


Figure 7

