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# Prostaglandins $E_2$ inhibits advanced glycation end products-induced adhesion molecule expression, cytokine production and lymphocyte proliferation in human peripheral blood mononuclear cells

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d) **Abbreviations: AGEs, advanced glycation end products ; BSA, bovine serum albumin ; COX, cyclooxygenase; cAMP, cyclic adenosine monophosphate ; dbcAMP, dibutyryl cAMP; ELISA, enzyme-linked immunosorbent assay ; FITC, fluorescein isothiocyanate ; ICAM, intercellular adhesion molecule ; IFN, interferon; m, monoclonal ; IL, interleukin, LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; $PGE_2$ ; prostaglandins $E_2$, PKA, protein kinase A ; RAGE, receptor for advanced glycation end products ; TNF, tumor necrosis factor**

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## ABSTRACT

Advanced glycation end products (AGEs) subtypes, proteins or lipids that become glycated after exposure to sugars, induce complications in diabetes. Among the various AGE subtypes, glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3) have been indicated to play roles in inflammation in diabetic patients. The engagement of AGEs and receptor for AGEs (RAGE) activates monocytes. Since the engagement of intercellular adhesion molecule-1 (ICAM-1), B7.1, B7.2 and CD40 on monocytes with their ligands on T-cells plays roles in cytokine production, we investigated the effects of AGE-2 and AGE-3 on the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α and the lymphocyte proliferation in human peripheral blood mononuclear cells (PBMC) and their modulation by prostaglandins $E_2$ ($PGE_2$). AGE-2 and AGE-3 induced the expressions of adhesion molecule, the cytokine production and the lymphocyte proliferation. $PGE_2$ concentration-dependently inhibited the actions of AGE-2 and AGE-3. The effects of $PGE_2$ were mimicked by an $EP_2$-receptor agonist, ONO-AE1-259-01, and an $EP_4$-receptor agonist, ONO-AE1-329. An $EP_2$-receptor antagonist, AH6809 and an $EP_4$-receptor antagonist, AH23848, inhibited the actions of $PGE_2$. The stimulation of

EP2- and EP4-receptors is reported to increase cyclic adenosine monophosphate (cAMP) levels. The effects of PGE2 were reversed by a protein kinase A (PKA) inhibitor, H89, and mimicked by a dibutyryl cAMP (dbcAMP) and an adenylate cyclase activator, forskolin. These results as a whole indicated that PGE2 inhibited the actions of AGE-2 and AGE-3 via EP2-/EP4-receptors and the cAMP/PKA pathway.

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## INTRODUCTION

It is known that sugars including glucose, fructose, and triose react with amino groups of proteins nonenzymatically, leading to the formation of AGE (Brownlee et al., 1988). AGEs, a heterogeneous group of complex structures, form non-enzymatically when reducing sugars react with free amino groups on proteins, lipids, or nucleic acids. The formation and accumulation of AGEs occur at an accelerated rate in diabetic patients and may participate in the pathogenesis of diabetic micro- and macrovascular complications (Bierhaus et al., 1998; Fukami et al., 2004). It is provided direct immunochemical evidence of the existence of four distinct AGE structures, including AGE-2, AGE-3, AGE-4, and AGE-5, within AGE-modified proteins and peptides (Takeuchi and Yamagishi, 2004). Among the various subtypes of AGE, it has been shown that glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3) are the main AGE structures detectable in the serum of diabetic patients. Toxic AGE structures, AGE-2 and AGE-3, have diverse biological activities on vascular wall cells, mesangial cells, Schwann cells, malignant melanoma cells and cortical neurons (Yamagishi et al., 2002; Okamoto et al., 2002). AGE-2 plays roles in the development of atherosclerosis (Takeuchi et al., 2000). The interaction between AGEs and the receptor for AGEs (RAGE), perturbs a variety of vascular homeostatic

functions and thus may contribute to diabetic vasculopathy (Schmidt et al., 1994; Wautier et al., 1996; Park et al., 1998). AGEs and RAGE are reported to be detected in atherosclerotic plaque of diabetic patients (Cuccurullo et al., 2006). A recent study reported that RAGE expression is associated with apoptotic smooth muscle cells and macrophages, suggesting that RAGE may promote plaque destabilization (Burke et al., 2004). It is reported that AGE-modified proteins can induce various proinflammatory and procoagulant cellular responses resulting from nuclear factor-κB (NF-κB) activation (Yan et al., 1994), including the expression of vascular cell adhesion molecule-1, TNF-α, interleukin (IL)-6, and tissue factor (Schmidt et al., 1994; Hofmann et al., 1999; Schmidt et al., 1995; Miyata et al., 1996; Bierhaus et al., 1997).

Microinflammation plays roles in the pathogenesis of diabetic vascular complications. It is reported that diabetes has more macrophage and T-cell infiltration in atherosclerotic plaques (Burke et al., 2004). Activation of monocytes/macrophages and T-cells induces the progression of inflammatory atherosclerotic plaques (Stoll and Bendszus, 2006). The enhanced expression of adhesion molecule, including ICAM-1, B7 and CD40, on monocytes results in the activation of T-cells (Camacho et al., 2001; Ranger et al. 1996; Durie et al., 1994). We also found that cell-to-cell interactions were brought about via the engagement between ICAM-1, B7.1, B7.2 and CD40 on

monocytes and their ligands, lymphocyte function-associated antigen (LFA)-1, CD28 and CD40 ligand (CD40L), on T-cells were involved in T-cell activation, inducing the production of TNF-α and IFN−γ in PBMC (Takahashi et al., 2003). Blockade of the engagement of adhesion molecules by antibodies against ICAM-1, B7.1, B7.2 and CD40 reduced cytokine production in PBMC. In a previous study, we found that AGE-2 and AGE-3, but not AGE-4 and AGE-5, induced the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the lymphocyte proliferation in PBMC (Takahashi et al., 2009; Wake et al., in press). We suggested that the activation of T-cells by the enhancement of adhesion molecule expression on monocytes might result in the development of diabetic microangiopathy.

PGE2, one of the major products of cyclooxygenase (COX)-initiated arachidonic acid metabolite released from monocytes, primes naive human T-cells for enhanced production of anti-inflammatory cytokines and the inhibition of pro-inflammatory cytokines through COX-2 (Hempel et al., 1994; Coleman et al., 1994). There are four subtypes of PGE2 receptors: prostanoid EP1-, EP2-, EP3- and EP4-receptors (Coleman et al., 1994). Activation of EP2- and EP4-receptors leads to an increase in cAMP levels (Coleman et al., 1994). However, little is known about the effect of PGE2 on the AGE-2- and AGE-3-induced adhesion molecule expressions on monocytes.

Therefore, we examined the effect of $PGE_2$ on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the lymphocyte proliferation in PBMC.

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## METHODS

### Reagents and drugs

$PGE_2$, AH6809 and AH23848 were purchased from Sigma Chemical (St. Louis, MO). ONO-D1-004, ONO-AE1-259-01, ONO-AE-248, ONO-AE1-329 and 11-deoxy-$PGE_1$ were kindly provided by Ono Pharmaceutical Co. Ltd. (Tokyo,Japan). Glyceraldehyde-derived AGE (AGE-2) and glycolaldehyde-derived AGE (AGE-3) were prepared as described previously (Cuccurullo et al., 2006). Briefly, AGEs-bovine serum albumin (BSA) was prepared by incubating BSA at 50mg/ml (Sigma Chemical) in $NaPO_4$ buffer (0.2M, pH 7.4) with D-glyceraldehyde (AGE-2) at 0.2 M and D-glycolaldehyde (AGE-3) at 0.2 M (Wako, Tokyo, Japan) at 37°C for 7 days in the presence of 1.5 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l EDTA and $1.0x10^5$ U/l penicillin under endotoxin-free conditions. Dibutyryl cAMP and forskolin were purchased from Wako (Tokyo,Japan). H-89 was purchased from Sigma Chemical. For flow cytometric analysis, an FITC-conjugated mouse $IgG_1$ monoclonal (m) Ab against ICAM-1/CD54 was purchased from DAKO (Glostrup, Denmark). FITC-conjugated mouse $IgG_1$ mAbs against B7.2 and CD40 were purchased from Pharmingen (San Diego, CA), and an FITC-conjugated an $IgG_1$ isotype-matched control was obtained from Sigma Chemical.

**Isolation of PBMC and monocytes**

Normal human PBMC were obtained from ten healthy volunteers after acquiring IRB approval (Okayama Univ. IRB No.106). Samples of 20 to 50 ml peripheral blood were withdrawn from a forearm vein, after which PBMC were prepared, and monocytes isolated from PBMC were separated by counterflow centrifugal elutriation as previously described (Takahashi et al., 2003). The PBMC and monocytes were then suspended at a final concentration of $1x10^6$ cells/ ml in the medium as previously described (Takahashi et al., 2003).

**Flow cytometric analysis**

Changes in the expression of human leukocyte antigens, ICAM-1, B7.1, B7.2 and CD40, on monocytes were examined by multi-color flow cytometry using a combination of anti-CD14 Ab with anti-ICAM-1, anti-B7.1, anti-B7.2 or anti-CD40 Ab. PBMC at $1x10^6$ cells/ml were incubated for 24 h. Cultured cells at $5x10^5$ cells/ml were prepared for flow cytometric analysis as previously described (Takahashi et al., 2003) and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA). The data were processed using the CELL QUEST program (BD Biosciences).

**Cytokine assay**

PBMC at $1x10^6$ cells/ml were used to analyze IFN-γ and TNF-α production. After culturing for 24 h at 37 $^oC$ in a 5%$CO_2$/air mixture, cell-free supernatant was assayed for IFN-γ, and TNF-α protein by ELISA employing the multiple Abs sandwich principle (R&D Systems, Minneapolis, MN). The detection limits of ELISA for IFN-γ and TNF-α were 10 pg/ml.

**Proliferation assay**

PBMC were treated with various conditions. Cultures were incubated for 48 h, during which they were pulsed with [$^3$H]-thymidine (3·3 Ci/well) for the final 16 h. Cells were then divided into 96-well microplates, 200 μl/well, resulting in 1μCi [$^3$H]-thymidine per well, and harvested by the Micro-Mate 196 Cell Harvester (Perkin Elmer Life Science Inc., Boston, MA, USA). Thymidine incorporation was measured by a beta-counter (Matrix 9600, Perkin Elmer Life Science Inc.).

**Measurement of cAMP production in monocytes.**

Monocytes at $1x10^6$ cells/ ml were incubated at 37 $^oC$ in a 5%$CO_2$/air mixture under

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different conditions. After the indicated periods, cells at $2x10^5$ cells/200 μl/well were supplemented with trichloroacetic acid to a final concentration of 5% and 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase at 100 μM and frozen at -80。C. Frozen samples were subsequently sonicated and assayed for cAMP using a cAMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions, for which no acetylation procedures were performed.

**Statistical examination**

Statistical significance was evaluated using ANOVA followed by Dunnett's test. A probability value of less than 0.05 was considered to indicate statistical significant. The results were expressed as the means ± S.E.M. of triplicate findings from five donors.

## RESULTS

### The effects of $PGE_2$ on AGE-2 and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the lymphocyte proliferation in PBMC

In the previous study, to evaluate the binding of AGE subtypes to RAGE, we established an *in vitro* assay by using the immobilized AGE subspecies and the His-tagged soluble form of RAGE (sRAGE) protein (Takahashi et al., 2009). AGE-2 and AGE-3 showed relatively high affinity binding for sRAGE, whereas AGE-4 and AGE-5 showed moderate affinity for sRAGE. The appropriate incubation time and concentration of AGEs were determined according to the study (Takahashi et al., 2009; Wake et al., in press.). AGE-2 and AGE-3 at 100 μg/ml significantly induced the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the proliferation at 16 h and thereafter up to 24 and 48 h. Moreover, the effects of AGE-2 and AGE-3 at concentrations ranging from 100 ng/ml to 100 μg/ml for 24 h were determined. AGE-2 and AGE-3 at 10 and 100 μg/ml significantly induced the expressions of adhesion molecule, the cytokine production and the lymphocyte proliferation.

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As shown in Figs. 1 and 2, we established the effect of $PGE_2$ at concentrations ranging from 1 nM to 1 μM on the expressions of ICAM-1, B7.1, B7.2 and CD40 and its impact on the production of IFN-γ and TNF-α and the lymphocyte proliferation in the presence of AGE-2 and AGE-3 at 100 μg/ml. $PGE_2$ concentration-dependently inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation. The $IC_{50}$ values for the inhibitory effect of $PGE_2$ on the expressions of ICAM-1, B7.1, B7.2 and CD40 and its impact on the production of IFN-γ and TNF-α and the lymphocyte proliferation in the presence of AGE-2 and AGE-3 were shown in Table 1. Moreover, we found that $PGE_2$ had no effect on the adhesion molecule expression and cytokine production in the presence of AGE-4 and AGE-5 (data not shown).

**The involvement of prostanoid $EP_2$- and $EP_4$-receptors in the actions of $PGE_2$**

To determine the involvement of $PGE_2$ receptor subtypes in the effects of $PGE_2$ on the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation, the effects of an $EP_1$-receptor agonist, ONO-D1-004 (Suzawa et al., 2000; Noguchi et al., 2001) an $EP_2$-receptor agonist, ONO-AE1-259-01 (Suzawa et al., 2000; Noguchi et al., 2001) an $EP_3$-receptor agonist, ONO-AE-248

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(Suzawa et al., 2000; Noguchi et al., 2001) and an $EP_4$-receptor agonist, ONO-AE1-329 (Suzawa et al., 2000; Noguchi et al., 2001) at concentrations ranging from 1 nM to 1 μM on the adhesion molecule expression, the cytokine production and the lymphocyte proliferation in the presence of AGE-2 and AGE-3 at 100 μM were determined (Figs. 3 and 4). The $IC_{50}$ values for the inhibitory effect of ONO-AE1-259-01 and ONO-AE1-329 on the expressions of ICAM-1, B7.1, B7.2 and CD40 and its impact on the production of IFN-γ and TNF-α and the lymphocyte proliferation in the presence of AGE-2 and AGE-3 were shown in Table 1. Apparently, the $EP_2$- and $EP_4$-receptor agonists concentration-dependently inhibited AGE-2- and AGE-3-induced effects on the adhesion molecule expression, the cytokine production and the lymphocyte proliferation, but $EP_1$- and $EP_3$-receptor agonists had no effect. Moreover, we confirmed that a mixed $EP_2$/$EP_4$-receptor agonist, 11-deoxy-$PGE_1$ (Suzawa et al., 2000; Noguchi et al., 2001), inhibited AGE-2- and AGE-3-induced adhesion molecule expression in a concentration-dependent manner (Fig. 5). Moreover, the effect of an $EP_2$-receptor antagonist, AH6809 (Kay et al, 2009) and an $EP_4$-receptor antagonist, AH23848 (Kay et al, 2009) at concentrations ranging from 0.1 to 100 μM on the adhesion molecule expression, cytokine production and lymphocyte proliferation were examined in the presence of $PGE_2$ at 1 μM (Figs. 6 and 7). AH6809 and AH23848 reversed the

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inhibitory effect of $PGE_2$ on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation in a concentration-dependent manner. On the other hand, AH6809 and AH23848 had no effect on the actions of AGE-2 and AGE-3 in the absence of $PGE_2$.

**The effects of $PGE_2$ on the production of cAMP in monocytes in the presence or absence of AGE-2 and AGE-3**

The effects of $PGE_2$ at 10 nM on the production of intracellular cAMP in monocytes isolated from PBMC in the presence (100 μg/ml) or absence of AGE-2 and AGE-3 were determined (Fig. 8). $PGE_2$ induced the production of cAMP in monocytes at a peak 30 min after stimulation. The presence of AGE-2 and AGE-3 did not influence the production of cAMP induced by $PGE_2$. $EP_2$- and $EP_4$-receptor agonists at 10 nM induced the production of cAMP (Fig. 8).

**The involvement of cAMP in the actions of $PGE_2$**

To investigate the involvement of the cAMP/PKA pathway in the effects of $PGE_2$ on the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation, the effect of a PKA inhibitor, H89, at concentrations

ranging from 0.1 to 100 μM on the actions of $PGE_2$ in the presence of AGE-2 and AGE-3 at 100 μg/ml was determined (Figs. 9 and 10). H89 reversed the inhibitory effect of $PGE_2$ on AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation. On the other hand, H89 had no effect on the actions of AGE-2 and AGE-3 in the absence of $PGE_2$. As shown in Figs. 11 and 12, the effects of a membrane-permeable cAMP analog, dbcAMP, and an adenylate cyclase activator, forskolin, at concentrations ranging from 0.1 to 100 μM on the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes, the production of IFN-γ and TNF-α and the lymphocyte prolioferation in PBMC in the presence of AGE-2 and AGE-3 at 100 μg/ml were examined. Both dbcAMP and forskolin inhibited AGE-2 and AGE-3-induced adhesion molecule expressions, the cytokine production and the lymphocyte proliferation in a concentration-dependent manner.

## DISCUSSION

The level of glyceraldehyde-derived AGE (AGE-2) is reported to be 17 μg/ml in the serum of patient with diabetes (Nakamura et al., 2007; Enomoto et al., 2006). It is reported that AGEs at the concentrations ranging from 50 to 200 μg/ml remarkably induce human monocyte adhesion to bovine retinal endothelial cells (Mamputu et al., 2004). AGEs at 200 μg/ml induce the expression of CD40, CD80 and CD86 and the production of IFN-γ in dendritic cells (Ge et al., 2005). In the previous study, we found that AGE-2 and AGE-3 at 10 and 100 μg/ml significantly up-regulated the expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ and TNF-α and the lymphocyte proliferation (Takahashi et al., 2009). Thus, the concentration used in the present study covers the pathological concentration of AGEs reported in the studies (Nakamura et al., 2007; Enomoto et al., 2006). Moreover, the accumulation of AGEs is demonstrated in the atherosclerotic lesion by immunohistochemistry (Nakamura et al., 1993). It is likely that higher concentrations of AGEs may be present in the specific inflammatory lesions. Therefore, we determined the effects of AGEs at rather high pharmacological concentration (100 μg/ml).

In the present study, we found, for the first time, that $PGE_2$ inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-γ

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and TNF-α and the lymphocyte proliferation (Figs. 1 and 2). It is suggested that $PGE_2$ modulates inflammation during atherogenesis and other inflammatory diseases by suppressing macrophage-derived chemokine production via the $EP_4$-receptor (Takayama et al., 2002). To investigate receptor subtypes involved in the action of $PGE_2$, we used selective agonists for respective receptors (Suzawa et al., 2000). The $EP_2$-receptor agonist, ONO-AE1-259-01 and the $EP_4$-receptor agonist, ONO-AE1-329, were demonstrated to be highly selective for mouse $EP_2$- and $EP_4$-receptors, respectively, using a receptor binding assay for Chinese hamster ovary cells transfected with each EP cDNA (Suzawa et al., 2000). It is reported that the selective $EP_1$-, $EP_2$-, $EP_3$- and $EP_4$-receptor agonists used in the present study were highly selective for their respective receptors (Suzawa et al., 2000). For example, the $EP_2$-receptor agonist, ONO-AE1-259, had at least 700-fold higher affinity for $EP_2$-receptors compared with other receptor agonists (Suzawa et al., 2000). As shown in Figs. 3 and 4, ONO-AE1-259 and ONO-AE-1-329 mimicked the effects of $PGE_2$ on the adhesion molecule expression, the cytokine production and the lymphocyte proliferation. In the present study, $IC_{50}$ values for the inhibitory effects of ONO-AE1-259 and ONO-AE-1-329 on the expression of ICAM-1 on monoctytes induced by AGE-2 and AGE-3 were similar, respectively (Table 1). It is unlikely that either receptor agonist

stimulated the other receptors at the concentration range used judging from the selectivity of each agonist. As shown in Fig. 5, the observation that the mixed EP2/EP4-receptor agonist, 11-deoxy-PGE1 (Noguchi et al., 2001) mimicked the inhibition of AGE-2- and AGE-3-induced adhesion molecule expression by PGE2 was consistent with the above conclusion. Since the $IC_{50}$ values of PGE2 to prevent the up-regulation of adhesion molecule expressions, cytokine production and lymphocyte proliferation were consistent with the affinity of those agonists to typical EP2- and EP4-receptors (Table 1; Takahashi et al., 2002). Moreover, the EP2-receptor antagonist, AH6809 and the EP4-receptor antagonist, AH23848 inhibited the actions of PGE2 (Figs. 6 and 7). Therefore, it was suggested that the inhibitory effect of PGE2 was mediated by the stimulation of EP2- and EP4-receptors but not EP1- and EP3-receptors.

It is known that the stimulation of EP2- and EP4-receptors induces the production of cAMP (Coleman et al., 1994). As shown in Fig. 8, PGE2, EP2- and EP4-receptors agonists induced the production of cAMP in monocytes irrespective of the presence of AGE-2 and AGE-3. The PKA inhibitor, H89, inhibited the action of PGE2 (Figs. 9 and 10) and the cAMP analog, dbcAMP, and the adenylate cyclase activator, forskolin, mimicked the effect of PGE2 (Figs. 11 and 12). These results suggested the involvement of the EP2/EP4-receptors-cAMP/PKA pathway in the actions of PGE2. In

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addition, the present data were consistent with the finding that the elevation of cAMP prevents the production of TNF-α in monocytes of diabetic patients (Jain et al., 2002). We observed a similar pattern of the inhibitory effects of $PGE_2$ on IL-18-induced activation of monocytes in human PBMC via $EP_2$- and $EP_4$-receptors (Takahashi et al., 2002). Thus, there may be a common pathway triggered by IL-18 and AGEs that was regulated by the $EP_2$/$EP_4$-receptor-cAMP/PKA system. Further work is necessary on this issue.

In the previous study, we found that AGE-2 and AGE-3 had higher affinity for RAGE than AGE-4 and AGE-5 using an *in vitro* binding assay (Takahashi et al., 2009). AGE-2 and AGE-3, but not AGE-4 and AGE-5, induced the up-regulation of their receptor RAGE expression on the cell surface of monocytes. $PGE_2$ had no effect on the expression of RAGE in the presence and absence of AGE-2 and AGE-3 (data not shown), suggesting that there might be distinct signal transduction pathways of RAGE activation, leading to enhanced expression of adhesion molecule and RAGE, which were differentially regulated by the cAMP-PKA system.

RAGE is predominantly localized with lesional macrophages in human carotid atherosclerotic plaques, where macrophages also represent the majority of COX-2-expressing cells (Cuccurullo et al., 2006). It is reported that AGEs ligate

cell-surface RAGE on the vascular endothelium, mononuclear phagocytes, vascular smooth muscle and neurons to activate cell signaling pathways such as P44/P42 mitogen-activated protein kinase and nuclear factor-kappa B (NF-κB) (Yan et al., 1994; Lander et al., 1997), redirectong cellular function in a manner linked to the expression of inflammatory and prothrombotic genes important in the pathogenesis of chronic disorders such as diabetic microvascular disease and amyloidosis (Schmidt et al., 1994; Miyata et al., 1996; Park et al., 1998). When stimulated with lipopolysaccharide (LPS), zymosan or polymerized bovine albumin (Penglis et al., 2000), the expression of COX-2 was specifically up-regulated, leading to enhanced production of $PGE_2$. LPS-treated monocytes/macrophages activate multiple signal transduction pathways, including the activation of NF-κB and JNK. Some of these pathways, in part, may be shared by RAGE signaling. However, we confirmed that AGE-2, AGE-3, AGE-4 and AGE-5 at 100 μg/ml had no effect on the expression of COX-2- mRNA and -protein in human monocytes (data not shown). In the present study, we examined the effect of a non-selective COX-2 inhibitor, indomethacin, and a selective COX-2 inhibitor, NS398, on the actions of $PGE_2$ in the presense or absence of AGE-2 and AGE-3. The COX-2 inhibitors had no effect on the expressions of adhesion molecule, the cytokine production and the lymphocyte proliferation (data not shown). In addition, AGE-2,

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AGE-3, AGE-4 and AGE-5 had no effect on $PGE_2$ production (data not shown). Therefore, it is likely that the endogenous production of $PGE_2$ in monocytes did not occur under the present conditions.

It is reported that $PGE_2$ induced by monocytes inhibits procollagen secretion by human vascular smooth muscle cells, leading to extracellular matrix remodeling and resistance to rupture during atherosclerosis (Fitzsimmons et al., 1999). Elevation of cAMP in endothelial cells inhibits proliferation, leading to the inhibition of atherosclerosis in patients with diabetes (Lorenowicz et al., 2007). Together with these results and our data, other extracellular stimuli, which induce intracellular cAMP production upon binding to their cognate G protein-coupled receptors, may regulate the activation of vascular smooth muscle cells and endothelial cells. In conclusion, $PGE_2$ inhibited AGE-2- and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40, the production of IFN-$\gamma$ and TNF-$\alpha$ and the lymphocyte proliferation via $EP_2$/$EP_4$-receptors and the cAMP/PKA pathway. Through the inhibition of toxic AGE-dependent responses in monocytes, the stimulation of $EP_2$- and $EP_4$-receptors may partially contribute to regulation of the development of atherosclerotic plaques in diabetes. The present study might lead to an exploration of the therapeutic potential of PGE2 on the systemic inflammatory response evoked by diabetes.

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**Footnotes**

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## LEGENDS FOR FIGURES

**Figure 1 The effects of $PGE_2$ on AGE-2 and AGE-3-induced expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes**

PBMC at $1x10^6$ cells/ml were incubated with $PGE_2$ at increasing concentrations from 1 nM to 1 μM in the presence of AGE-2 (A), AGE-3 (B) and BSA (C) at 100 μg/ml for 24 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Isotype-matched control represents FITC-conjugated $IgG_1$. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. ***P< 0.01* compared with the value for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

**Figure 2 The effects of $PGE_2$ on AGE-2 and AGE-3-induced production of IFN-γ and TNF-α and the lymphocyte proliferation in PBMC**

The effect of $PGE_2$ at increasing concentrations from 1 nM to 1 μM in the presence of AGE-2 (A), AGE-3 (B) and BSA (C) at 100 μg/ml on IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [$^3$H]-thymidine uptake as described in Methods. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. ***P*<

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*0.01* compared with the value for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

**Figure 3 The effect of prostanoid receptor agonists on AGE2- and AGE3-induced expressions of ICAM-1, B7.1, B7.2 and CD40**

PBMC at $1x10^6$ cells/ml were incubated with the $EP_1$-receptor agonist, ONO-D1-004 (A), the $EP_2$-receptor agonist, ONO-AE1-259-01 (B), the $EP_3$-receptor agonist, ONO-AE-248 (C) and the $EP_4$-receptor agonist, ONO-AE1-329 (D) at increasing concentrations from 1 nM to 1 μM in the presence of AGE-2 (filled circles;●) and AGE-3 (open circles;○) at 100 μg/ml for 24 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *$P< 0.05$,**$P< 0.01$ compared with the value for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

**Figure 4 The effect of prostanoid receptor agonists on AGE2- and AGE3-induced production of IFN-γ and TNF-α and the lymphocyte proliferation**

PBMC at $1x10^6$ cells/ml were incubated with the $EP_1$-receptor agonist, ONO-D1-004

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(A), the EP2-receptor agonist, ONO-AE1-259-01 (B), the EP3-receptor agonist, ONO-AE-248 (C) and the EP4-receptor agonist, ONO-AE1-329 (D) at increasing concentrations from 1 nM to 1 μM in the presence of AGE-2 (filled circles;●) and AGE-3 (open circles;○) at 100 μg/ml for 24 h. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [$^3$H]-thymidine uptake as described in Methods. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. *$P< 0.05$,**$P< 0.01$ compared with the values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

**Figure 5 The effects of 11-deoxy-PGE1 on AGE2- and AGE3-induced ICAM-1, B7.1, B7.2 and CD40 expressions on human monocytes.**

PBMC at 1x10$^6$ cells/ml were incubated with increasing concentrations of the EP2/ EP4-receptor agonist, 11-deoxy-PGE1 at increasing concentrations from 1 nM to 1 μM in the presence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml for 24 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. **$P< 0.01$ compared with the values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

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**Figure 6 The effects of prostanoid receptor antagonists on the inhibitiory effect of PGE2 on the expressions of ICAM-1, B7.1, B7.2 and CD40**

PBMC at $1x10^6$ cells/ml treated with PGE2 at 1 μM were incubated with the EP2-receptor antagonist, AH6809 (A) and the EP4-receptor antagonist, AH23848 (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. Filled circles ( ● ) represent the effect of antagonists on PGE2-inhibited adhesion molecule expression in the presence of AGE-2. Open circles ( ○ ) represent the effect of antagonists on PGE2-inhibited adhesion molecule expression in the presence of AGE-3. Filled squares ( ■ ) represent the effect of antagonists on the actions of AGE-2 in the absence of PGE2. Open squares ( □ ) represent the effect of antagonists on the actions of AGE-3 in the absence of PGE2. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. ***P< 0.01* compared with the values for PGE2 in the presence of AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

**Figure 7 The effects of prostanoid receptor antagonists on the inhibitiory effect of**

**PGE2 on the production of IFN-γ and TNF-α and the lymphocyte proliferation**

PBMC at $1x10^6$ cells/ml treated with PGE2 at 1 μM were incubated with the EP2-receptor antagonist, AH6809 (A) and the EP4-receptor antagonist, AH23848 (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 and AGE-3 at 100 μg/ml. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [$^3$H]-thymidine uptake as described in Methods. Filled circles ( ● ) represent the effect of antagonists on PGE2-inhibited adhesion molecule expression in the presence of AGE-2. Open circles ( ○ ) represent the effect of antagonists on PGE2-inhibited adhesion molecule expression in the presence of AGE-3. Filled squares ( ■ ) represent the effect of antagonists on the actions of AGE-2 in the absence of PGE2. Open squares ( □ ) represent the effect of antagonists on the actions of AGE-3 in the absence of PGE2. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. ***P< 0.01* compared with the values for PGE2 in the presence of AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

**Figure 8 The effects of PGE2 on the production of cAMP in monocytes in the presence or absence of AGE-2 and AGE-3**

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(A) Monocytes at $1x10^6$ cells/ml were incubated with $PGE_2$ at 10 nM in the presence (filled circles;●) and absence (open circles;○) of AGE-2 (A) and AGE-3 (B) at 100 μg/ml, and the time course changes in the levels of cAMP in monocytes were determined at the indicated time points. (C) The effect of $PGE_2$, the $EP_2$-receptor agonist, ONO-AE1-259-01 and the $EP_3$-receptor agonist, ONO-AE-248 at 10 nM on the production of cAMP in the presence or absence of AGE-2 and AGE-3 at 30 min was determined. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. ND, not detected. When an error bar was within a symbol, the bar was omitted.

**Figure 9 The effects of PKA inhibitor on $PGE_2$-inhibited ICAM-1, B7.1, B7.2 and CD40 expressions**

The effect of a PKA inhibitor, H89 at increasing concentrations from 0.1 to 100 μM on the expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes treated with $PGE_2$ at 10 nM in the presence of the AGE-2 (A) and AGE-3 (B) at 100 μg/ml was determined by flow cytometry. Filled circles (●) represent the effects of H89 on the $PGE_2$-induced inhibition of responses in the presence of AGE-2 and AGE-3. Open circles (○) represent the effects of H89 in the presence of AGE-2 and AGE-3 without

PGE2 stimulation. The results are expressed as the means ± S.E.M. of triplicate findings from five donors. ***P< 0.01* compared with the value for PGE2. When an error bar was within a symbol, the bar was omitted.

**Figure 10 The effects of PKA inhibitor on PGE2-inhibited TNF-α and IFN-γ production and the lymphocyte proliferation**

The effect of a PKA inhibitor, H89 at increasing concentrations from 0.1 to 100 μM on the production of TNF-α and IFN-γ in PBMC treated with PGE2 at 10 nM in the presence of AGE-2 (A) and AGE-3 (B) at 100 μg/ml was determined by ELISA. The lymphocyte proliferation was determined by [$^3$H]-thymidine uptake as described in Methods. Filled circles (●) represent the effects of H89 on the PGE2-induced inhibition of responses in the presence of AGE-2 and AGE-3. Open circles (○) represent the effects of H89 in the presence of AGE-2 and AGE-3 without PGE2 stimulation. The results are expressed as the means ± S.E.M. of triplicate findings from five donors. ***P< 0.01* compared with the value for PGE2. When an error bar was within a symbol, the bar was omitted.

**Figure 11 The effects of forskolin and dbcAMP on AGEs-induced ICAM-1, B7.1, B7.2 and CD40 expressions on human monocytes.**

PBMC at $1x10^6$/ml were incubated with an adenylate cyclase activator, forskolin (A) and a cAMP analog, dbcAMP (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 (filled circles;●) and AGE-3 (open circles;○) at 100 μg/ml for 24 h. The expressions of ICAM-1, B7.1, B7.2 and CD40 on monocytes were determined by flow cytometry. The results are expressed as the means ± S.E.M. of five donors with triplicate determinations. ***P< 0.01* compared with the values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

**Figure 12 The effects of forskolin and dbcAMP on AGEs-induced IFN-γ and TNF-α production and the lymphocyte proliferation in PBMC**

PBMC at $1x10^6$/ml were incubated with an adenylate cyclase activator, forskolin (A) and a cAMP analog, dbcAMP (B) at increasing concentrations from 0.1 to 100 μM in the presence of AGE-2 (filled circles;●) and AGE-3 (open circles;○) at 100 μg/ml for 24 h. IFN-γ and TNF-α concentrations in conditioned media were determined by ELISA. The lymphocyte proliferation was determined by [$^3$H]-thymidine uptake as described in Methods. The results are expressed as the means ± S.E.M. of five donors

with triplicate determinations. $^{**}P < 0.01$ compared with the values for AGE-2 and AGE-3. When an error bar was within a symbol, the bar was omitted.

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**Table 1 The $IC_{50}$ values for the inhibitory effect of $PGE_2$ and EP-2/4 receptor agonists in the presence of AGE-2 and AGE-3**

A) AGE-2

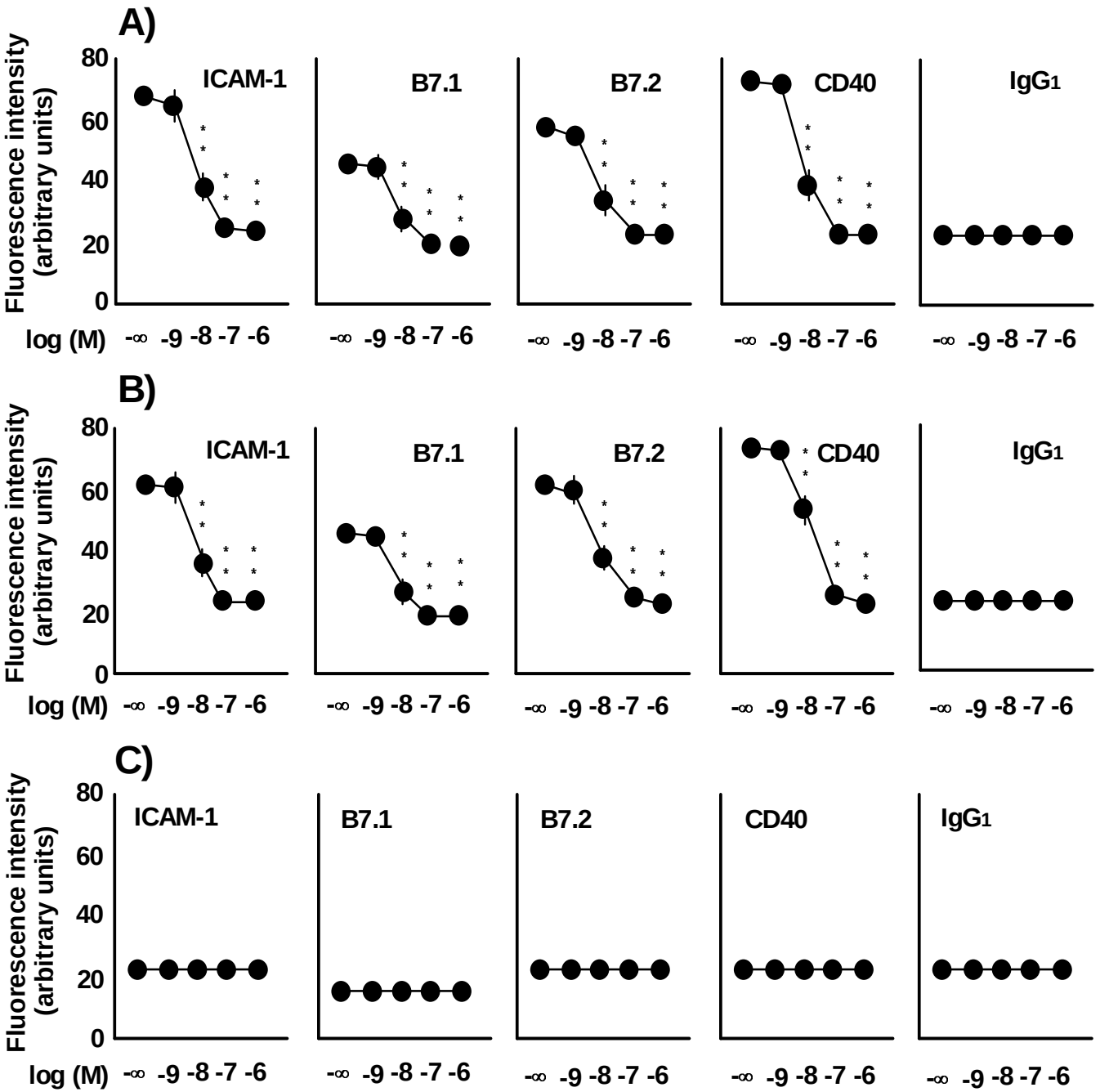
| (nM) | ICAM-1 | B7.1 | B7.2 | CD40 | TNF-α | IFN-γ | proliferation |
|---|---|---|---|---|---|---|---|
| $PGE_2$ | 7±0.2 | 7±0.2 | 8±0.3 | 8±0.2 | 7±0.2 | 8±0.3 | 7±0.3 |
| ONO-AE1-259-01 | 9±0.2 | 10±0.1 | 10±0.1 | 8±0.3 | 7±0.2 | 8±0.2 | 7±0.2 |
| ONO-AE1-329 | 9±0.2 | 10±0.2 | 10±0.1 | 10±0.2 | 10±0.2 | 10±0.2 | 10±0.3 |

B) AGE-3

| (nM) | ICAM-1 | B7.1 | B7.2 | CD40 | TNF-α | IFN-γ | proliferation |
|---|---|---|---|---|---|---|---|
| $PGE_2$ | 8±0.3 | 8±0.1 | 10±0.2 | 15±0.3 | 8±0.3 | 8±0.1 | 7±0.4 |
| ONO-AE1-259-01 | 8±0.2 | 10±0.1 | 10±0.2 | 8±0.2 | 7±0.2 | 8±0.2 | 8±0.2 |
| ONO-AE1-329 | 9±0.2 | 10±0.1 | 10±0.2 | 10±0.1 | 10±0.3 | 10±0.1 | 10±0.2 |

*The results are expressed as the means ± S.E.M. of five donors with triplicate determinations.*

# Figure 1



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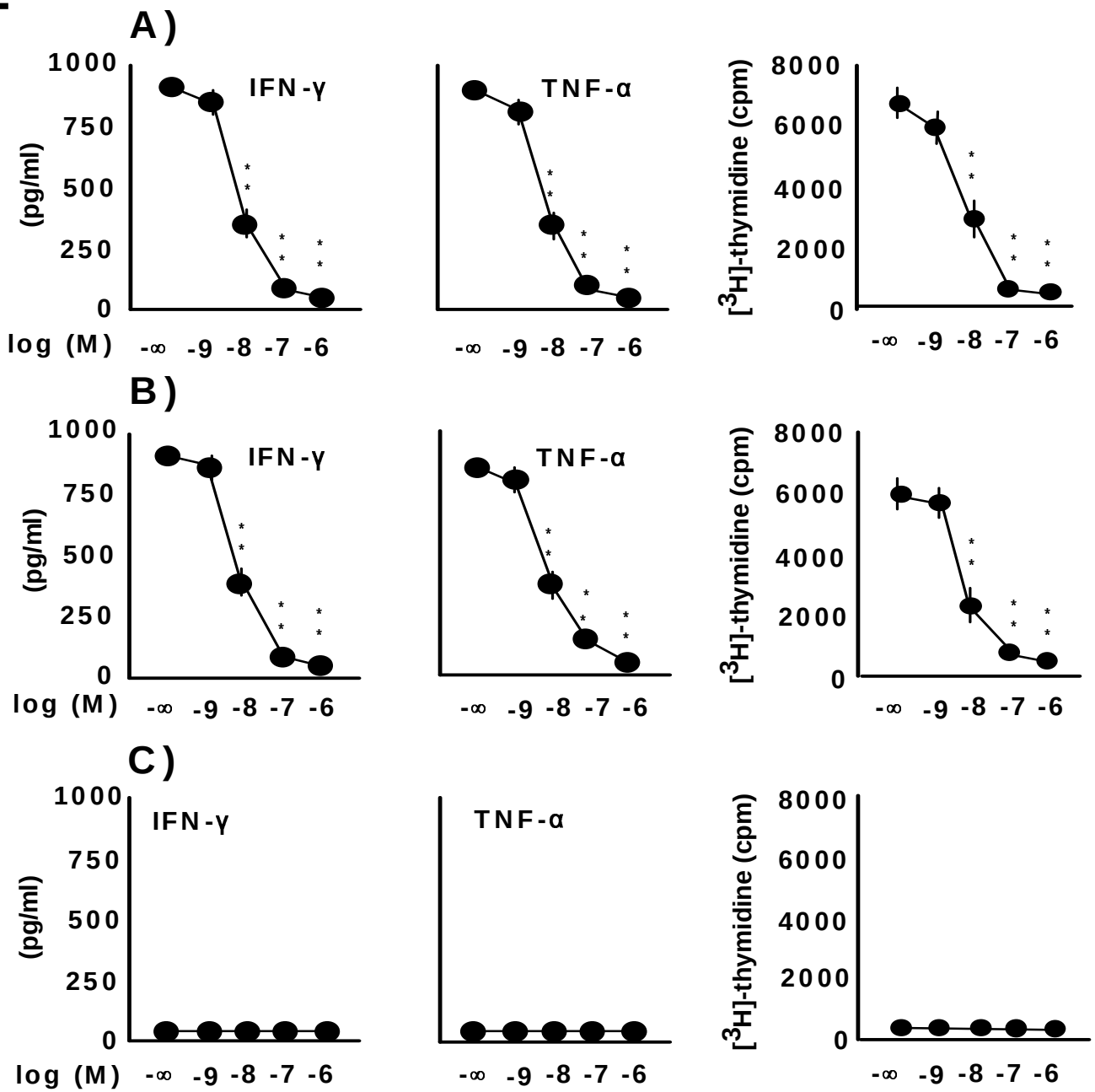

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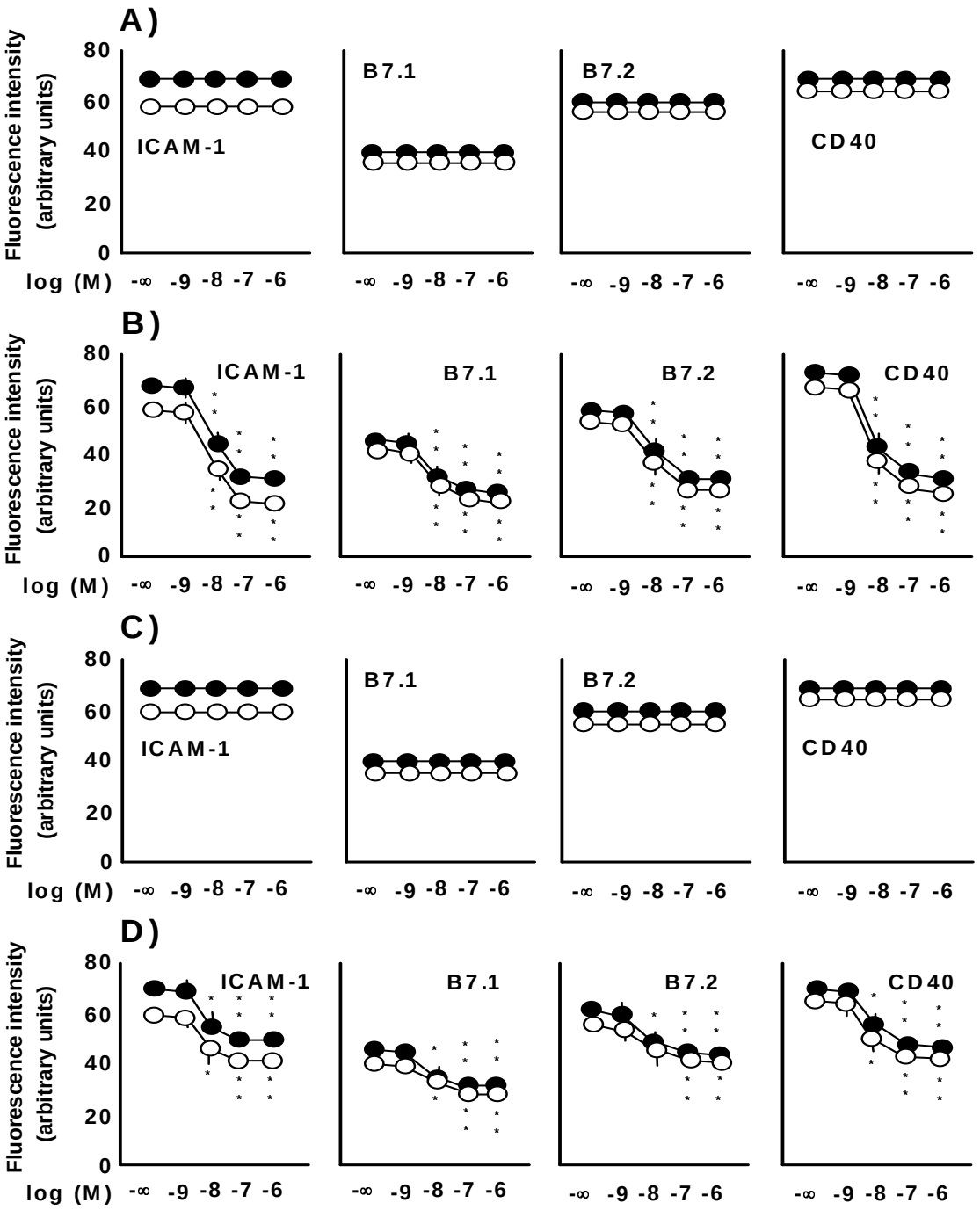

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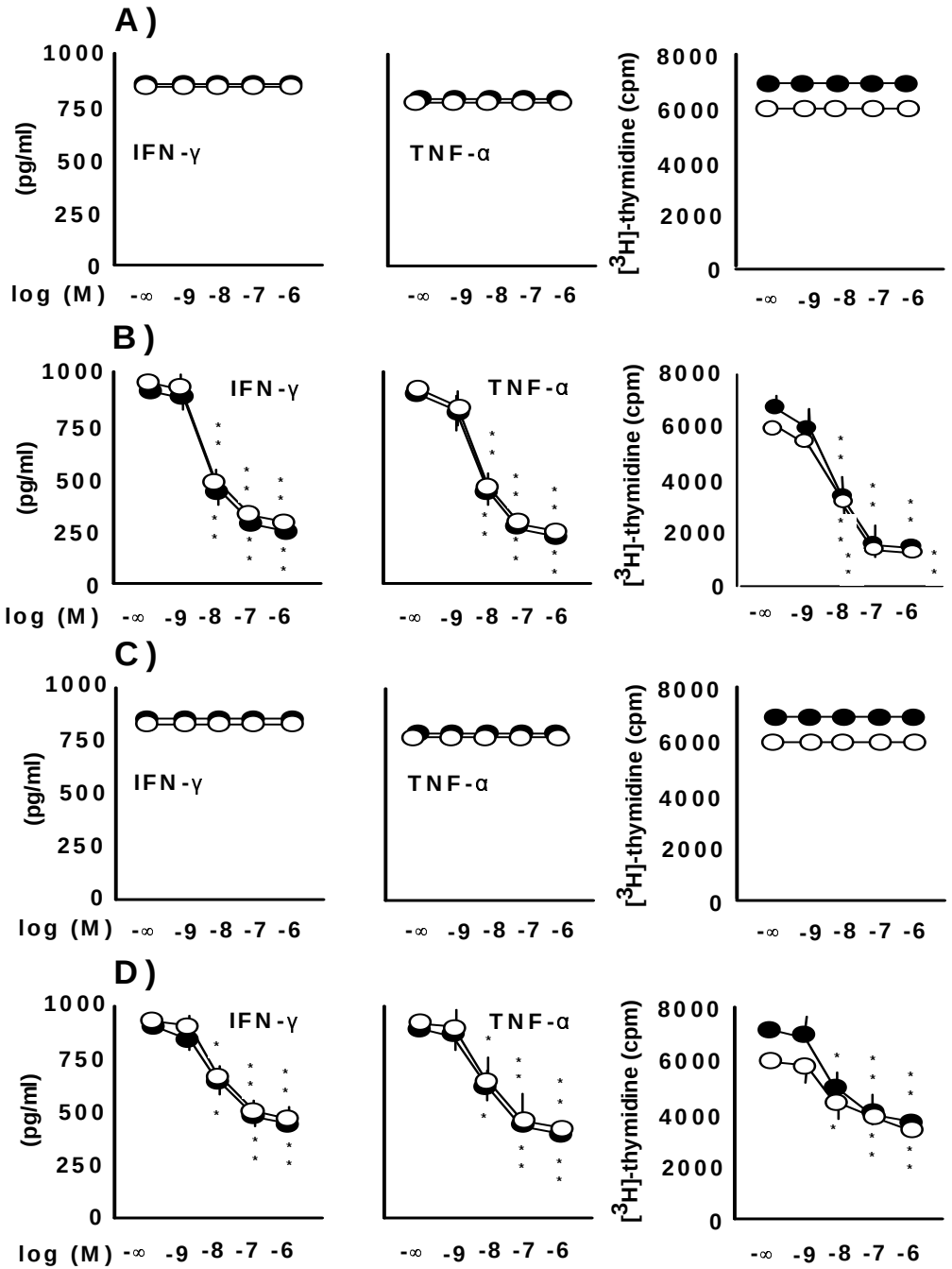

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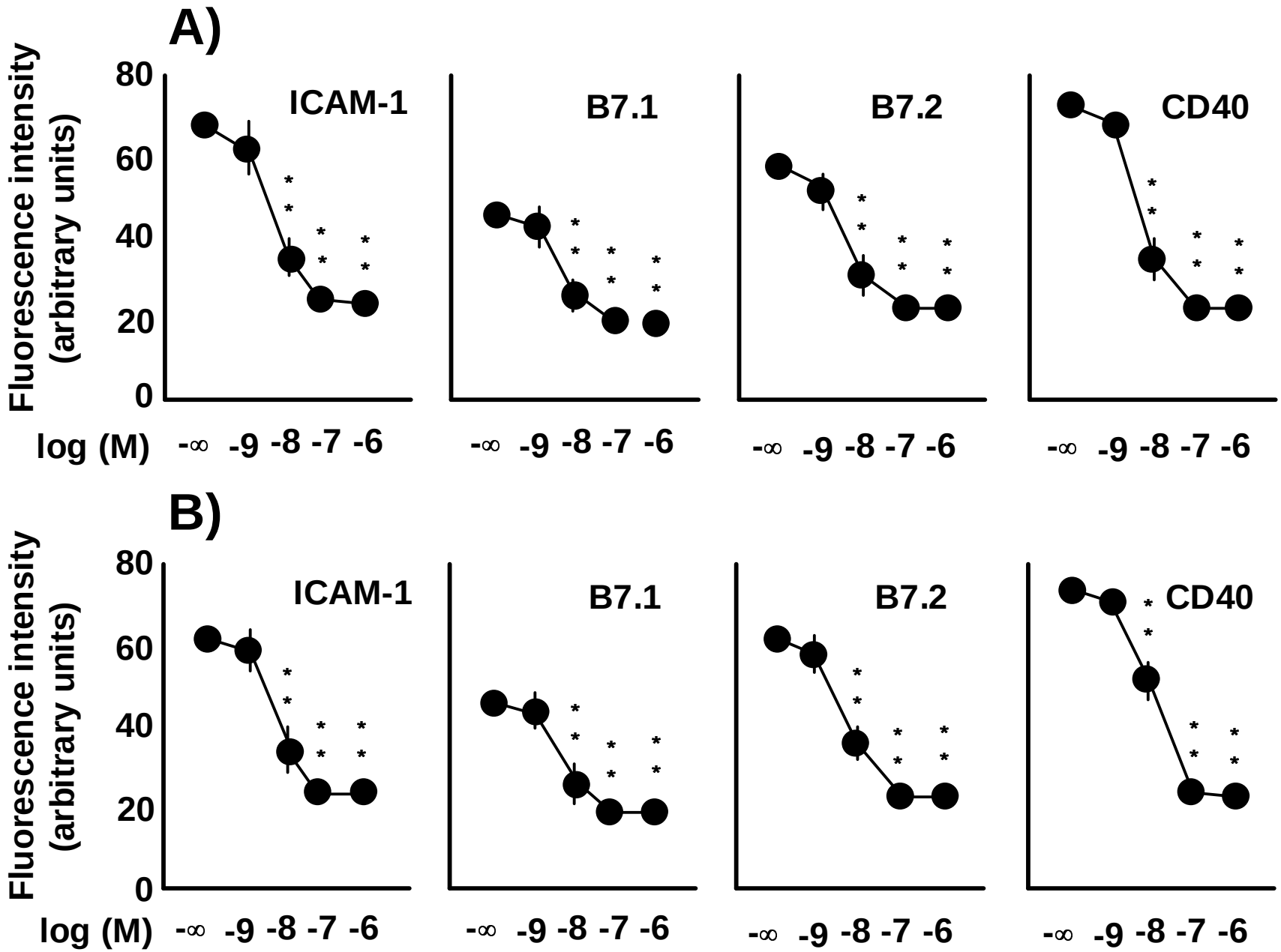

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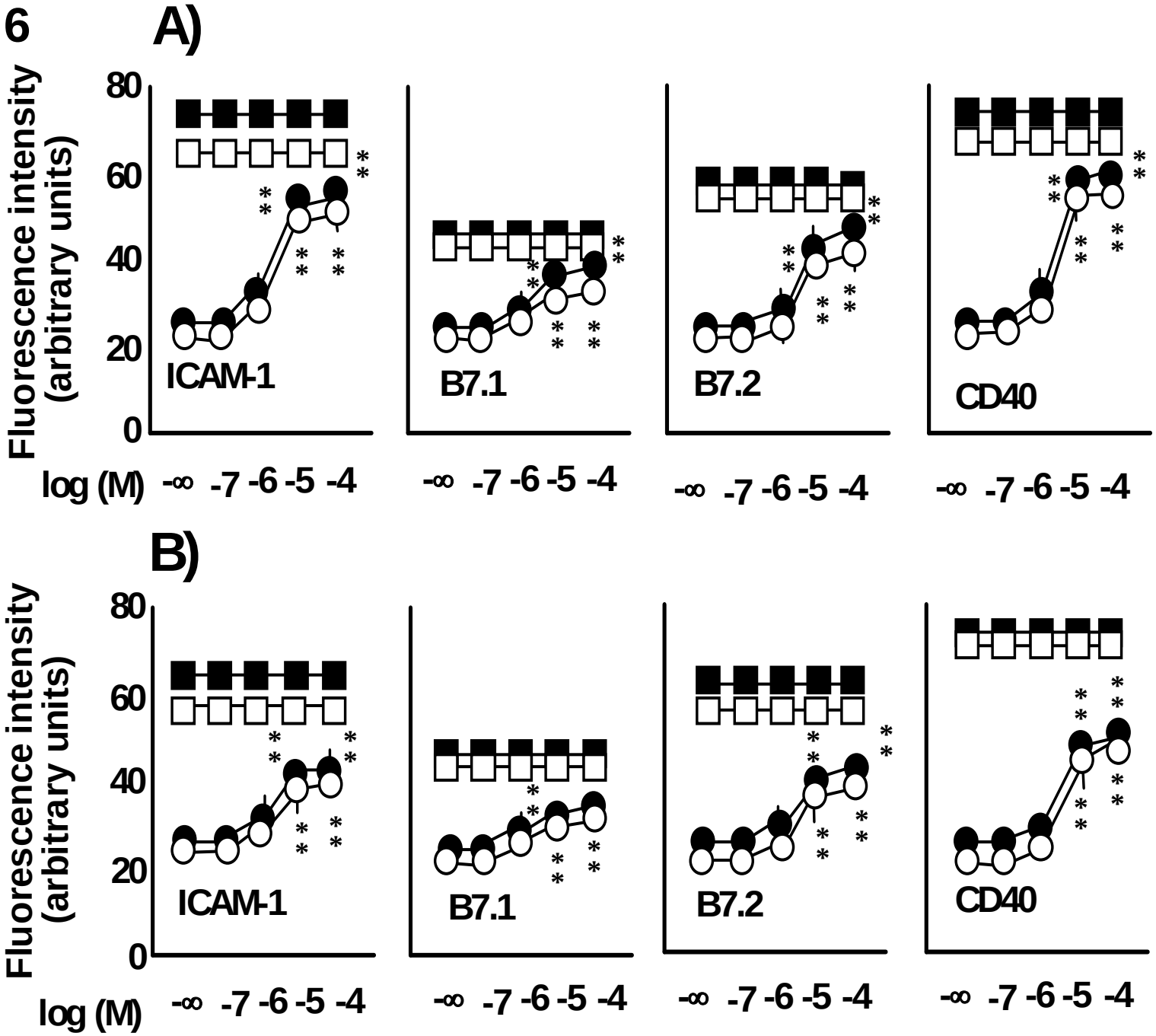
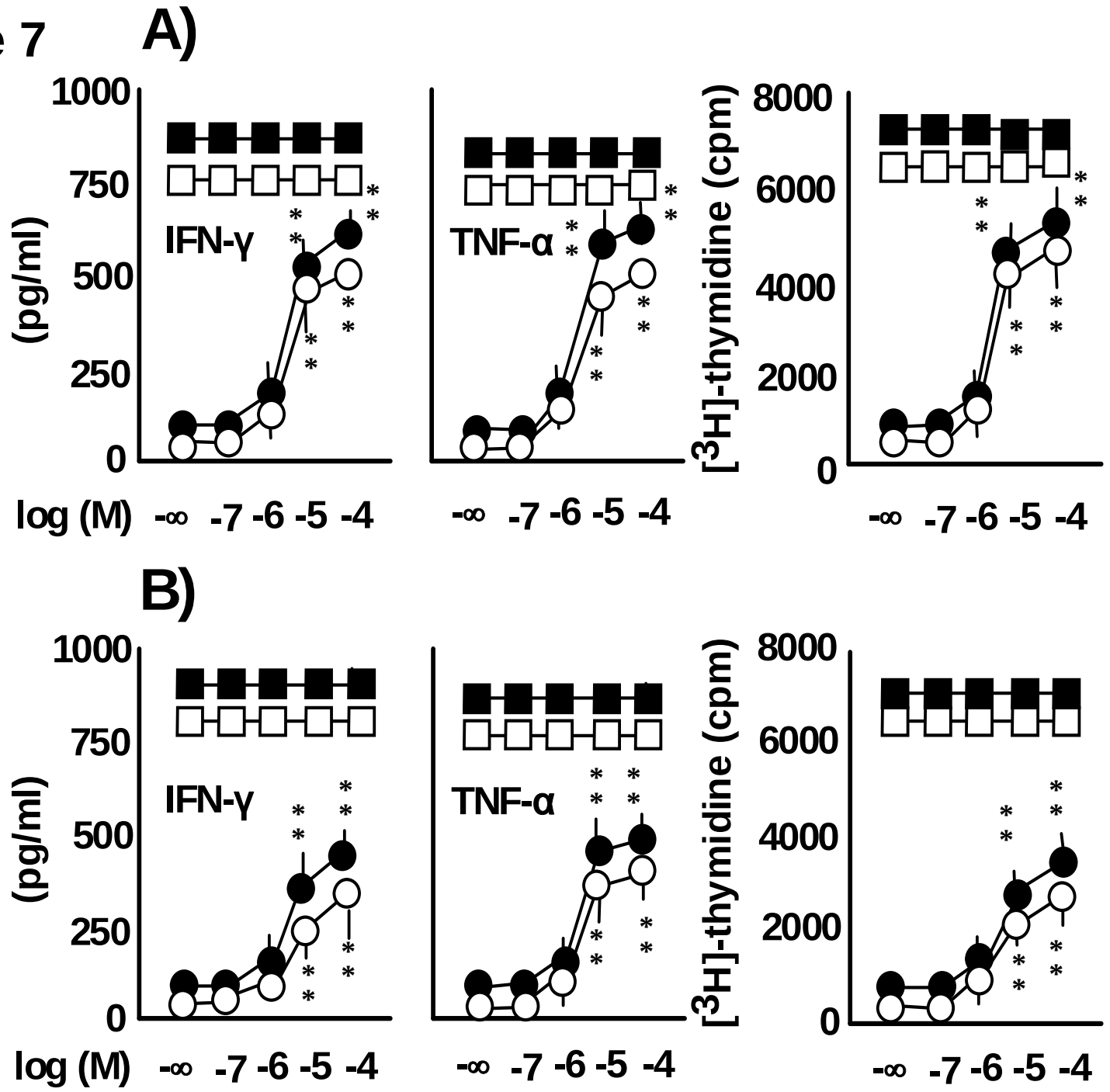


Figure 7



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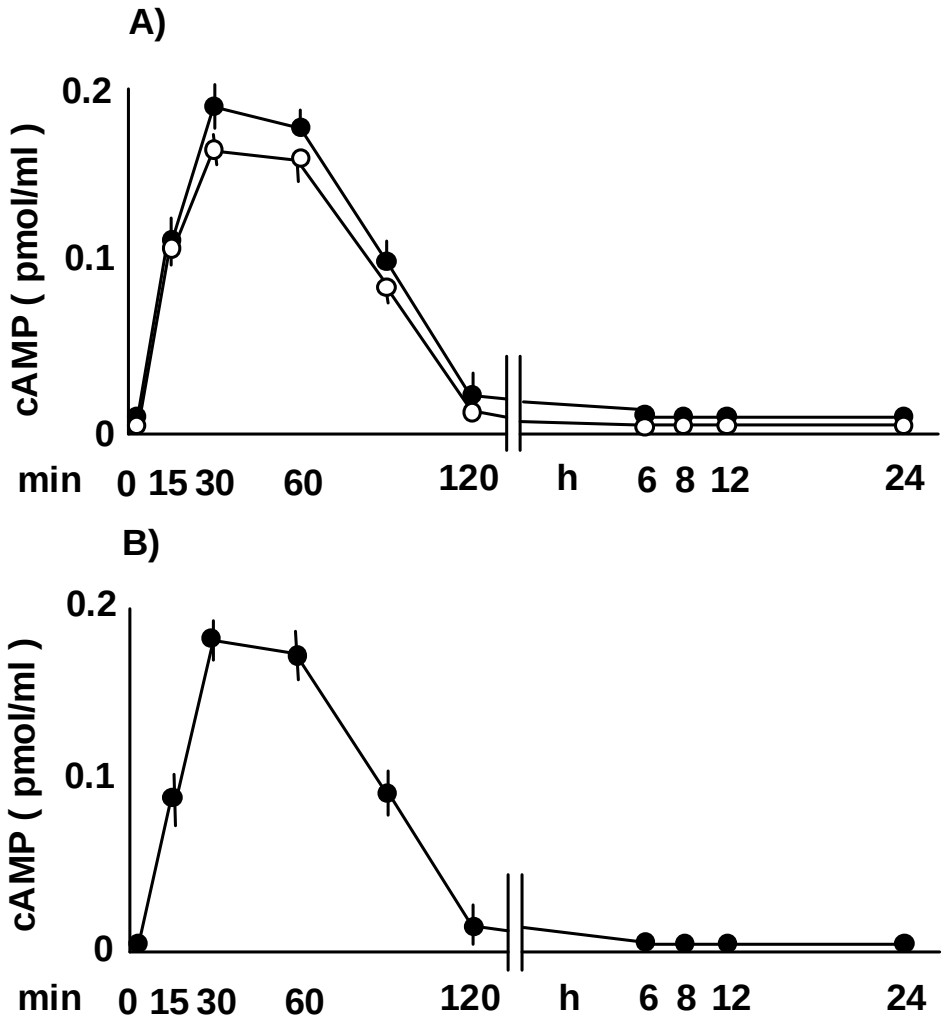
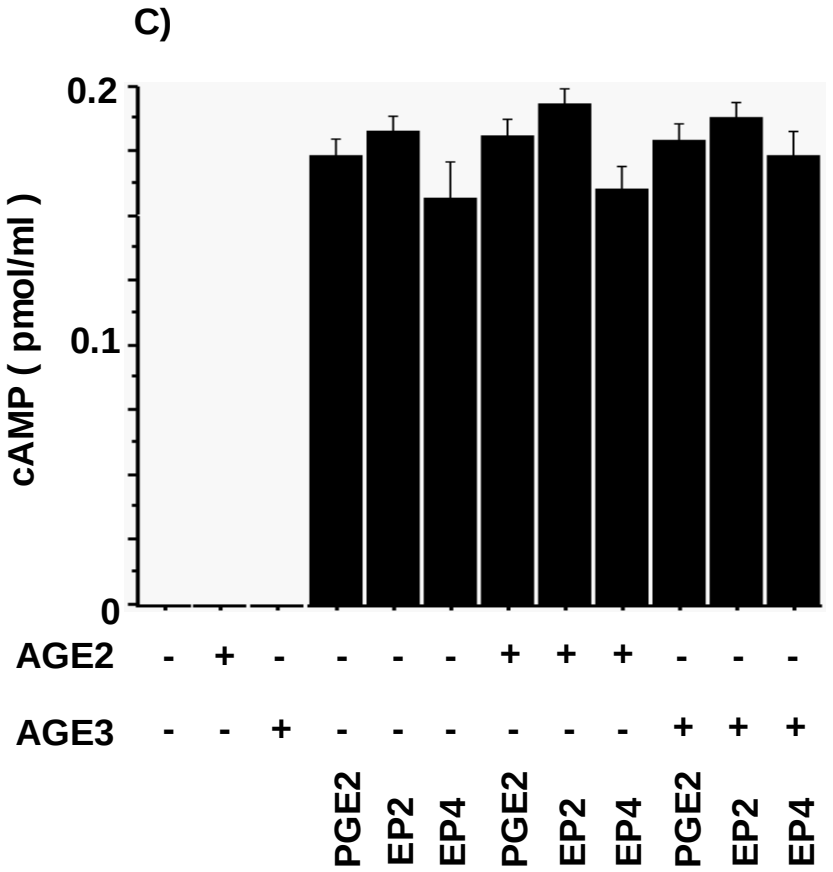
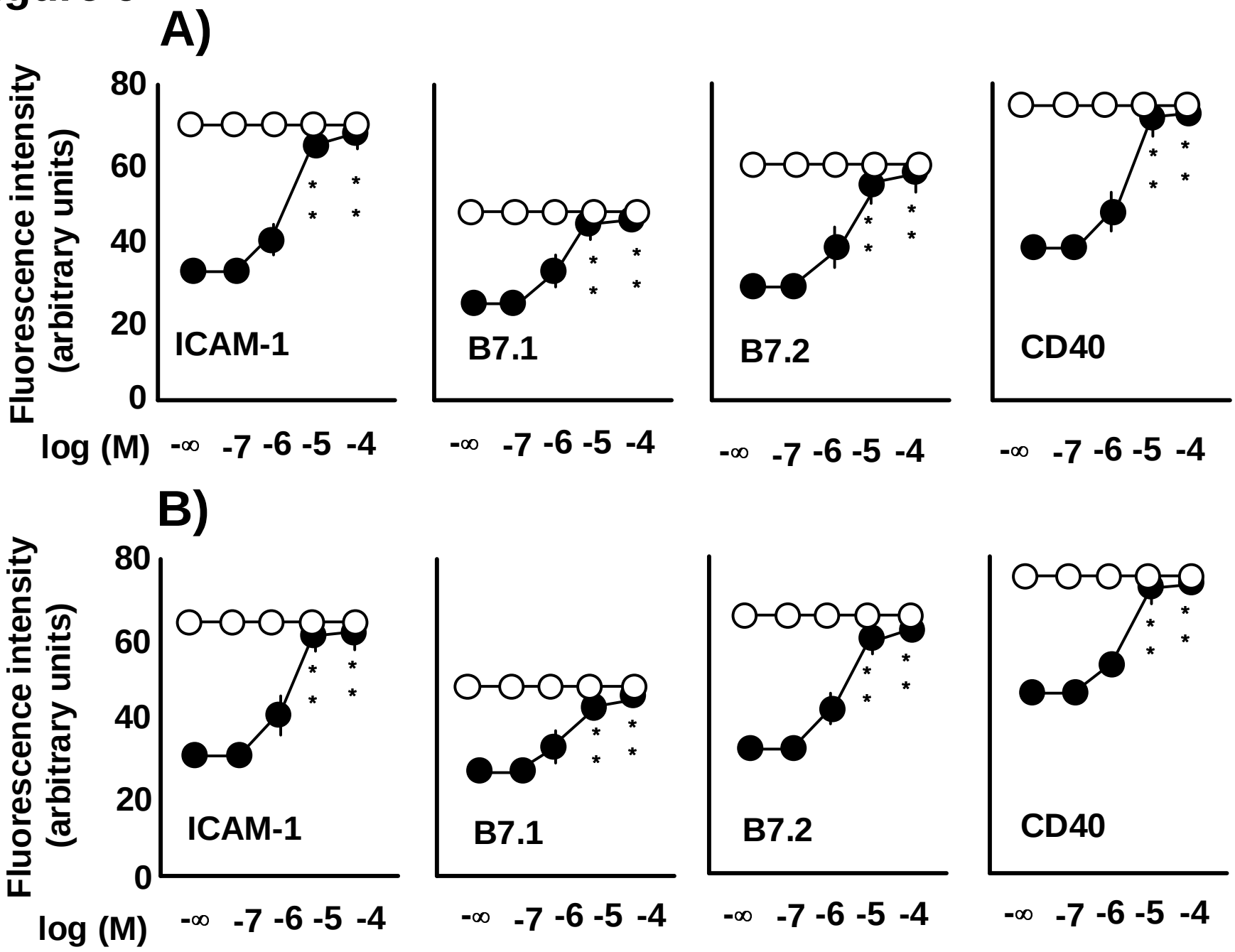
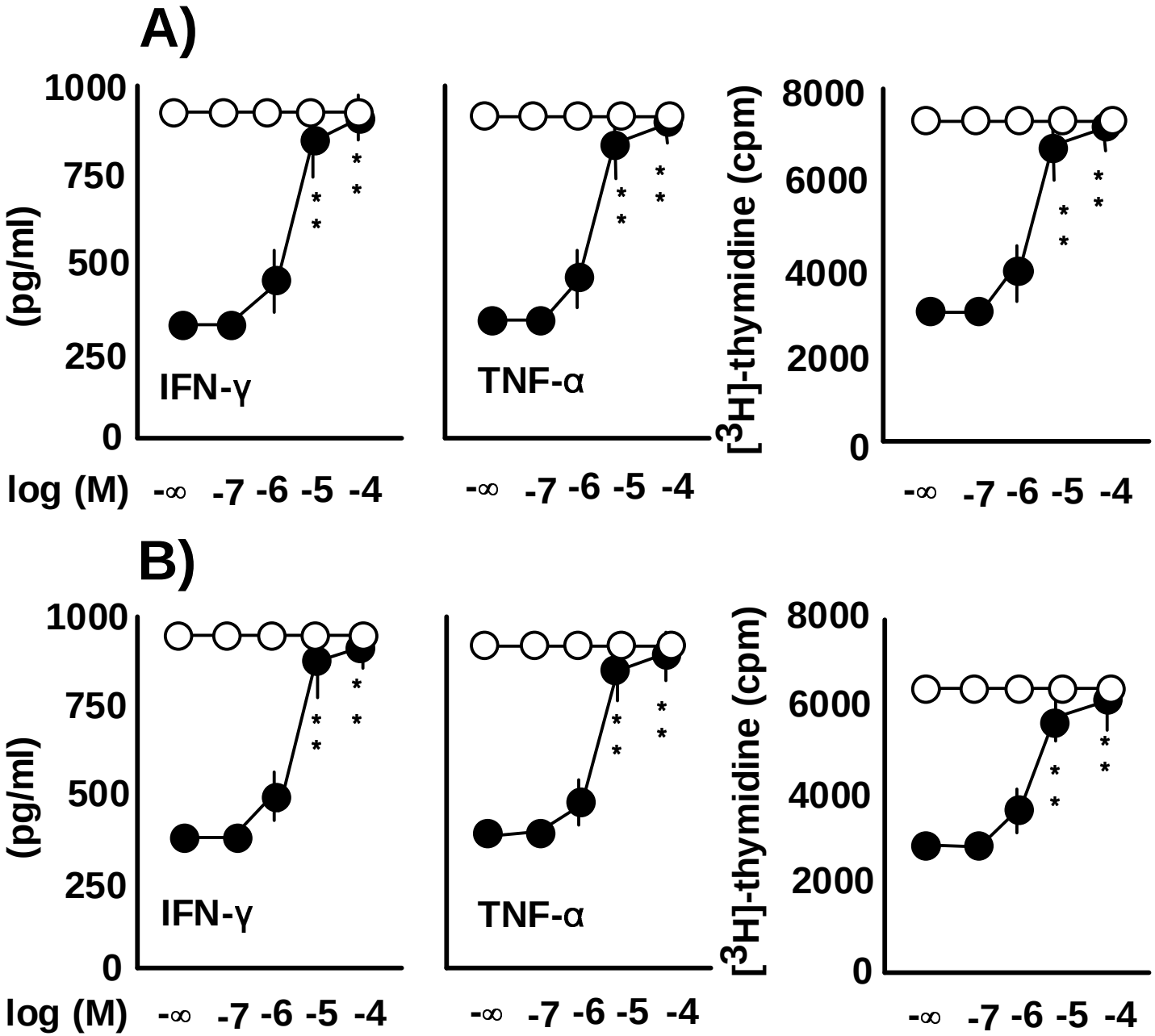


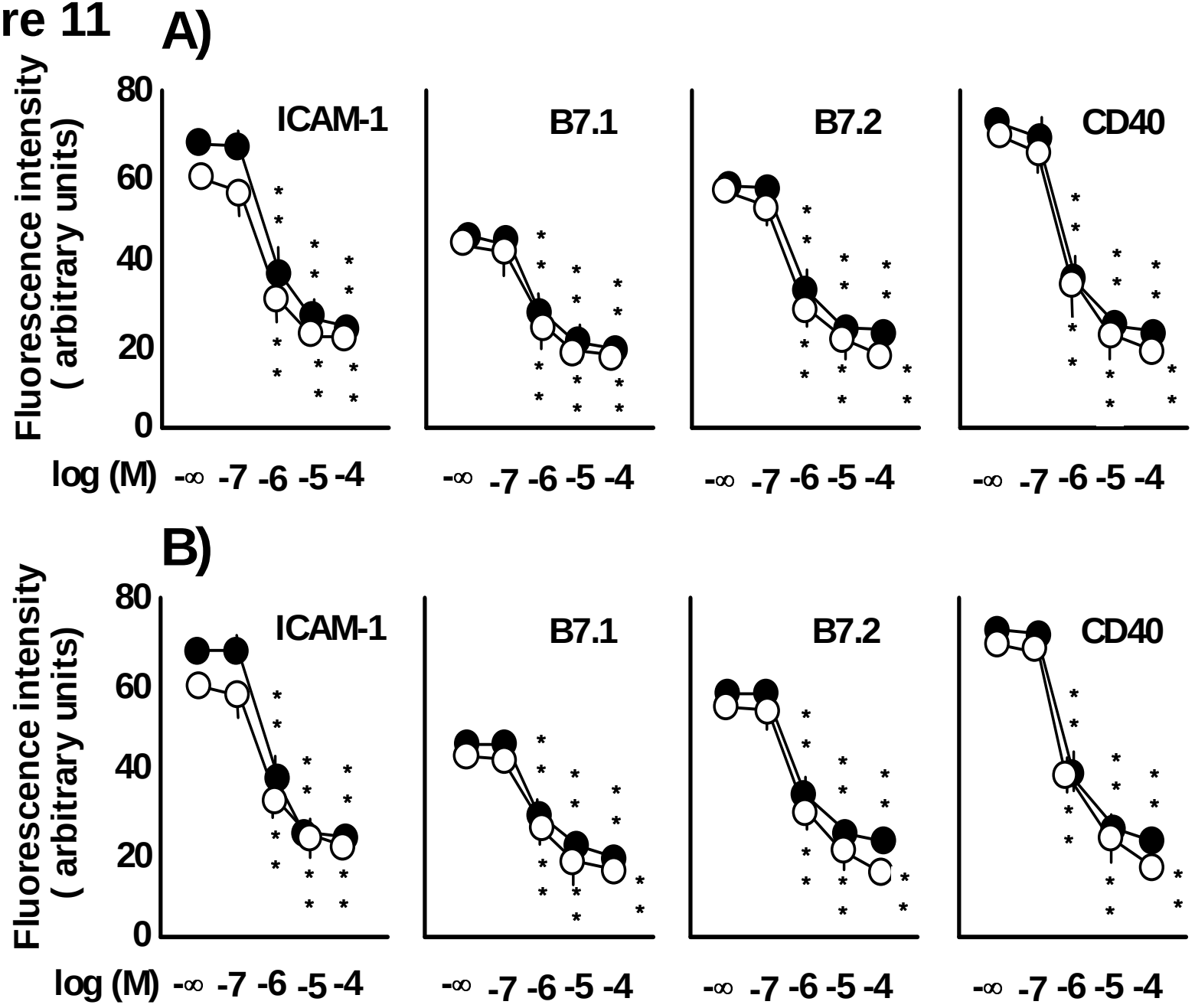
# Figure 9



# Figure 10



# Figure 11



# Figure 12