

## **Adenosine Inhibits TNF- $\alpha$ Release from Mouse Peritoneal Macrophages via A<sub>2A</sub> and A<sub>2B</sub>, but not the A<sub>3</sub> Adenosine Receptor**

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**Running Title:** A<sub>2A</sub> and A<sub>2B</sub> Receptors Inhibit TNF- $\alpha$  Release from Macrophages

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[<sup>125</sup>I]AB-MECA, *N*<sup>6</sup>-(4-amino-3-[<sup>125</sup>I]iodobenzyl)adenosine-5'-*N*-methylcarboxamide; AR, adenosine receptor; cAMP, cyclic adenosine monophosphate; C<sub>T</sub>, cycle threshold; CPX, 1,3-dipropyl-8-cyclopentylxanthine; CCPA, 2-chloro-*N*<sup>6</sup>-cyclopentyladenosine; DMPX, 3,7-dimethyl-1-propargylxanthine; DMSO, dimethylsulfoxide; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; I-ABA, *N*<sup>6</sup>-(4-amino-3-iodobenzyl)adenosine; IB-MECA, *N*<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide; IL, interleukin; KO, knock-out; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; NECA, adenosine-5'-*N*-ethylcarboxamide; NF- $\kappa$ B, nuclear factor  $\kappa$  B; PAF, platelet-activating factor; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; TLR, Toll-like receptor; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; WT, wild-type; ZAS, zymosan-activated serum.

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## ABSTRACT

Adenosine is elaborated in injured tissues where it suppresses inflammatory responses of essentially all immune cells, including production of pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Most of the anti-inflammatory actions of adenosine have been attributed to signaling through the A<sub>2A</sub> adenosine receptor (A<sub>2A</sub>AR). Previously, however, it has been shown that the A<sub>3</sub>AR agonist *N*<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide (IB-MECA) potently inhibited TNF- $\alpha$  release from macrophages obtained from A<sub>2A</sub>AR 'knock-out' (A<sub>2A</sub>KO) mice, suggesting that the A<sub>3</sub>AR may also regulate cytokine expression. Here, we confirmed that the A<sub>2A</sub>AR is the primary AR subtype that suppresses TNF- $\alpha$  release from thioglycollate-elicited mouse peritoneal macrophages induced by both Toll-like receptor-dependent (TLR) and TLR-independent stimuli, but determined that the A<sub>2B</sub>AR rather than the A<sub>3</sub>AR mediates the non-A<sub>2A</sub>AR actions of adenosine since: 1) the ability of IB-MECA to inhibit TNF- $\alpha$  release was not altered in macrophages isolated from A<sub>3</sub>KO mice, and 2) the A<sub>2B</sub>AR antagonist MRS 1754 blocked the ability of the non-selective AR agonist adenosine-5'-*N*-ethylcarboxamide (NECA) to inhibit TNF- $\alpha$  release from macrophages isolated from A<sub>2A</sub>KO mice. Although A<sub>2B</sub>ARs appear capable of inhibiting TNF- $\alpha$  release, the A<sub>2A</sub>AR plays a dominant suppressive role since MRS 1754 did not block the ability of NECA to inhibit TNF- $\alpha$  release from macrophages isolated from wild-type (WT) mice. Furthermore, the potency and efficacy of adenosine to inhibit TNF- $\alpha$  release from WT macrophages were not influenced by blocking A<sub>2B</sub>ARs with MRS 1754. The data indicate that adenosine

suppresses TNF- $\alpha$  release from macrophages primarily via A<sub>2A</sub>ARs, although the A<sub>2B</sub>AR appears to play an underlying inhibitory role that may contribute to the anti-inflammatory actions of adenosine under select circumstances.

## INTRODUCTION

Pro-inflammatory cytokines including tumor necrosis- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, and IL-6 are generated in tissues infected by microbial pathogens as well as in tissues subjected to generalized trauma such as ischemia/reperfusion injury. Generation of inflammatory mediators serves a necessary function to facilitate wound healing, in part by recruiting various immune cell populations. Nevertheless, excessive or chronic inflammation can also lead to additional tissue injury and is the target for therapy in a wide variety of diseases (Rankin, 2004). In the context of microbial invasion and sepsis, pro-inflammatory cytokine expression is induced by engagement of Toll-like receptors (TLR) by bacterial components such as lipopolysaccharide (LPS) (Beutler et al., 2003). During generalized tissue injury, cytokine expression is induced by lipid mediators (e.g., leukotrienes, platelet-activating factor [PAF]), oxygen-derived free radicals, complement components, and possibly by activation of TLR or *N*-formylmethionyl-leucyl-phenylalanine (fMLP) receptors activated by intracellular debris released from necrotic cells (Beutler et al., 2003; Frangogiannis et al., 2002; Le et al., 2002).

Adenosine is a purine nucleoside generated by metabolically stressed or inflamed tissues that is recognized as an endogenous anti-inflammatory agent due to its potent suppressive action on virtually all cells of the immune system (Hasko and Cronstein, 2004). Of the four adenosine receptor (AR) subtypes ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ ), the  $G_s$  protein-coupled  $A_{2A}$ AR is most widely recognized to attenuate inflammation via a cAMP-mediated pathway (Hasko and Cronstein,

2004; Sitkovsky, 2003). A critical role has been established for the A<sub>2A</sub>AR in inhibiting neutrophil superoxide production, degranulation, and adhesion (Hasko and Cronstein, 2004); T-cell expansion and differentiation (Huang et al., 1997); platelet aggregation (Paul et al., 1990), and endothelial cell adhesion molecule expression (Bouma et al., 1996). The use of A<sub>2A</sub>AR deficient mice (A<sub>2A</sub>KO) has confirmed the importance of the A<sub>2A</sub>AR in limiting pro-inflammatory responses (Ohta and Sitkovsky, 2001).

Adenosine also potently inhibits the expression of pro-inflammatory cytokines, including TNF- $\alpha$ , from several different cell types in response to TLR activation (Hasko and Cronstein, 2004). Previous work has shown that the A<sub>2A</sub>AR is the primary receptor subtype responsible for this effect acting by a variety of mechanisms such as inhibition of nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Bshesh et al., 2002; Lukashev et al., 2004; Majumdar and Aggarwal, 2003; Sands et al., 2004) and inhibition of p38-induced promotion of RNA stability (Fotheringham et al., 2004). However, it has been suggested that the A<sub>3</sub>AR is also capable of suppressing pro-inflammatory cytokine expression (Hasko and Cronstein, 2004; Hasko et al., 2000). Furthermore, several studies have shown that A<sub>3</sub>AR agonists are beneficial using *in vivo* models of inflammation (Baharav et al., 2005; Hasko et al., 1998; Mabley et al., 2003). Our laboratory has shown that A<sub>3</sub>AR agonists are effective at protecting against myocardial ischemia/reperfusion injury when administered at the time of reperfusion (Auchampach et al., 2003), a therapeutic window associated with a powerful inflammatory reaction involving cytokine production initiated by free radical

generation, complement activation, calcium overload, and tissue necrosis (Frangogiannis et al., 2002).

The goal of this investigation was to further characterize the AR subtypes involved in regulating pro-inflammatory cytokine expression, focusing on the potential involvement of the A<sub>3</sub>AR. Specifically, we sought to determine the AR subtypes involved in inhibiting TNF- $\alpha$  expression from mouse peritoneal macrophages in response to TLR agonists as well as non-TLR stimuli generated in the context of ischemia/reperfusion injury. Our experimental approach involved the use of thioglycollate-elicited peritoneal macrophages obtained from A<sub>2A</sub> and A<sub>3</sub>KO mice, and newly developed antagonists for rodent ARs. Our results indicate that the A<sub>3</sub>AR plays no role in regulating TNF- $\alpha$  expression. Rather, we demonstrate that the A<sub>2A</sub>AR is the predominant receptor subtype responsible for inhibiting TNF- $\alpha$  production, and that the A<sub>2B</sub>AR plays an underlying inhibitory role. Previous work implicating the A<sub>3</sub>AR is explained by the use of high concentrations of A<sub>3</sub>AR agonists capable of activating A<sub>2A</sub> and A<sub>2B</sub>ARs.

## MATERIALS AND METHODS

**Materials.** Cell culture reagents, TRIzol™ reagent, pcDNA3.1, G418, Lipofectamine, and ThermoScript RT-PCR kits were purchased from Invitrogen Life Technologies (Carlsbad, CA). TNF-α ELISA kits were purchased from BD Biosciences (San Jose, CA). SYBR Green supermix and Bradford reagent were purchased from BioRad (Hercules, CA). ZM 241385 was from Tocris Cookson Inc. (Ellisville, MO), adenosine deaminase from Roche Applied Science (Indianapolis, IN), BG 9928 was a gift from Dr. Barry Ticho (Biogen Inc), and all remaining drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO). *N*<sup>6</sup>-(4-amino-3-[<sup>125</sup>I]iodobenzyl)adenosine-5'-*N*-methylcarboxamide ([<sup>125</sup>I]AB-MECA) and [<sup>125</sup>I]ZM 241385 were synthesized and purified by HPLC, as described previously (Auchampach et al., 1997; Olah et al., 1994; Palmer et al., 1995). [<sup>3</sup>H]MRS 1754 was custom synthesized according to the procedure of Ji et al. (Ji et al., 2001).

**Mice.** C57BL/6 wild-type (WT) mice were purchased from Taconic Laboratories. Homozygous A<sub>3</sub>KO mice were a kind gift from Dr. Marlene Jacobson (Merck Research Laboratories), and A<sub>2A</sub>KO mice were kindly provided by Dr. Jiang-Fan Chen (Boston University). All of the A<sub>2A</sub> and A<sub>3</sub>KO mice used in these studies were back-crossed to the C57BL/6 genetic background.

**Isolation of Mouse Peritoneal Macrophages.** Mice were injected intraperitoneally with 2 ml of 2% thioglycollate. After 4 days, peritoneal cells

collected by lavage were seeded onto 24-well plates in RPMI with 10% calf serum and gentamicin (50 µg/ml) for 4 hours to allow the macrophages to adhere to the plates. Non-adherent cells were subsequently removed by washing with RPMI, and the adherent macrophages were refed with RPMI with 10% calf serum and gentamicin. Macrophages were used for experiments immediately following isolation.

**Treatment with Adenosine Agonists and Antagonists.** Macrophages were pretreated with AR agonists for 30 minutes at 37°C, followed by stimulation with various activating agents including LPS, zymosan-activated serum (ZAS), A23187, or phorbol 12-myristate 13-acetate (PMA) at the concentrations indicated. ZAS was prepared by incubating mouse serum with zymosan A (15 mg/ml) followed by removal of zymosan by centrifugation, as described by Salamone et al. (Salamone *et al.*, 2001). Antagonists were given 30 minutes prior to treatment with the AR agonists. After 24 hours, culture media was collected and assayed for TNF-α by ELISA. Subsequently, the cells were lysed with 0.4N NaOH and assayed for total protein by the Bradford assay (Bradford, 1976). TNF-α released was expressed as pg/mg of protein, or as a percentage of maximal TNF-α released from vehicle-treated cells.

**Quantitative Real-time RT-PCR.** Total RNA was isolated from macrophages using TRIzol™ reagent. Subsequently, 1 µg of total macrophage RNA was reverse transcribed using a mixture of random and poly-T primers

according to the manufacturer's protocol (Invitrogen). Primers were designed for the mouse A<sub>1</sub> (FWD, 5' TGGCTCTGCTTGCTATTG 3'; REV, 5' GGCTATCCAGGCTTGTTC 3'), A<sub>2A</sub> (FWD, 5' TCAGCCTCCGCCTCAATG 3'; REV, 5' CCTTCCTGGTGCTCCTGG 3'), A<sub>2B</sub> (FWD, 5' TTGGCATTGGATTGACTC 3'; REV, 5' TATGAGCAGTGGAGGAAG 3'), and A<sub>3</sub>AR (FWD, 5' CGACAACACCACGGAGAC 3'; REV, 5' GCTTGACCACCCAGATGAC 3') using Beacon Design software (BioRad). PCR amplification (in SYBR green Supermix) was performed utilizing an iCycler iQ™ thermocycler (BioRad) for 40 cycles of 25 seconds at 95°C followed by 45 seconds at an optimized annealing temperature for each AR. The cycle threshold (C<sub>T</sub>), determined as the initial increase in fluorescence above background, was ascertained for each sample. Melt curves were performed upon completion of the cycles to assure that non-specific products were absent. For quantification of AR transcripts, a standard curve plotting C<sub>T</sub> versus copy number was constructed for each receptor subtype by analyzing 10-fold serial dilutions of plasmids containing the full-length mouse AR clones. AR transcript levels were expressed as copies/50 ng of total RNA.

**Radioligand Binding Assays.** Binding assays were conducted with membranes prepared from isolated macrophages. Briefly, macrophages cultured on 150 mm plates were washed with PBS, scraped into cold homogenization buffer (10 mM Na-HEPES [pH=7.4], 10 mM EDTA, and 0.1 mM benzamidine), homogenized in a glass Dounce homogenizer, and then centrifuged at 20,000 X

g for 30 minutes. Cell pellets were washed in binding buffer (10 mM Na-HEPES [pH=7.4], 1 mM EDTA, and 0.1 mM benzamidine) and then resuspended in binding buffer containing 10% (w/v) sucrose. Membranes were stored in aliquots at -20°C until used for binding assays.

For radioligand binding studies, 50 µg of membrane protein was incubated in a final volume of 100 µl of binding buffer containing 5 mM MgCl<sub>2</sub>, 1 unit/ml adenosine deaminase, and either ~0.5 nM [<sup>125</sup>I]ZM 241385 to label A<sub>2A</sub>ARs (Palmer et al., 1995), ~0.5 nM [<sup>125</sup>I]AB-MECA to label A<sub>1</sub> and A<sub>3</sub>ARs (Auchampach et al., 1997; Olah et al., 1994), or 10 nM [<sup>3</sup>H]MRS 1754 to label A<sub>2B</sub>ARs (Ji et al., 2001). In competition experiments, inhibitors were included in the reactions at the concentrations indicated. After incubating at 21° C for 3 hours, the incubations were terminated by rapid filtration over glass-fiber filters using a 48-well Brandel cell harvester. Filter discs containing trapped membranes bound with radioligand were quantified using a gamma or liquid scintillation counter. Non-specific binding was determined in the presence of 1 µM ZM 241385, 1 µM I-AB-MECA, or 100 µM adenosine-5'-*N*-ethylcarboxamide (NECA), respectively.

Competition radioligand binding assays were also conducted with membranes prepared from HEK 293 cells expressing recombinant mouse ARs to determine the affinity of the antagonists used in the study for mouse ARs. The full-length cDNA sequences of the four mouse ARs were obtained by RT-PCR using total RNA isolated from mouse brain tissue. The cDNA clones were verified by sequencing and then subcloned into the mammalian expression

vector pcDNA3.1, transfected into HEK 293 cells using Lipofectamine, and selected with 2 mg/ml G418. After antibiotic selection, the cells were maintained in DMEM cell culture media containing 10% fetal bovine serum with 0.6 mg/ml G418. Cell membranes were prepared and then incubated with radioligands ( $[^{125}\text{I}]\text{AB-MECA}$  for  $A_1$  and  $A_3\text{ARs}$ ,  $[^{125}\text{I}]\text{ZM 241385}$  for  $A_{2A}\text{ARs}$ , and  $[^3\text{H}]\text{MRS 1754}$  for  $A_{2B}\text{ARs}$ ) and antagonist competitors. The radioligand binding data were analyzed as described previously (Auchampach et al., 1997).

**Data Analysis.** Data are reported as means  $\pm$  SEM. Differences between groups were analyzed by one-way ANOVA followed by post-hoc analyses with unpaired Student's *t*-test with the Bonferroni correction. A *p* value  $< 0.05$  was considered statistically significant.

## RESULTS

**Antagonist pharmacology of mouse ARs.** Preliminary radioligand binding studies were conducted to assess the affinity and selectivity of the A<sub>1</sub>AR antagonists CPX and BG 9928, the A<sub>2A</sub>AR antagonist ZM 241385, the A<sub>2B</sub>AR antagonist MRS 1754, and the A<sub>3</sub>AR antagonist MRS 1523 for recombinant mouse ARs expressed in HEK 293 cells. The data from these studies are presented in Table 1 and were used to choose appropriate concentrations of the chemicals in subsequent studies with macrophages.

**AR expression in mouse peritoneal macrophages.** Our initial goal was to determine which AR subtypes are expressed in mouse peritoneal macrophages. To begin, we quantified AR transcript levels by quantitative real-time RT-PCR. The absolute copy numbers of the AR transcripts were calculated based on standard curves generated with mouse AR cDNA clones. As illustrated in FIGURE 1A, we detected mRNA expression of A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>ARs in mouse peritoneal macrophages. mRNA expression of the A<sub>2A</sub>AR was highest (5,395 ± 657 copies/ 50 ng RNA), followed by the A<sub>2B</sub>AR (649 ± 92) and the A<sub>3</sub>AR (455 ± 59). We did not detect expression of A<sub>1</sub>AR mRNA above background levels.

We subsequently conducted radioligand binding assays with crude membrane preparations to assess expression of ARs at the protein level in mouse peritoneal macrophages. Membranes were incubated with ~0.5 nM of the A<sub>2A</sub>AR antagonist [<sup>125</sup>I]ZM 241385, ~0.5 nM of the A<sub>1</sub>/A<sub>3</sub>AR agonist [<sup>125</sup>I]AB-MECA, or 10 nM of the A<sub>2B</sub>AR antagonist [<sup>3</sup>H]MRS 1754. We detected specific binding of [<sup>125</sup>I]ZM 241385 to membranes, defined by inclusion of 1 μM ZM

241385 (FIGURE 1B). Specific binding of [ $^{125}$ I]ZM 241385 was not displaced by the A<sub>1</sub>AR antagonist BG 9928 (100 nM), the A<sub>2B</sub>AR antagonist MRS 1754 (100 nM), or the A<sub>3</sub>AR antagonist MRS 1523 (5  $\mu$ M), indicating that [ $^{125}$ I]ZM 241385 was specifically labeling A<sub>2A</sub>ARs. Given that we included [ $^{125}$ I]ZM 241385 in our assays at a concentration equal to its K<sub>d</sub> value for the A<sub>2A</sub>AR (Table 1), we estimated the B<sub>max</sub> of [ $^{125}$ I]ZM 241385 to be ~40 fmol/mg protein.

We also detected specific binding of [ $^{125}$ I]AB-MECA to membranes prepared from mouse peritoneal macrophages (FIGURE 1C), defined by inclusion of 1  $\mu$ M nonradiolabeled I-AB-MECA. Since [ $^{125}$ I]AB-MECA binds with relatively high affinity to both A<sub>1</sub> and A<sub>3</sub>ARs (Auchampach et al., 1997; Olah et al., 1994), it could have labeled either of these AR subtypes in our assays. However, specific binding of [ $^{125}$ I]AB-MECA was displaced solely by the A<sub>3</sub>AR antagonist MRS 1523 (5  $\mu$ M), and not by BG 9928 (A<sub>1</sub>AR antagonist, 100 nM) or ZM 241385 (A<sub>2A</sub>AR antagonist, 100 nM), indicating that [ $^{125}$ I]AB-MECA was binding to the A<sub>3</sub>AR. We estimated the B<sub>max</sub> of [ $^{125}$ I]AB-MECA binding to mouse peritoneal macrophages to be ~18 fmol/mg protein.

[ $^3$ H]MRS 1754 has recently been characterized as a useful high affinity antagonist radioligand for recombinant A<sub>2B</sub>ARs (Ji et al., 2001). Therefore, we attempted to use [ $^3$ H]MRS 1754 to detect protein expression of A<sub>2B</sub>ARs in mouse peritoneal macrophages. However, we were unable to detect specific binding of [ $^3$ H]MRS 1754 with macrophage membranes (data not shown) most likely due to a combination of low specific activity of [ $^3$ H]MRS 1754, high non-specific binding,

and the potentially low expression of endogenous A<sub>2B</sub>ARs in mouse peritoneal macrophages.

In summary, our quantitative real-time RT-PCR and radioligand binding data indicate that A<sub>2A</sub> and A<sub>3</sub>ARs are expressed in mouse peritoneal macrophages, correlating with previous work implicating these two receptor subtypes in the regulation of TNF- $\alpha$  release (Hasko and Cronstein, 2004; Hasko et al., 2000). Our PCR data also suggest that A<sub>2B</sub>ARs are expressed in mouse macrophages.

**AR activation inhibits TNF- $\alpha$  release from macrophages in response to TLR and non-TLR stimuli.** Pro-inflammatory cytokine production by macrophages is classically known to be stimulated by TLR agonists such as LPS (Beutler et al., 2003). In preliminary studies, we tested a panel of agents associated with ischemia/reperfusion injury for their ability to stimulate TNF- $\alpha$  release from peritoneal macrophages. Among those chosen were hydrogen peroxide as a source of oxygen-derived free radicals, PAF, PMA as an activator of protein kinase C (PKC), ZAS as a source of complement factor C5a, A23187 to mimic calcium overload, and TNF- $\alpha$ . Of these, LPS, PMA, A23187, and ZAS induced TNF- $\alpha$  release from isolated peritoneal macrophages (FIGURE 2). LPS at a concentration of 10  $\mu$ g/ml was the most potent stimulant increasing TNF- $\alpha$  from a basal level of  $130 \pm 35$  pg/mg in vehicle treated cells to  $557,936 \pm 172,636$  pg/mg protein (~5,000-fold increase), whereas ZAS (6  $\mu$ l) was the least potent producing increases of ~5-fold above basal levels.

We subsequently assessed the ability of AR stimulation to inhibit TNF- $\alpha$  release from mouse peritoneal macrophages. Isolated macrophages were pre-incubated with either 1 or 10  $\mu$ M of the non-selective AR agonist NECA for 30 minutes prior to stimulation with LPS, PMA, A23187, or ZAS. As shown in FIGURE 2, pretreatment with NECA inhibited LPS-, A23187-, and ZAS-induced TNF- $\alpha$  release with varying efficacies (~95%, 50%, and 70%, respectively). Although there appeared to be a trend, NECA did not significantly inhibit PMA-induced TNF- $\alpha$  release (FIGURE 2C). Thus, the results demonstrate that AR activation inhibits TNF- $\alpha$  release from peritoneal macrophages induced by both TLR-dependent and TLR-independent stimuli.

**The A<sub>3</sub>AR plays no role in regulating TNF- $\alpha$  production in mouse peritoneal macrophages.** To determine the potential involvement of the A<sub>3</sub>AR in inhibiting TNF- $\alpha$  release, we next examined the effect of the AR agonist *N*<sup>6</sup>-3-iodobenzyladenosine-5'-*N*-methyluronamide (IB-MECA). IB-MECA is an *N*<sup>6</sup>-substituted 5'-methyluronamide derivative of adenosine developed as a potent and selective agonist for the A<sub>3</sub>AR (Gallo-Rodriguez et al., 1994). As shown in FIGURE 3, treatment with IB-MECA at a concentration of 1  $\mu$ M potently inhibited LPS-stimulated TNF- $\alpha$  release ( $64 \pm 7\%$ ). However, when the cells were pretreated with the A<sub>3</sub>AR antagonist MRS 1523 (10  $\mu$ M) or the A<sub>1</sub>AR antagonist 1,3-dipropyl-8-cyclopentylxanthine (CPX, 30 nM) the inhibitory action of IB-MECA on TNF- $\alpha$  release was not affected, whereas it was successfully blocked by ZM 241385 at concentrations (30 and 100 nM) capable of blocking A<sub>2A</sub> and A<sub>2B</sub>ARs

(FIGURE 3). These results suggest that IB-MECA inhibited TNF- $\alpha$  release via the A<sub>2A</sub> and/or A<sub>2B</sub>AR rather than the A<sub>3</sub>AR.

To conclusively exclude a role of the A<sub>3</sub>AR in regulating TNF- $\alpha$  release, we compared concentration-response curves generated with IB-MECA using macrophages isolated from either WT or A<sub>3</sub>KO mice. For purpose of comparison, concentration-response curves were also conducted with the A<sub>1</sub>AR agonist 2-chloro-*N*<sup>6</sup>-cyclopentyladenosine (CCPA) and the A<sub>2A</sub>AR agonist CGS 21680. As revealed in Figure 4, the concentration-response curves generated with IB-MECA and the other subtype-selective AR agonists were similar when macrophages from WT and A<sub>3</sub>KO mice were compared. EC<sub>50</sub> values calculated for each AR agonist are presented in Table 2. The potency order of the agonists to inhibit TNF- $\alpha$  release from both WT and A<sub>3</sub>KO macrophages was CGS 21680 > IB-MECA > CCPA, which is indicative of an effect mediated by the A<sub>2A</sub>AR.

**A<sub>2B</sub>ARs mediate inhibition of TNF- $\alpha$  release in macrophages from A<sub>2A</sub>KO mice.** The data suggest that the A<sub>2A</sub>AR is the primary AR subtype that inhibits TNF- $\alpha$  release from mouse peritoneal macrophages. However, to examine the potential contribution of additional AR subtypes, we evaluated the effect of the non-selective AR agonist NECA on TNF- $\alpha$  released by macrophages isolated from A<sub>2A</sub>KO mice. As presented in FIGURE 5, NECA continued to suppress TNF- $\alpha$  released in response to LPS, ZAS, and A23187 in macrophages isolated from A<sub>2A</sub>KO mice. These results illustrating an A<sub>2A</sub>AR-independent suppression of TNF- $\alpha$  release from mouse peritoneal macrophages concur with

those reported earlier by Hasko and colleagues (Hasko et al., 2000). Since the  $A_1$ AR does not appear to be expressed in mouse peritoneal macrophages and since our data do not support the involvement of the  $A_3$ AR, we predicted that the  $A_{2B}$ AR may be suppressing TNF- $\alpha$  production in macrophages from  $A_{2A}$ KO mice. To address this theory, we examined the effect of blocking  $A_{2B}$ ARs with MRS 1754. As shown in FIGURE 6, MRS 1754 (but not the  $A_3$ AR antagonist MRS 1523) completely blocked the inhibitory effect of NECA on LPS-, ZAS-, and A23187-induced TNF- $\alpha$  release from  $A_{2A}$ KO macrophages.

**The  $A_{2B}$ AR does not influence suppression of LPS-induced TNF- $\alpha$  release from WT macrophages.** We next questioned the relative contribution of the  $A_{2B}$ AR in suppressing TNF- $\alpha$  release when all ARs are present at physiological densities, i.e., in macrophages isolated from WT mice. To address this issue, two experiments were performed. First, we examined whether blockade of the  $A_{2B}$ AR with MRS 1754 (300 nM) reduces the ability of the non-selective AR agonist NECA to inhibit LPS-induced TNF- $\alpha$  release. In the second experiment, we examined whether the concentration-response relationship with adenosine, the endogenous AR ligand, is shifted in the presence of MRS 1754 (100 nM). As shown in FIGURE 7A, the ability of NECA to inhibit LPS-induced TNF- $\alpha$  production was not antagonized by MRS 1754. MRS 1754 also failed to appreciably shift the concentration-response curve generated with adenosine to inhibit LPS-induced TNF- $\alpha$  release in macrophages isolated from WT mice

(FIGURE 7B).  $EC_{50}$  values in the absence and presence of MRS 1754 were  $20.6 \pm 5.6 \mu\text{M}$  and  $48.8 \pm 13.7 \mu\text{M}$  ( $p > 0.05$ ), respectively.

## DISCUSSION

The results of the present study indicate that the A<sub>2A</sub>AR is the predominant AR subtype that suppresses TNF- $\alpha$  production from murine peritoneal macrophages in response to TLR-dependent and TLR-independent stimuli, and that the A<sub>2B</sub>AR plays an underlying inhibitory role. Although we detected the expression of the A<sub>3</sub>AR at both the mRNA and protein level in mouse macrophages, our experiments using AR gene KO mice and specific AR antagonists argue against a role for the A<sub>3</sub>AR in regulating TNF- $\alpha$  expression.

An earlier study by Hasko and colleagues (Hasko et al., 2000) suggested that the A<sub>3</sub>AR may regulate pro-inflammatory cytokine expression. These investigators demonstrated that adenosine continued to suppress LPS-induced TNF- $\alpha$  and IL-12 production from murine peritoneal macrophages isolated from A<sub>2A</sub>KO mice. Since it was observed that the A<sub>3</sub>AR agonist IB-MECA (but not the A<sub>2A</sub>AR agonist CGS 21680) was also capable of suppressing cytokine expression from A<sub>2A</sub>KO macrophages, it was cautiously postulated that the A<sub>3</sub>AR may be responsible for the A<sub>2A</sub>AR-independent actions of adenosine (Hasko and Cronstein, 2004; Hasko et al., 2000). In the present investigation, however, we have determined that IB-MECA inhibits TNF- $\alpha$  release from macrophages devoid of A<sub>2A</sub>ARs via the A<sub>2B</sub>AR rather than the A<sub>3</sub>AR. This conclusion is based on the following pieces of evidence: 1) the A<sub>2B</sub>AR antagonist MRS 1754 blocked the ability of the non-selective AR agonist NECA to inhibit TNF- $\alpha$  production from A<sub>2A</sub>KO macrophages whereas the A<sub>3</sub>AR antagonist MRS 1523 was ineffective, and 2) the potency and efficacy of three different AR agonists to inhibit LPS-

induced TNF- $\alpha$  release were essentially identical in macrophages isolated from WT and A<sub>3</sub>KO mice. The low potency of IB-MECA to inhibit TNF- $\alpha$  production ( $EC_{50} = 467 \pm 13$  nM) is also evidence to suggest that it is not acting via the A<sub>3</sub>AR in our studies. Notably, Sajjadi and colleagues (Sajjadi et al., 1996) reported several years ago that activation of the A<sub>3</sub>AR inhibited LPS-induced TNF- $\alpha$  production from PMA-differentiated U937 human monocytic cells based on the agonist potency order of IB-MECA = N<sup>6</sup>-(3-iodo-4-aminobenzyladenosine (I-ABA) > CGS 21680 and on the inability of CPX or the A<sub>2A</sub>AR antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) to block the inhibitory effect of I-ABA on LPS-induced TNF- $\alpha$  production. In retrospect, however, it seems likely that the inhibitory AR involved was also the A<sub>2B</sub>AR, since all of the agonists used in the study displayed very low potency ( $EC_{50} > 1$   $\mu$ M; Sajjadi et al. 1996).

We only observed the A<sub>2B</sub>AR to functionally inhibit LPS-induced TNF- $\alpha$  production in studies with macrophages isolated from A<sub>2A</sub>KO mice, but not from WT mice. Specifically, we found that blockade of A<sub>2B</sub>ARs with MRS 1754 did not reduce the ability of NECA or adenosine to inhibit LPS-induced TNF- $\alpha$  release from WT macrophages (Figure 7). These data suggest that the A<sub>2B</sub>AR plays relatively little role in regulating cytokine production in macrophages that express the A<sub>2A</sub>AR at normal levels, likely due to the fact that abundantly expressed A<sub>2A</sub>ARs mask the inhibitory actions of the A<sub>2B</sub>AR. However, it is important to consider that our studies were performed with macrophages that had been stimulated previously in the isolation process using thioglycollate. Since it has been shown that expression of the A<sub>2A</sub>AR, and to a lesser extent the A<sub>2B</sub>AR, is

induced in response to inflammatory stimuli (Murphree et al., 2005), it is possible that the A<sub>2B</sub>AR plays a more important role in the initial stages of inflammation prior to the induction of the A<sub>2A</sub>AR. Future studies with unstimulated macrophages are necessary to test this theory and to exclude the possibility that genetic deletion of the A<sub>2A</sub>AR produces adaptive changes that may increase the influence of A<sub>2B</sub>AR signaling in macrophages.

It is interesting that the A<sub>2B</sub>AR has generally been considered to be a pro-inflammatory receptor. Activation of the A<sub>2B</sub>AR has been shown to stimulate IL-8 production by the human mast cell line HMC-1 (Feoktistov and Biaggioni, 1995) and to stimulate IL-6 and monocyte chemoattractant protein-1 (MCP-1) production from human primary bronchial smooth muscle cells (Zhong et al., 2004). In the gut, the A<sub>2B</sub>AR has been shown to be expressed at high levels in epithelial cells, which promotes chloride/water secretion as well as production of IL-6 in response to adenosine released from infiltrating inflammatory cells (Strohmeier et al., 1995). Finally, we have previously shown that the A<sub>2B</sub>AR mediates degranulation of dog BR mastocytoma cells (Auchampach et al., 1997). Based on these observations, it has been theorized that specific antagonists of the A<sub>2B</sub>AR may be effective anti-inflammatory agents. It has also been speculated that the mechanism of action of theophylline and enprofylline for the treatment of asthma may be due, in part, to its relatively high potency as an A<sub>2B</sub>AR antagonist (Feoktistov and Biaggioni, 1995; Auchampach et al., 1997). Nevertheless, the results of the present study suggest that the A<sub>2B</sub>AR is also capable of suppressing TNF- $\alpha$  expression in macrophages, supporting the alternative view

that selective agonists of the A<sub>2B</sub>AR may be useful anti-inflammatory agents in certain disease states.

We did not address the specific mechanism by which A<sub>2</sub>AR signaling suppressed TNF- $\alpha$  release. However, A<sub>2</sub>AR activation is likely to interfere with central components controlling cytokine expression since we observed that AR stimulation inhibited TNF- $\alpha$  release induced by a variety of stimuli including LPS, A23187, and ZAS. A<sub>2A</sub> and A<sub>2B</sub>ARs are both G<sub>s</sub> protein-coupled receptors that increase intracellular cAMP levels upon activation. Previous studies have suggested that, in some cell types (endothelial cells, C6 glioma cells, myeloid cells, and lymphoid cells), the A<sub>2A</sub>AR/cAMP pathway inhibits NF- $\kappa$ B activation, an important transcription factor that drives the expression of many inflammatory genes including TNF- $\alpha$  (Bshesh et al., 2002; Lukashev et al., 2004; Majumdar and Aggarwal, 2003; Sands et al., 2004). In other cell types, it has been suggested that A<sub>2A</sub>AR signaling interferes with stimulus-induced p38 kinase activation, which along with NF- $\kappa$ B promotes transcription of pro-inflammatory genes (including TNF- $\alpha$ ) and promotes mRNA stability by interfering with destabilizing AU-rich elements in the 3' untranslated region (Fotheringham et al., 2004). It appears that the signaling pathways by which ARs inhibit cytokine expression vary depending on the specific cell type and the pro-inflammatory stimulus. Although we observed that AR stimulation suppressed TNF- $\alpha$  release in response to a LPS, A23185, and ZAS, it was ineffective when PMA was used as the stimulus. PMA may increase TNF- $\alpha$  release by post-translational mechanisms involving activation of tumor necrosis factor converting enzyme

(TACE) that cleaves soluble TNF- $\alpha$  from preformed parent molecules within the cell membrane (Doedens et al., 2003). Thus, the lack of effect of NECA on PMA-induced TNF- $\alpha$  appears to support the theory that AR signaling suppresses TNF- $\alpha$  release at the level of gene expression or RNA stability.

A<sub>3</sub>AR agonists have been shown to be effective in several different experimental animal models of inflammation. For instance, IB-MECA or its 2-chloro derivative CI-IB-MECA have been shown to prevent lethality induced by endotoxemia (Hasko et al., 1998), to reduce the severity of arthritis in adjuvant-induced arthritis (Baharav et al., 2005), to lessen intestinal damage in experimental colitis (Mabley et al., 2003), and to diminish myocardial ischemia/reperfusion injury (Auchampach et al., 2003). Interestingly, in some of these studies the beneficial effects of the agonists correlated with reduced expression of inflammatory cytokines. Based on the results of the present experiments, it is conceivable that IB-MECA and CI-IB-MECA provided benefit in these *in vivo* studies by activating the A<sub>2A</sub>AR instead of the A<sub>3</sub>AR, especially since recent studies have demonstrated that IB-MECA and CI-IB-MECA have higher affinity for the A<sub>2A</sub>AR than originally appreciated (Murphree et al., 2002). However, it is also possible that A<sub>3</sub>AR agonists effectively reduce inflammation *in vivo* via an A<sub>3</sub>AR-mediated mechanism that suppresses cytokine production from cell types other than the macrophage, or by reducing the expression of alternative pro-inflammatory mediators. In this regard, G<sub>i</sub> protein-coupled receptors including the A<sub>3</sub>AR have been suggested to inhibit the expression of IL-12 from human monocytes (la Sala et al., 2005), an important cytokine that links

innate and adaptive immunity by activating macrophages and promoting Th1 versus Th2 development. A final possibility is that A<sub>3</sub>AR agonists are effective *in vivo* by inhibiting other pro-inflammatory responses of immune cells such as chemotaxis (Knight et al., 1997) or superoxide production (Gessi et al., 2002). Additional studies using more selective A<sub>3</sub>AR agonists in models of inflammation are warranted.

In summary, we have conclusively determined that the A<sub>2A</sub>AR is the primary AR subtype that mediates inhibition of TNF- $\alpha$  release from mouse peritoneal macrophages induced by both TLR-dependent and TLR-independent stimuli. Additionally, we have identified a previously unrecognized function of the A<sub>2B</sub>AR to inhibit TNF- $\alpha$  expression, suggesting that this often-considered pro-inflammatory receptor may be suppressive under select circumstances. Overall, the results of our study highlight the broad anti-inflammatory potential of AR agonists in treatment of inflammatory disorders.

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## FOOTNOTES

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## FIGURE LEGENDS

**FIGURE 1:** Expression of AR subtypes in macrophages isolated from WT mice.

**A:** mRNA levels quantified by real-time RT-PCR. **B** and **C:** Total binding (cpm) of [<sup>125</sup>I]ZM 241385 (~0.5 nM; **B**) or [<sup>125</sup>I]AB-MECA (~0.5 nM; **C**) to macrophage membranes (50 µg) in the presence of vehicle or various competitors at the concentrations indicated. \**p* < 0.05 versus the vehicle group, n=3.

**FIGURE 2:** Inhibition of TNF-α release from WT macrophages treated with LPS (10 µg/ml, **A**), ZAS (2 or 6 µl, **B**), PMA (100 or 300 nM, **C**), or A23187 (300 or 1,000 nM, **D**) by the non-selective AR agonist NECA (1 or 10 µM). The basal level of TNF-α production in unstimulated cells was 130 ± 35 pg/mg. \**p* < 0.05 versus the vehicle group, n=3-6.

**FIGURE 3:** TNF-α released from LPS-stimulated (10 µg/ml) WT macrophages pretreated with the A<sub>3</sub>AR agonist IB-MECA (1, 1 µM) in the presence or absence of the A<sub>1</sub>AR-selective antagonist CPX (30 nM), the A<sub>3</sub> antagonist MRS 1523 (10 µM), or the A<sub>2A</sub>AR antagonist ZM 241385 (30 or 100 nM). Results are displayed as the percentage of TNF-α released from vehicle-treated cells. \**p* < 0.05 versus the vehicle group, n=6.

**FIGURE 4:** Inhibition of LPS-induced (10 µg/ml) TNF-α release from macrophages isolated from WT or A<sub>3</sub>KO mice by increasing concentrations of

subtype-selective AR agonists. **A:** CCPA; A<sub>1</sub>AR. **B:** CGS 21680; A<sub>2A</sub>AR. **C:** IB-MECA; A<sub>3</sub>AR. Results are displayed as the percentage of TNF- $\alpha$  released from vehicle-treated cells. n=6.

**FIGURE 5:** Inhibition of LPS- (10  $\mu$ g/ml, **A**), ZAS- (6  $\mu$ l, **B**), or A23187-induced (1  $\mu$ M, **C**) TNF- $\alpha$  release from macrophages isolated from A<sub>2A</sub>KO mice by the non-selective AR agonist NECA. \* $p$  < 0.05 versus the vehicle group; n=5-10.

**FIGURE 6:** TNF- $\alpha$  released from LPS- (10  $\mu$ g/ml, **A**), ZAS- (6  $\mu$ l, **B**), or A23187- (1  $\mu$ M, **C**) stimulated macrophages isolated from A<sub>2A</sub>KO mice by 1  $\mu$ M NECA (N) in the presence or absence of the A<sub>2B</sub>AR antagonist MRS 1754 (1  $\mu$ M) or the A<sub>3</sub>AR antagonist MRS 1523 (10  $\mu$ M). \* $p$  < 0.05 versus the vehicle group; n=4-10.

**FIGURE 7:** TNF- $\alpha$  released from LPS-stimulated (10  $\mu$ g/ml) macrophages isolated from WT mice pretreated with 1  $\mu$ M NECA (N) in the presence or absence of the A<sub>2B</sub>AR antagonist MRS 1754 (300 nM) (**A**), or with increasing concentrations of adenosine (**B**) in the presence or absence of MRS 1754 (100 nM). Results are displayed as the percentage of TNF- $\alpha$  released from vehicle-treated cells. \* $p$  < 0.05 versus the vehicle group; n=6.

**Table 1:** Antagonist dissociation constants (nM) for recombinant mouse ARs.

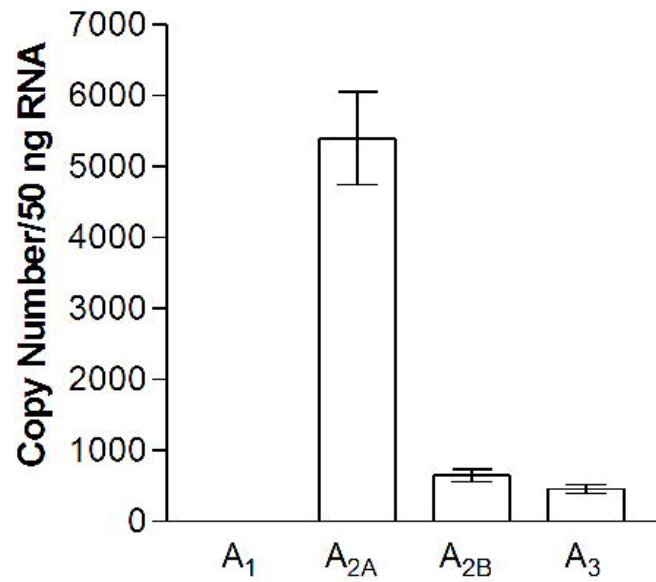
	<b>A<sub>1</sub></b>	<b>A<sub>2A</sub></b>	<b>A<sub>2B</sub></b>	<b>A<sub>3</sub></b>
<b>CPX</b>	1.5 ± 0.5	598 ± 71	86 ± 36	0%*
<b>BG 9928</b>	1.7 ± 0.8	40 ± 5%**	2,160 ± 840	7 ± 4%**
<b>MRS 1754</b>	8.9 ± 1.2	15 ± 2%*	3.4 ± 0.2	0%*
<b>ZM 241385</b>	249 ± 33	0.72 ± 0.22	31 ± 11	10 ± 2%**
<b>MRS 1523</b>	5,330 ± 462	0%**	0%**	702 ± 51

Percent inhibition at 10 μM\* or 100 μM\*\*.

**Table 2:** EC<sub>50</sub> (nM) values of subtype-selective AR agonists to inhibit LPS-induced TNF- $\alpha$  release from macrophages isolated from WT or A<sub>3</sub>KO mice; n=6.

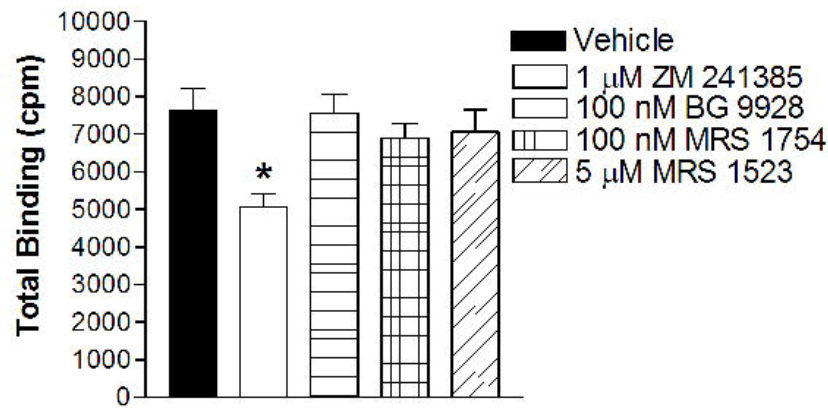
	<b>CCPA</b>	<b>CGS 21680</b>	<b>IB-MECA</b>
<b>WT</b>	4,605 $\pm$ 1,220	38 $\pm$ 15	529 $\pm$ 182
<b>A<sub>3</sub>KO</b>	3,526 $\pm$ 1,976	56 $\pm$ 19	849 $\pm$ 240

**A.**



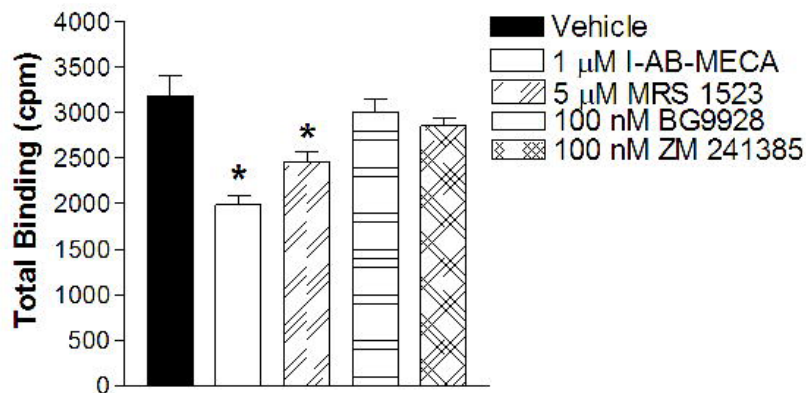
**B.**

**[<sup>125</sup>I]ZM 241385**



**C.**

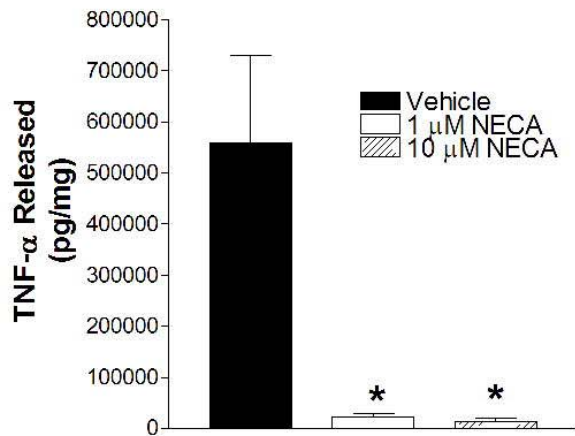
**[<sup>125</sup>I]AB-MECA**



**FIGURE 1**

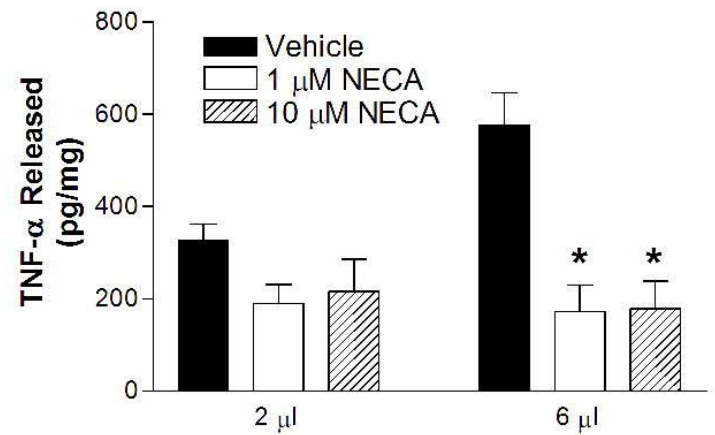
A.

LPS



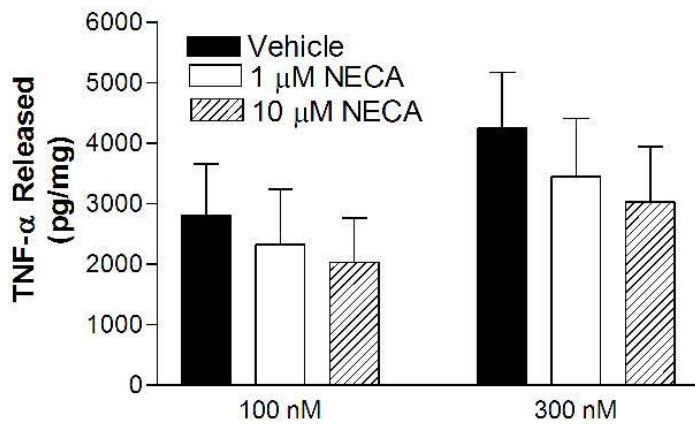
B.

ZAS



C.

PMA



D.

A23187

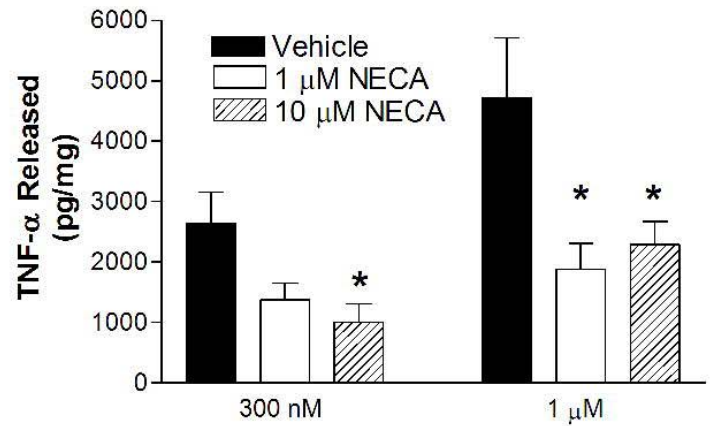


FIGURE 2

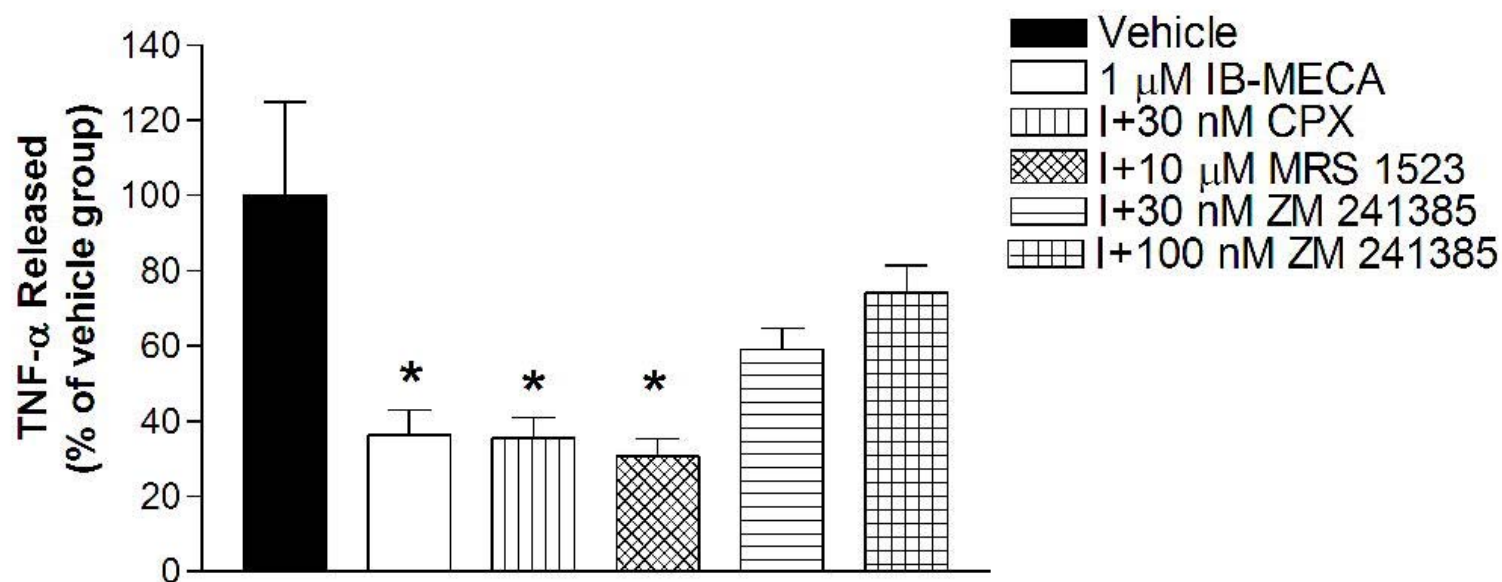
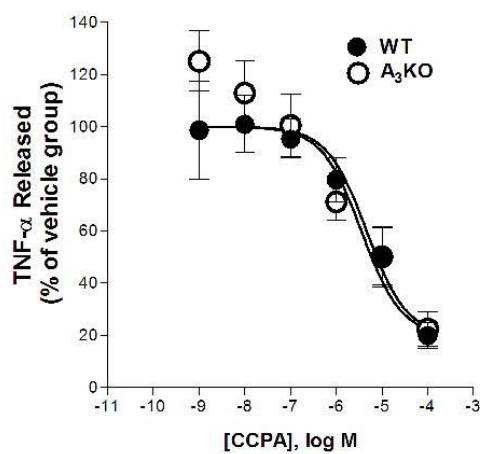
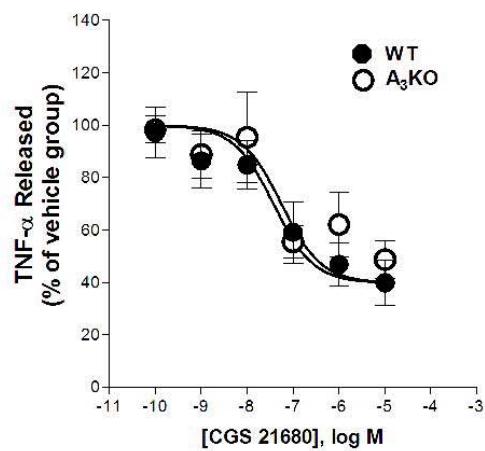


FIGURE 3

A.



B.



C.

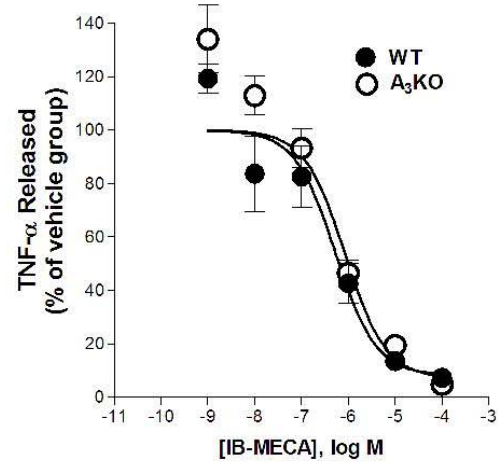
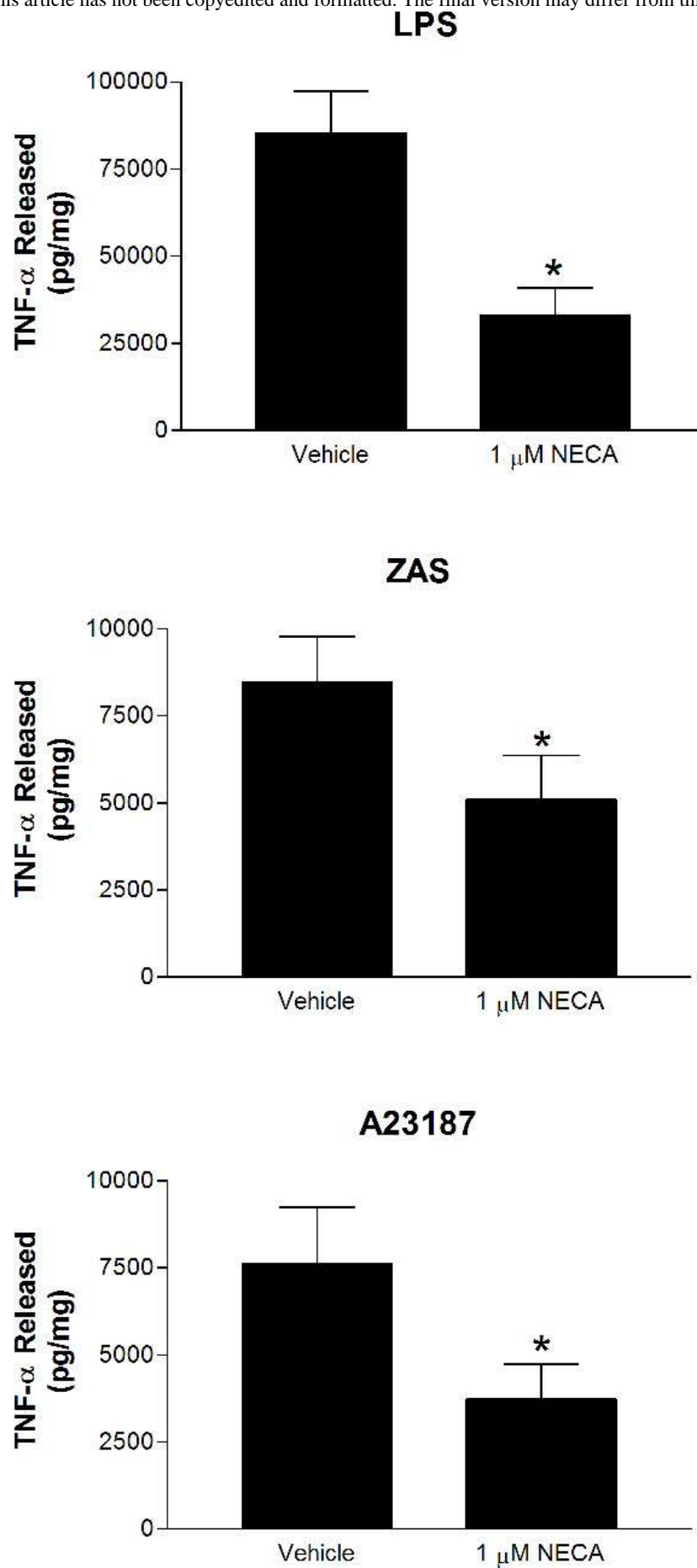
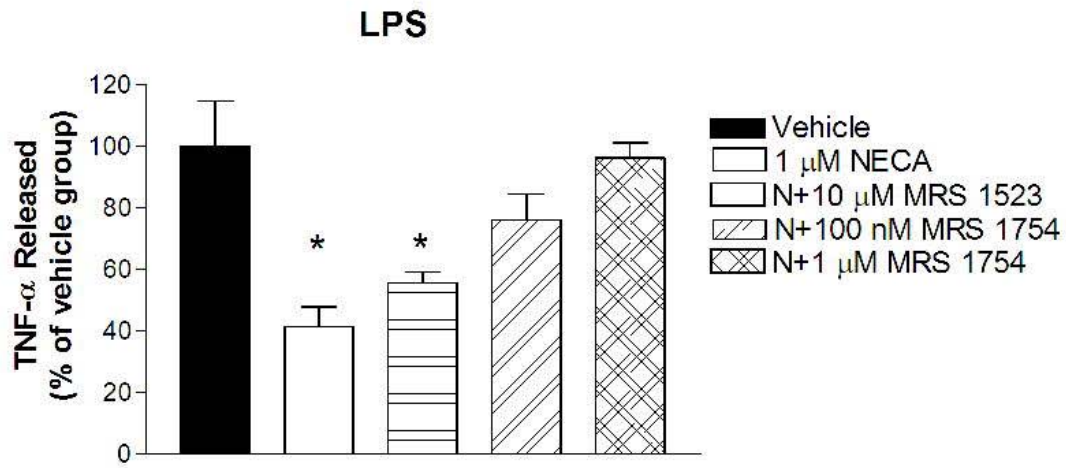


FIGURE 4

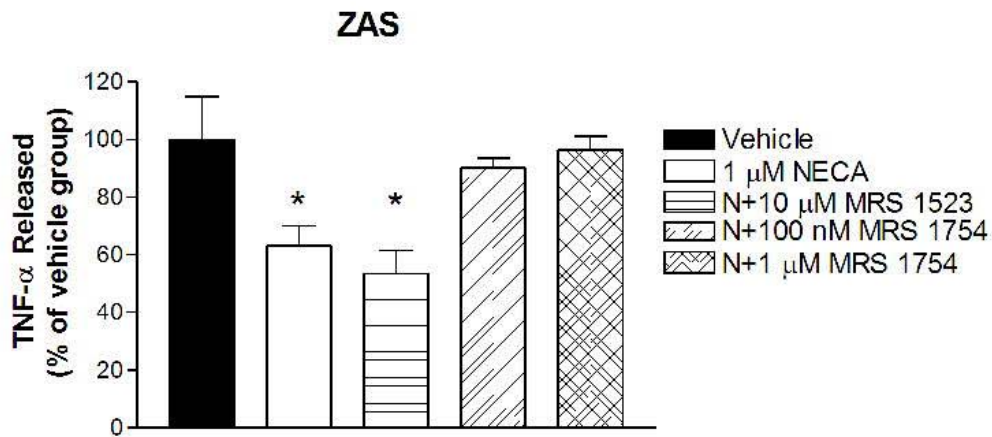


**FIGURE 5**

A.



B.



C.

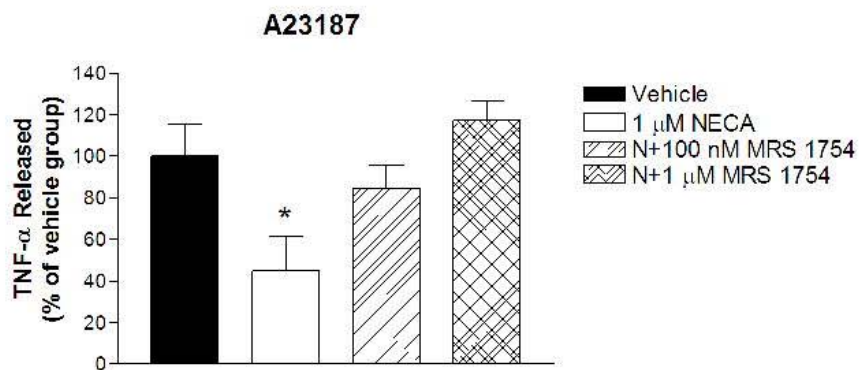
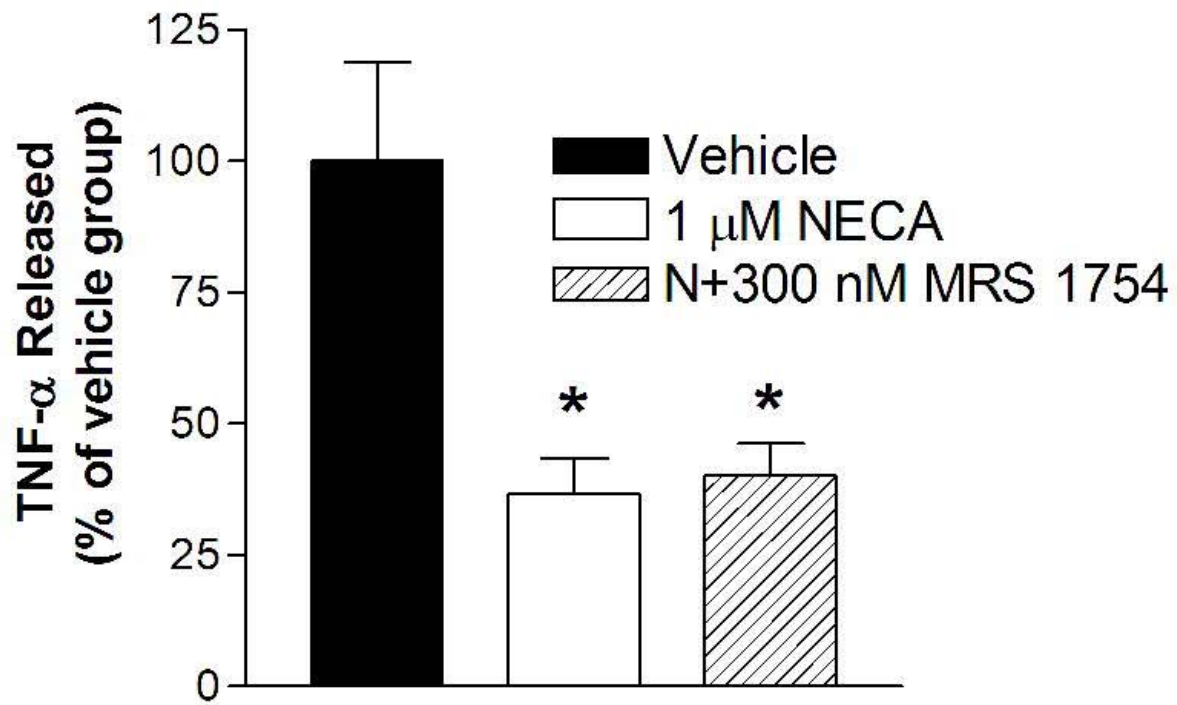


FIGURE 6

A.



B.

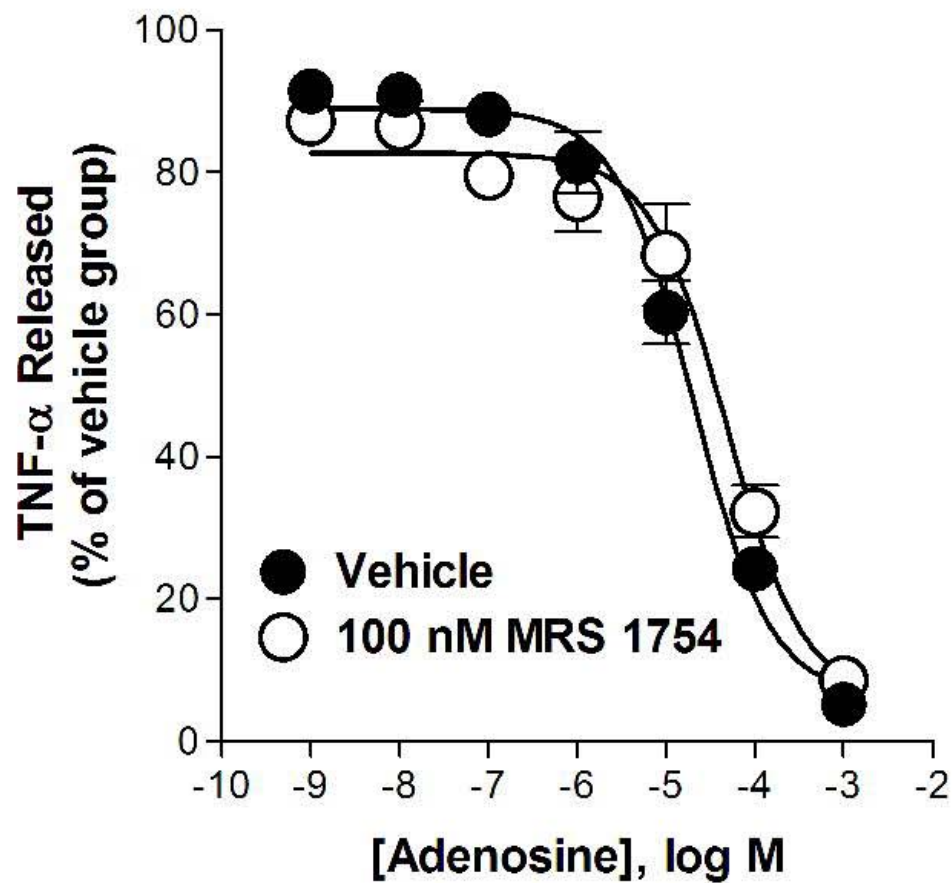


FIGURE 7