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Activation of the macrophage A2b adenosine receptor regulates tumor necrosis factor-α levels following vascular injury

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Abstract

Objective—The control of expression of Tumor Necrosis Factor-alpha (TNF-α) impacts a variety of processes during a stress response. Macrophages are a major source of TNF-α, the level of which is known to be regulated by adenosine. Previous studies highlighted the role of the A2a adenosine receptor (A2aAR) in this process, while the role of the A2b adenosine receptor (A2bAR) has not been clearly identified. Here, we examined the contribution of the A2bAR to TNF-α regulation by macrophages at base line and under vascular stress.

Materials and Methods—We employed a newer A2bAR selective ligand, BAY 60-6583 in-vitro and in-vivo, and an A2bAR antagonist CVT-6883, as well as examined macrophages derived from control or A2bAR knockout (KO) mice.

Results—We found that the expression of the A2bAR is upregulated in macrophages derived from wild type mice subjected to arterial injury, and this receptor activity controls the level of TNF-α released from macrophages.

Conclusion—We identified a significant role for the A2bAR in the regulation TNF-α, which would contribute to the anti-inflammatory actions of adenosine under vascular stress. This conclusion could focus attention on this receptor as a therapeutic target.

Keywords

A2b adenosine receptor; macrophage; Tumor Necrosis Factor-alpha; vascular injury

Introduction

Adenosine, a purine nucleoside released from cells, acts on four G protein-coupled receptors: A1, A2a, A2b, and A3 adenosine receptors [1]. From all these receptors, the A2b

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adenosine receptor (A2bAR) requires higher concentrations of adenosine to be activated. Adenosine level is augmented under cellular distress, such as hypoxic, ischemic, or inflamed environments [1]. Adenosine reduces tissue damage and promotes tissue repair through receptor-mediated signaling, leading to anti-inflammatory effects [1,2]. These have been assigned mostly to the activation of the A2a adenosine receptor (A2aAR) [3]. Our recent studies involving the use of A2bAR knockout (KO) mice show that the A2bAR too mediates some aspects of a systemic anti-inflammatory response [4,5], particularly after vascular injury [6].

Macrophages play a key role in dictating inflammatory responses [7] and inflammatory resolution [8]. In response to Toll-like receptor ligands, such as lipopolysaccharide (LPS) of Gram-negative bacteria, macrophages secret inflammatory cytokines during early phases of the immune response [9,10]. Tumor Necrosis Factor-alpha (TNF-α) is a multifunctional cytokine that mediates significant roles in acute and chronic inflammation, and infection [11,12]. Adenosine is a strong inhibitor of TNF-α production by LPS-challenged human monocytes or mouse macrophages [7]. Using A2aAR KO mice and A3 adenosine receptor (A3AR) KO mice, it was shown that the A2aAR mediates adenosine suppression of TNF-α production by macrophages, while the A3AR is not involved in this effect [7,13].

A2bAR prevents vascular lesion formation in a mouse femoral artery injury model that resembles human restenosis after angioplasty [6], and this is accompanied by changes in plasma TNF-α level. In addition, we reported the inducibility of the A2bAR gene in vascular smooth muscle cells exposed to mitogenic stimuli *in vitro* [14] and *in vivo* [6], and more specifically by TNF-α [15]. However, it has not been clear whether the macrophage A2bAR contributes to the regulation of expression of TNF-α, in a looping back mechanism. In a study by Ryzhov et al., [16] it was concluded that the expression of the mouse A2bAR, but not its activation is important for the control of TNF-α expression by macrophages. On the other hand, Kreckler et al., [13] reported that A2bAR activity controls, although mildly, TNF-α release by macrophages. Here, we focused on exploring the role of A2bAR expression and activation in the control of TNF-α release by macrophage, utilizing A2bAR KO vs. control cells, and BAY 60-6583 [17], a newer selective A2bAR agonist, and CVT-6883 [18], a specific A2bAR antagonist. We clearly identified an inhibitory role of A2bAR activation in the control of TNF-α production in macrophages at base line and following vascular injury. This latter condition also resulted in upregulation of expression of the A2bAR in macrophages.

Materials and Methods

Isolation of Mouse Macrophages and Treatments

All procedures were performed according to the Guidelines for Care and Use of Laboratory Animals published by the National Institutes of Health. Macrophages were isolated from peritoneal cavities of A2bAR KO/β-galactosidase knock in mice on C57BL/6 background [4], and age- (8-12 weeks old), gender-matched C57BL/6 wild-type (WT) mice as described in [4]. Briefly, mice were injected with 50 ml/kg Brewer thioglycollate medium (Cat. No. B2551, Sigma-Aldrich, St. Louis, MO). After 3 days, peritoneal macrophages were collected by lavage and plated onto 24-well plates (10^6 cells/well) in Macrophage-SFM medium (Cat.

No. 12065, Invitrogen, Carlsbad, CA) with 0.1% Penicillin-Streptomycin (Cat. No. 15070063, Invitrogen, Carlsbad, CA). Non-adherent cells were removed 3 hours later by washing with phosphate-buffered saline (PBS) (Cat. No. 21030CV, Mediatech, Manassas, VA) twice and the adherent macrophages were re-fed with Macrophage-SFM medium with 0.1% Penicillin-Streptomycin. Following isolation, macrophages were used immediately for experiments. They were incubated at 37°C in Macrophage-SFM medium with 0.1% Penicillin-Streptomycin containing 1 U/ml Adenosine Deaminase (Cat. No. 10102105001, Roche Applied Science, Indianapolis, IN). First, macrophages were pre-incubated with adenosine receptor antagonists for 30 min. MRS1754 (Cat. No. 2752, Tocris Bioscience, Ellisville, MO) or CVT-6883 (Cat. No. CT-CV001, ChemieTek, Indianapolis, IN) were used as selective A2bAR antagonists. DPCPX (Cat. No. C101, Sigma-Aldrich, St. Louis, MO) [19], SCH442416 (Cat. No. 2463, Tocris Bioscience, Ellisville, MO) [20], and MRS1191 (Cat. No. M227, Sigma-Aldrich, St. Louis, MO) [21] were used as antagonists for the A1, A2a, and A3 adenosine receptors, respectively [22]. Macrophages were then incubated with or without the A2bAR specific agonist BAY 60-6583 for 30 min, followed by treatment with 10 μg/ml lipopolysaccharide (LPS) (*E. coli.* serotype 0127:B8; Cat. No. L-4516, Sigma-Aldrich, St. Louis, MO) to stimulate macrophages and be able to monitor appreciable levels of cytokine release. Culture media were collected within 120 min and assayed for released TNF-α by enzyme-linked immunosorbent assay (ELISA Ready-SET-Go!, Cat. No. 88-7324, eBioscience, San Diego, CA) according to the manufacturer's instruction. Subsequently, the cells were lysed with 0.4 N NaOH and assayed for total protein using the Bradford assay (Protein Assay Kit, Cat. No. 500-0121, Bio-Rad Laboratories, Inc., Hercules, CA).

Western Blot Analysis

Macrophages on the plate were washed three times with cold 1 X PBS, followed by addition of ice-cold Radioimmunoprecipitation Assay (RIPA) buffer (1 X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)), freshly supplemented with 1 X protease inhibitor cocktail (Cat. No. 11697498001, Roche Applied Science, Indianapolis, IN). The lysed cells were collected and centrifuged at maximum speed for 10 min, at 4 °C. The supernatants were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western Blotting as previously described [4]. The membranes were reacted with anti-VASP (1:2000 dilution; Cat. No. 3112, Cell signaling Technology Inc., Danvers, MA) and re-probed with anti-β-actin (1:10,000 dilution, Cat. No. A5441, Sigma-Aldrich, St. Louis, MO) as loading control.

Animal Procedures

Wild type or A2bAR KO mice were given an intraperitoneal (i.p.) injection of BAY 60-6583 dissolved in Polyethylene Glycol (PEG) 400 (provided by Dr. Eltzschig via Bayer HealthCare AG, Wuppertal, Germany) and diluted with saline at 100 μg/kg body weight or an equivalent volume of diluted PEG 400 with saline. After 2 hours, mice received i.p. injection of LPS at 1 μg/g body weight to stimulate macrophages and TNF-α production. Blood was collected by cardiac puncture one hour later, and TNF-α concentration was determined from the isolated plasma by ELISA as above. Peritoneal macrophages were separated as described as above.

The femoral artery injury model

In other experiments, we applied the mouse femoral artery injury model (involving endothelial denudation) or sham injury as control, as described in our earlier study [6]. Briefly, twelve-week-old male A2bAR KO or age, sex, and strain-matched C67BL/6 control mice were anaesthetized, an incision was made in the groin and a clamp was used to occlude the femoral artery below the inguinal ligament. A cut was made distal to the epigastric artery, and a 0.25 mm angioplasty guidewire was introduced. The clamp was then removed and the guidewire advanced 3 cm, 10 times. After removal of the guidewire, the artery was ligated and the incision was closed. Sham surgery included all of the procedures described except that no guidewire was introduced. One week after femoral artery injury, mice were anaesthetized and subjected to macrophage collection as described above. Similar procedures were employed in our core with other set of mice, which after injury were subjected to analysis of the femoral artery as described in [6].

Quantitative PCR

Total RNA from macrophages was prepared with RNeasy mini kit (Cat. No. 74104, Qiagen, Valencia, CA) according to the manufacturer's instruction. One μg of RNA was reverse transcribed using a mixture of poly-T and 18s primers [23] with High capacity cDNA reverse transcription kit (Cat. No. 4374966, Applied Biosystems (ABI), Foster City, CA) according to the manufacturer's instruction. TNF-α and adenosine receptor mRNAs were quantified using ABI TaqMan® Gene Expression Assays and the following primers: TNF-α, Cat. No. Mm99999068-m1; A1AR, Cat. No. Mm01308023-m1; A2aAR, Cat. No. Mm00802075-m1; A2bAR, Cat. No. Mm00839292-m1; A3AR, Cat. No. Mm00802076-m1). Reactions were carried out with the TaqMan® Gene Expression Master Mix (Cat. No. 4369016), using the ABI7300 Real-Time PCR System. 18s rRNA was used as the endogenous control amplified with ABI TaqMan® Gene Expression Assay (Cat. No. 4319413E).

Data Analysis

Comparisons between multi-groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-tests. Two-tailed Student's *t* test were used to compare two groups. A $p < 0.05$ was considered statistically significant.

Results and Discussion

The selectivity of a new A2bAR agonist

We first characterized the specificity of a more recent A2bAR ligand BAY 60-6583 [17] in macrophages by testing its effect on the phosphoryplation of vasodilater-stimulated phosphoprotein (VASP), a well-characterized substrate of cAMP-dependent protein kinase A (PKA) [24]. As Shown in Figure 1A, the majority of VASP was phosphorylated in macrophages after treatment with BAY 60-6583 at 1 μM, a concentration used based on preliminary titration experiments. The effect of BAY 60-6583 on VASP phosphorylation was ablated by two selective A2bAR antagonists, MRS1754 or CVT-6883, but not by antagonists of other adenosine receptors, including DPCPX, which antagonizes the A1AR, SCH442416

the A2aAR, and MRS1191 the A3AR. Furthermore, BAY 60-6583 treatment did not influence VASP phosphorylation in cells derived from A2bAR KO mice (Figure 1B). Figure 1B shows a marginal phosphorylated band in vehicle-treated KO cells, the level of which is not significantly different in the BAY 60-6583-treated KO cells (based on three different biological samples). A similar conclusion was derived based on testing VASP phosphorylation as function of exposure time to this ligand (Figure 1C). Nevertheless, based on the sensitivity of this assay we deduce that BAY 60-6583 is selective at this concentration range, but we cannot conclude that it is absolutely specific.

Effect of A2bAR activation on the control of TNF-α expression by macrophages

With a specific ligand at hand, the effect of A2bAR activation on TNF-α release from macrophages was examined. As shown in Figure 2A, BAY 60-6583 treatment led to a reduction in TNF-α level, and all the effect of BAY 60-6583 was reversed by the A2bAR antagonist CVT-6883. In contrast, BAY 60-6583 had no influence on TNF-α level in A2bAR KO macrophages (Figure 2B). Of note, in the A2bAR KO macrophages, the level of TNF-α was 1.4 fold higher than in control cells (based on analysis of 8 mice in each group), in accordance with [4],[16]. The selection of BAY 60-6583 concentration was guided by the data shown in Figure 1B, namely, a dose that is effective in WT but not KO cells (≤ 1 μM; data not shown). Hence, by using a selective A2bAR agonist and control vs. A2bAR KO cells we identified a suppressing role for A2bAR activation in TNF-α release by macrophages. In another report on mouse peritoneal macrophages, the A2bAR antagonist MRS1754 was used and it did not significantly reverse the effect of the A2-type (A2a and A2b) adenosine receptor agonist adenosine-5′-N-ethylcarboxamide (NECA) on TNF-α release [16], leading the authors to conclude that A2bAR expression, and not activation, are important for TNF-α regulation. In our system, MRS1754 yielded a similar trend as CVT-6883 on BAY 60-6583-treated cells (Figure 3), although one needed to pay careful attention to preparations in which MRS1754 came out of solution (due to partial solubility). BAY 60-6583 is a selective ligand, the specific ablation of which (by a selective antagonist) could be more readily detectable than when both A2-type adenosine receptors are activated. Plus, in our study the exposure to the ligand is only for 2 hours rather than 16 hours in [16]. We selected shorter incubation time in order to limit potential side effects on expression of other genes. For instance, prolonged incubation with NECA can upregulate the expression of the adenylyl cyclase inhibitory receptor, the A3AR [25].

To further validate the *in vitro* studies, BAY 60-6583 was administered *in vivo* to activate the A2bAR, following a protocol described by Eckle et al [17]. Injection of BAY 60-6583 into mice inhibited TNF-α production by macrophages at mRNA level (Figure 4A), as also manifested in the plasma (Figure 4B). The *in vivo* effects of BAY 60-6583 are moderate (about two fold on mRNA level; Figure 4A), but significant. BAY 60-6583 was used here to demonstrate that TNF-α level is regulated by A2bAR activation, also considering that this ligand was not effective in A2bAR null cells. For therapeutic application, repeated administration of BAY 60-6583 might be attempted particularly as this compound becomes widely available.

The A2bAR controls TNF-α levels in macrophages from mice subjected to injury

We reported that A2bAR KO mice display plasma levels of TNF-α that are higher than in control mice (5.64+/-2.3 pg/ml compared to 12.0+/-3.0 pg/ml) [6]. Augmented plasma levels of TNF-α in A2bAR KO mice was also reported by [16]. At one day post femoral artery injury, there is a sharp increase in TNF-α plasma level in both wild type and A2bAR KO mice (106.8+/-18.1 pg/ml in WT and 144.8+/-23.9 pg/ml in KO mice) compared to sham injury and maintain a high plasma TNF-α level after one week [6]. Therefore, we examined whether macrophages derived from injured mice are as responsive to A2bAR activation with regard to the control of TNF-α level. C57BL/6 mice were subjected to femoral artery injury, as we described in [6] and under Methods. It was found that the reducing effect of BAY 60-6583 on TNF-α release from macrophages is as significant as in the non-injured control system (Figure 5A). The effect of BAY 60-6583 was reversed by the A2bAR antagonist CVT-6883, suggesting that even under challenge the macrophage A2bAR is a significant contributor to the attenuation of TNF-α release. TNF-α is known to promote inflammatory and mitogenic effects [11,12], as well as to upregulate the A2bAR gene in smooth muscle cells [6]. Macrophages derived from the injured mice express higher levels of the A2bAR as compared to non-injured (Figure 5B), which could explain the ability of this receptor to reduce TNF-α production even under conditions where its level is upregulated.

Taken together, our study is novel in that it shows the potential of the A2bAR to regulate TNF-α production by macrophages *in vitro* and *in vivo* at base line and under stress. Clearly, the A2aAR is a regulator of TNF-α production by macrophages [2,3], as also concluded when mouse peritoneal macrophages from A2aAR KO mice were compared to control ones [13]. This latter study also eluted to the possibility that the A2bAR might be involved. Our investigations indicate that the expression and activation of the A2bAR significantly contribute to the control of this cytokine production in macrophages. It is anticipated that under conditions where the A2bAR is upregulated, whether during injury [6] or under oxidative stress [15], the contribution of this receptor to the above regulation will be sustained. Our findings and conclusions would be important for the potential design and application of a combined A2aAR- A2bAR-based therapy, rather than singly targeting the A2aAR.

Acknowledgments

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References

1. Fredholm BB. Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ. 2007; 14:1315–1323. [PubMed: 17396131]
2. Linden J. Adenosine in tissue protection and tissue regeneration. Mol Pharmacol. 2005; 67:1385–1387. [PubMed: 15703375]
3. Sitkovsky M, Lukashev D, Deaglio S, Dwyer K, Robson SC, Ohta A. Adenosine A2A receptor antagonists: blockade of adenosinergic effects and T regulatory cells. Br J Pharmacol. 2008; 153 Suppl 1:S457–464. [PubMed: 18311159]

4. Yang D, Zhang Y, Nguyen HG, et al. The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. J Clin Invest. 2006; 116:1913–1923. [PubMed: 16823489]
5. Eckle T, Grenz A, Laucher S, Eltzschig HK. A2B adenosine receptor signaling attenuates acute lung injury by enhancing alveolar fluid clearance in mice. J Clin Invest. 2008; 118:3301–3315. [PubMed: 18787641]
6. Yang D, Koupenova M, McCrann DJ, et al. The A2b adenosine receptor protects against vascular injury. Proc Natl Acad Sci U S A. 2008; 105:792–796. [PubMed: 18184815]
7. Hasko G, Pacher P, Deitch EA, Vizi ES. Shaping of monocyte and macrophage function by adenosine receptors. Pharmacol Ther. 2007; 113:264–275. [PubMed: 17056121]
8. Gilroy DW, Lawrence T, Perretti M, Rossi AG. Inflammatory resolution: new opportunities for drug discovery. Nat Rev Drug Discov. 2004; 3:401–416. [PubMed: 15136788]
9. Gordon S. Pattern recognition receptors: doubling up for the innate immune response. Cell. 2002; 111:927–930. [PubMed: 12507420]
10. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002; 20:197–216. [PubMed: 11861602]
11. Bradley JR. TNF-mediated inflammatory disease. J Pathol. 2008; 214:149–160. [PubMed: 18161752]
12. Aringer M, Smolen JS. The role of tumor necrosis factor-alpha in systemic lupus erythematosus. Arthritis Res Ther. 2008; 10:202. [PubMed: 18226185]
13. Kreckler LM, Wan TC, Ge ZD, Auchampach JA. Adenosine inhibits tumor necrosis factor-alpha release from mouse peritoneal macrophages via A2A and A2B but not the A3 adenosine receptor. J Pharmacol Exp Ther. 2006; 317:172–180. [PubMed: 16339914]
14. St Hilaire C, Yang D, Schreiber BM, Ravid K. B-Myb regulates the A(2B) adenosine receptor in vascular smooth muscle cells. J Cell Biochem. 2008; 103:1962–1974. [PubMed: 17979185]
15. St Hilaire C, Koupenova M, Carroll SH, Smith BD, Ravid K. TNF-alpha upregulates the A(2B) adenosine receptor gene: The role of NAD(P)H oxidase 4. Biochem Biophys Res Commun. 2008; 375:292–296. [PubMed: 18647598]
16. Ryzhov S, Zaynagetdinov R, Goldstein AE, et al. Effect of A2B adenosine receptor gene ablation on adenosine-dependent regulation of proinflammatory cytokines. J Pharmacol Exp Ther. 2008; 324:694–700. [PubMed: 17965229]
17. Eckle T, Krahn T, Grenz A, et al. Cardioprotection by ecto-5′-nucleotidase (CD73) and A2B adenosine receptors. Circulation. 2007; 115:1581–1590. [PubMed: 17353435]
18. Sun CX, Zhong H, Mohsenin A, et al. Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. J Clin Invest. 2006; 116:2173–2182. [PubMed: 16841096]
19. von der Leyen H, Schmitz W, Scholz H, Scholz J, Lohse MJ, Schwabe U. Effects of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), a highly selective adenosine receptor antagonist, on force of contraction in guinea-pig atrial and ventricular cardiac preparations. Naunyn Schmiedebergs Arch Pharmacol. 1989; 340:204–209. [PubMed: 2554151]
20. Moresco RM, Todde S, Belloli S, et al. In vivo imaging of adenosine A2A receptors in rat and primate brain using [11C]SCH442416. Eur J Nucl Med Mol Imaging. 2005; 32:405–413. [PubMed: 15549298]
21. Jacobson KA, Park KS, Jiang JL, et al. Pharmacological characterization of novel A3 adenosine receptor-selective antagonists. Neuropharmacology. 1997; 36:1157–1165. [PubMed: 9364471]
22. Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov. 2006; 5:247–264. [PubMed: 16518376]
23. Zhu LJ, Altmann SW. mRNA and 18S-RNA coapplication-reverse transcription for quantitative gene expression analysis. Anal Biochem. 2005; 345:102–109. [PubMed: 16139233]
24. Krause M, Dent EW, Bear JE, Loureiro JJ, Gertler FB. Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. Annu Rev Cell Dev Biol. 2003; 19:541–564. [PubMed: 14570581]
25. Yaar R, Cataldo LM, Tzatsos A, Francis CE, Zhao Z, Ravid K. Regulation of the A3 adenosine receptor gene in vascular smooth muscle cells: role of a cAMP and GATA element. Mol Pharmacol. 2002; 62:1167–1176. [PubMed: 12391281]

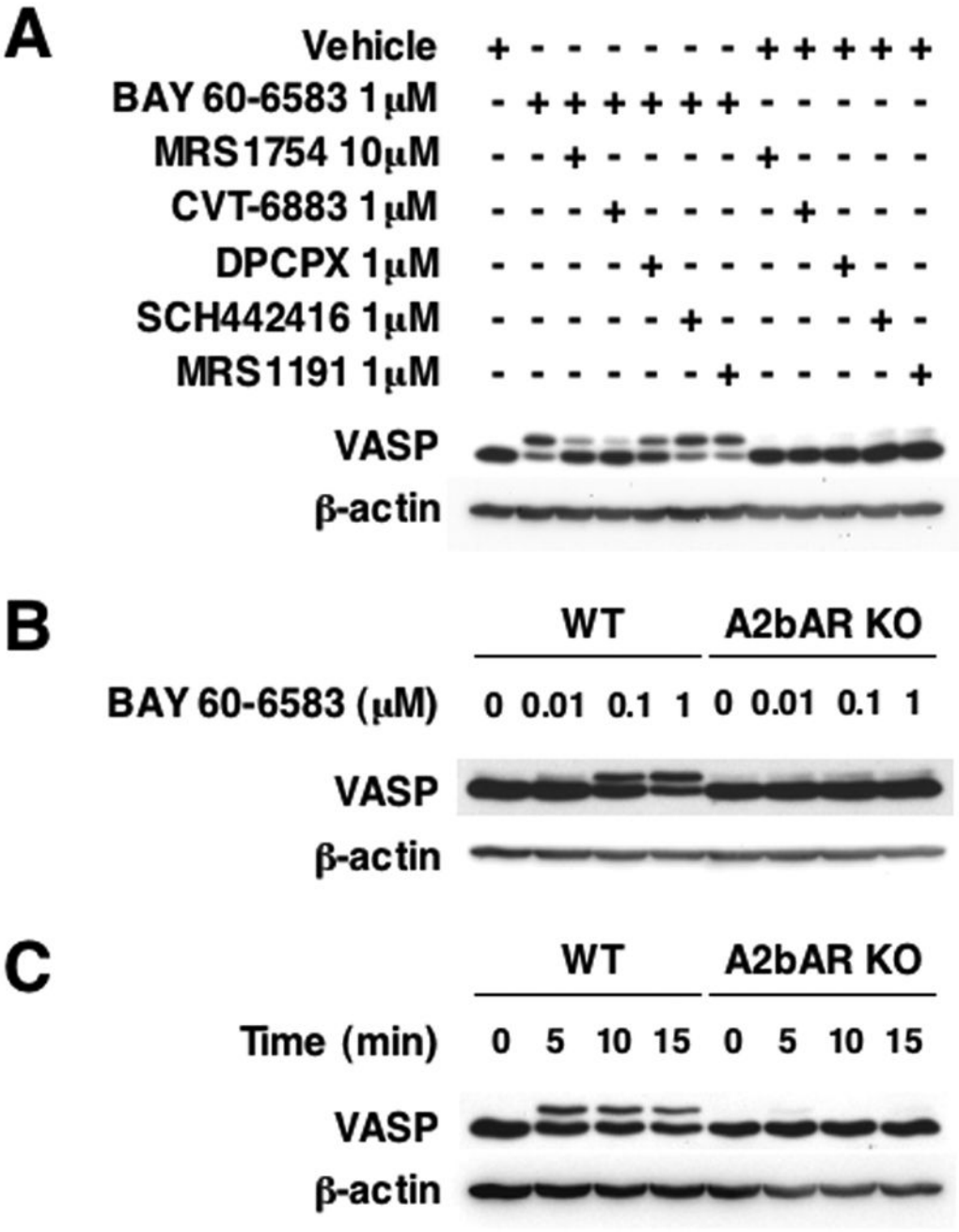

Figure 1.

The selectivity of BAY 60-6583 as an A2bAR agonist. (A) Western blotting of VASP in WT peritoneal macrophages treated with BAY 60-6583 at 1 μM for 10 min after 30 min pre-incubation with the following antagonists, MRS1754 (10 μM) to A2bAR; CVT-6883 (1 μM) to A2bAR; DPCPX (1 μM) to A1AR, SCH442416 (1 μM) to A2aAR, and MRS1191 (1 μM) to A3AR. PKA phosphorylates VASP at serine 157 and as a result VASP shifts from 46 to 50 kDa in SDS-PAGE [24]. BAY 60-6583 was used at 1 μM following preliminary experiments with lower and higher concentrations. The membrane was also reacted with

anti-β-actin as loading control. Shown is a representative blot from three experiments. (B) Western blotting of VASP in peritoneal macrophages from WT and A2bAR KO mice treated with BAY 60-6583 at different doses, as indicated. Shown is a representative blot from three experiments. (C) Western blotting of VASP in peritoneal macrophages from WT and A2bAR KO mice treated with 1 μM BAY 60-6583 for different times, as indicated.

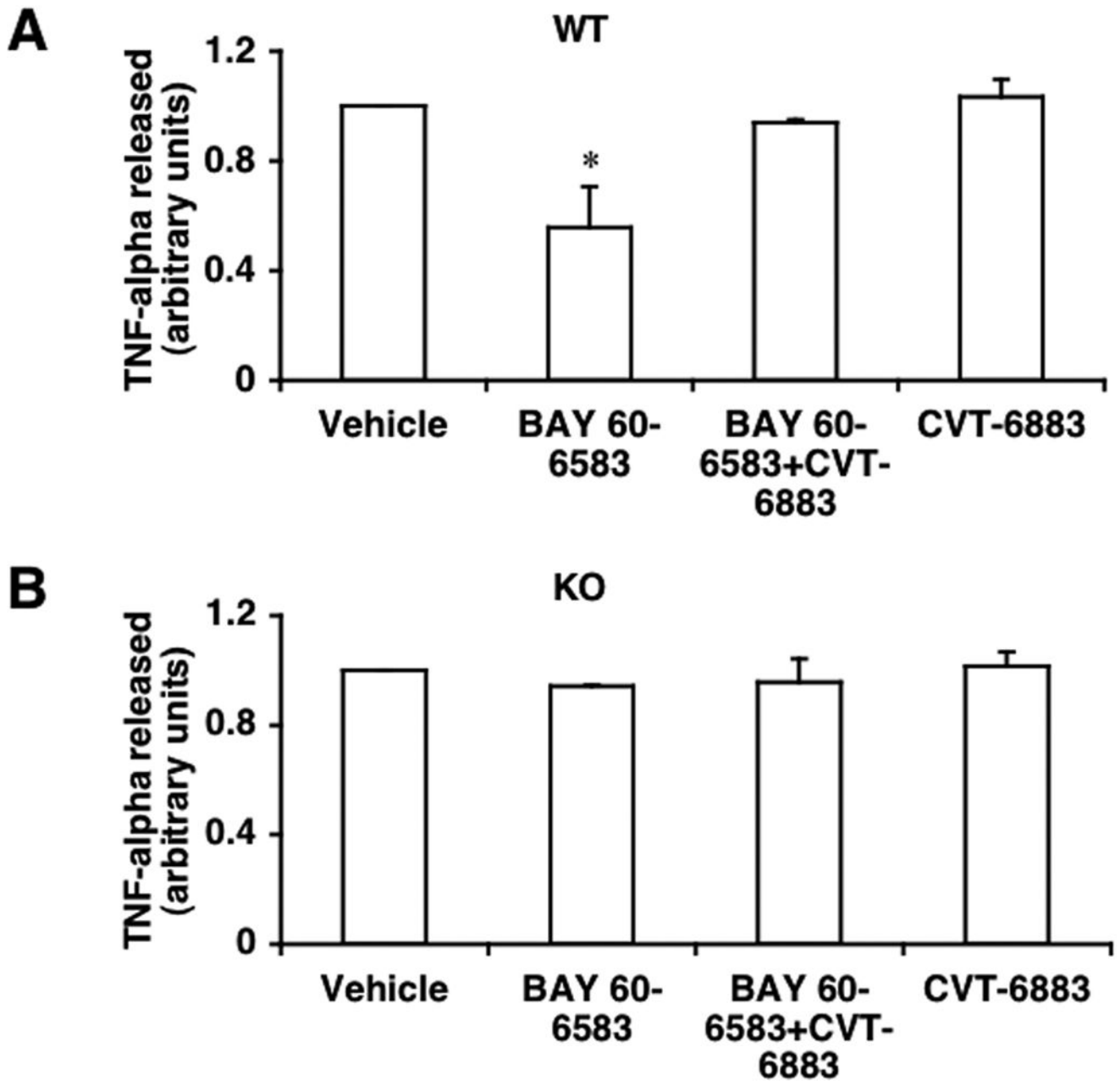

Figure 2.

The effect of A2bAR activation or deletion on the release of TNF-α from macrophages. (A) TNF-α released from WT macrophages. Cells were treated with the A2bAR antagonist CVT-6883 (1 μM) and then incubated with the A2bAR agonist BAY 60-6583 (1 μM) and followed by LPS (10 μg/ml) challenge for a total of 2 hours, as described under Methods. TNF-α released from cells is shown as a ratio to the maximal TNF-α level released from vehicle-treated cells (designated as 1). Shown are averages +/- SD (n=3). (B) TNF-α released from A2bAR KO macrophages. Cells were treated as in (A). Shown are averages +/- SD (n=3). *, $p < 0.05$ for vehicle-treated cells as compared to BAY 60-6583-treated cells,

and BAY 60-6583-treated cells as compared to BAY 60-6583 plus CVT-6883-treated cells or just CVT-6883-treated cells.

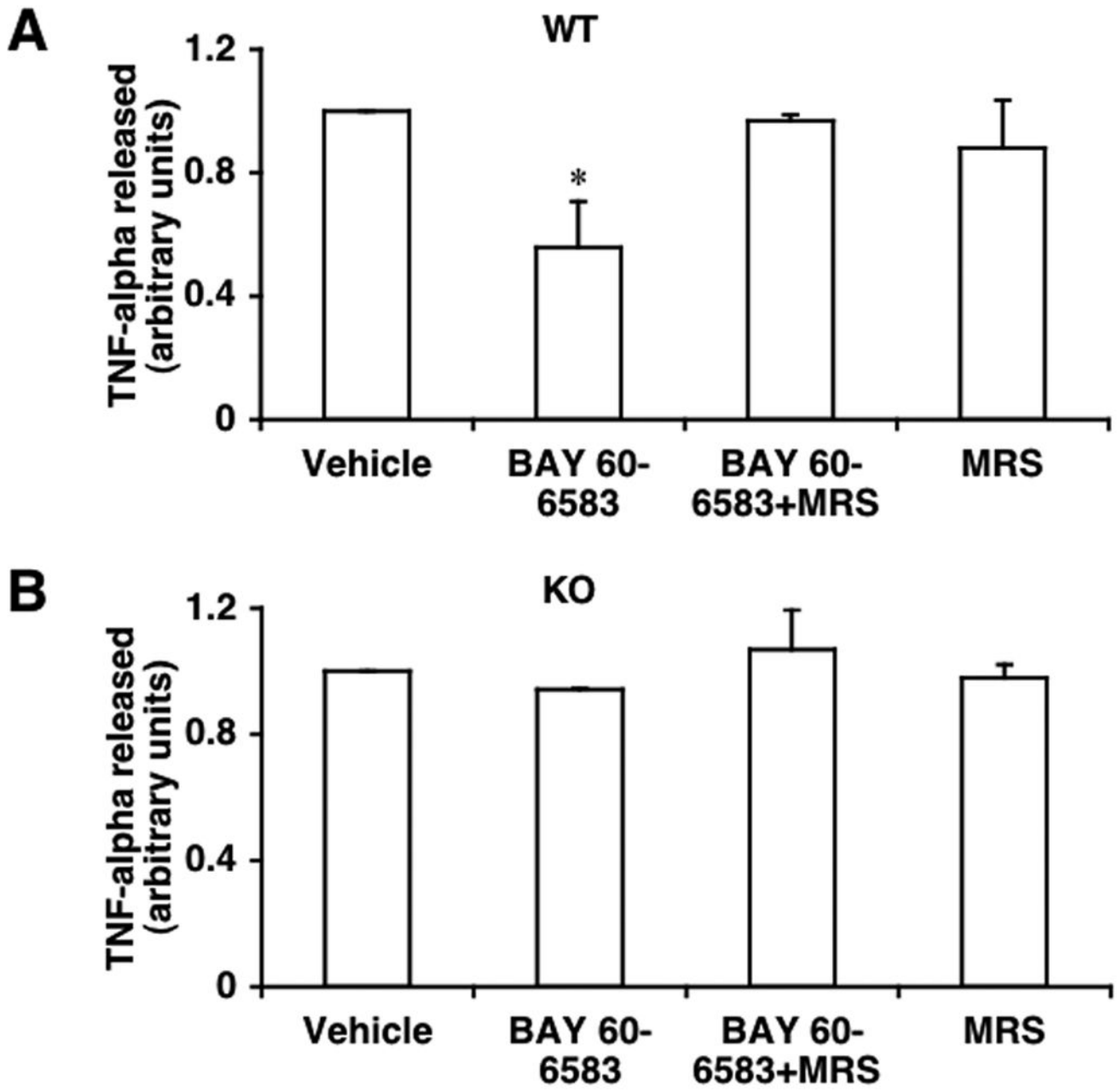

Figure 3.

The effect of A2bAR antagonist, MRS1754, on TNF-α release from macrophages treated with BAY 60-6583. (A) TNF-α released from WT macrophages pre-incubated with MRS1754 (10 μM) and incubated with BAY 60-6583 (1 μM) and followed by LPS (10 μg/ml) challenge as indicated under Methods. TNF-α released is shown as a ratio to the maximal TNF-α level in vehicle-treated cells (designated as 1). Shown are averages +/- SD (n=3). (B) TNF-α released from A2bAR KO peritoneal macrophages treated as in (A). Shown are averages +/- SD (n=3). *, $p < 0.05$ for vehicle-treated cells as compared to BAY

60-6583-treated cells, and BAY 60-6583-treated cells as compared to BAY 60-6583 plus MRS1754-treated cells or just MRS1754-treated cells.

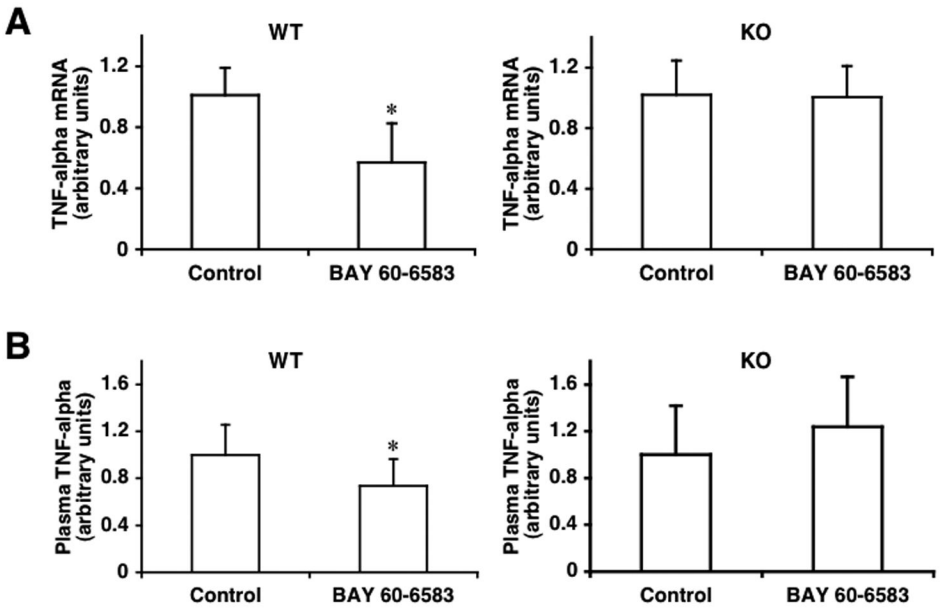

Figure 4.

In vivo effects of BAY 60-6583. (A) Quantitative PCR analysis of TNF-α mRNA levels in macrophages from WT or A2bAR KO mice injected with BAY 60-6583 (100 μg/kg body weight) or its vehicle PEG 400 (control) for 2 hours then injected with LPS (1 μg/g body weight; to allow induction and clear detection of this cytokine level) for another 1 hour as described in Methods. TNF-α mRNA level in ligand-treated mice is expressed as a ratio to TNF-α mRNA levels in vehicle-injected mice in the WT or the A2bAR KO group (each set at 1). The rationale for selecting the above dose of BAY 60-6583 is as follows: in other studies we noted that injection of higher concentrations (stepwise by log scale) yields some effects in KO mice as well, while lower concentrations are not as effective. (B) Plasma TNF-α levels in the WT or A2bAR KO mice presented in panel (A). The values in WT or the A2bAR KO group treated with vehicle are each set at 1 for normalization within each experimental group. The data in (A) and (B) are averages +/- SD (n=5). *, $p < 0.05$ for vehicle-treated cells as compared to BAY 60-6583-treated cells. Of note, the plasma level of TNF-α is 1.4 fold greater in the KO macrophages as compared to control (n=8), in general agreement with [4],[16].

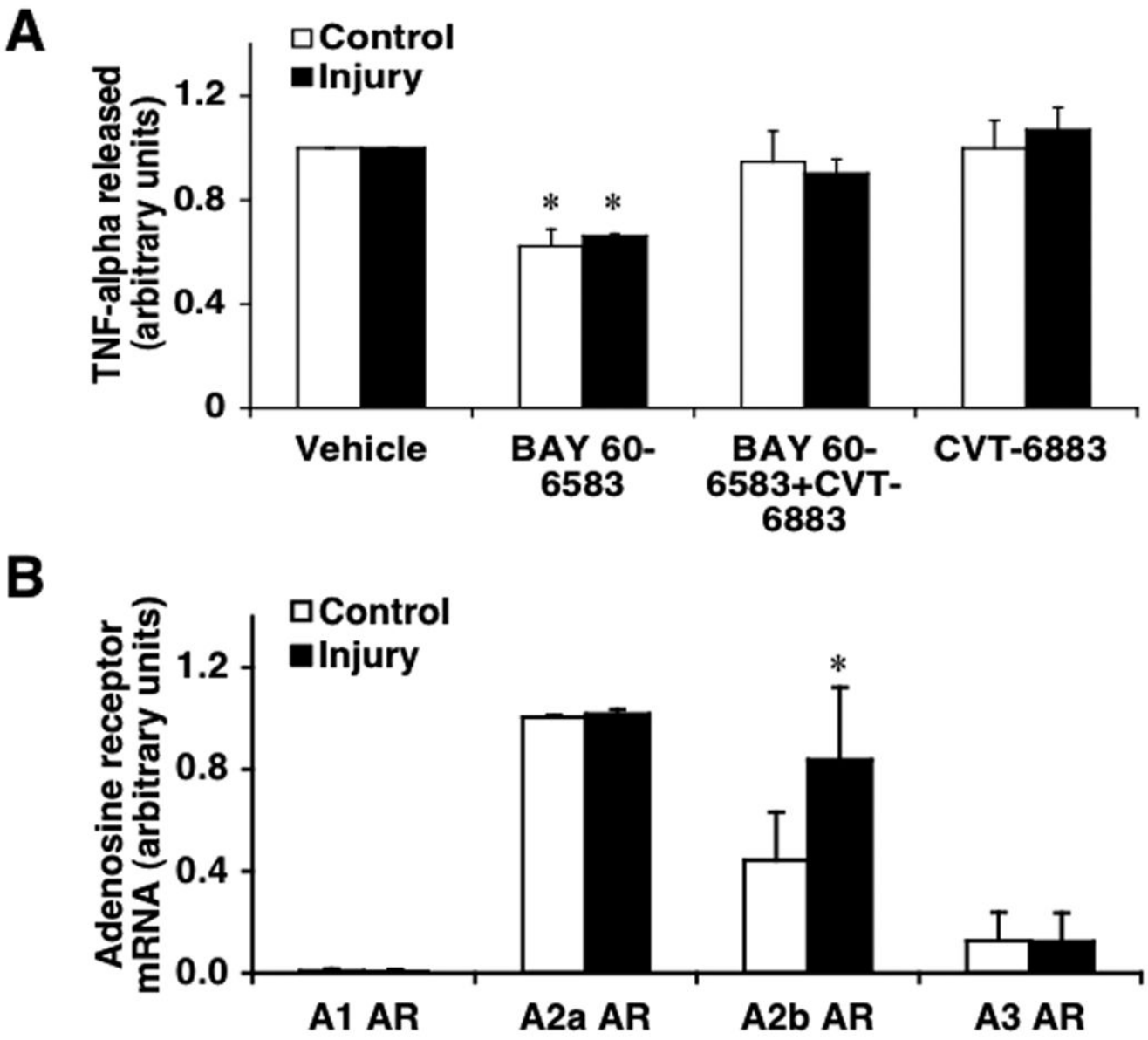

Figure 5.

The A2bAR controls TNF-α levels in macrophages from mice subjected to injury. (A) Peritoneal macrophages were derived form WT mice at one week after femoral artery injury (Injury), or from sham-injured mice (Control), and subjected to measurement of TNF-α release as described under Methods. Each of the values in the control and injury was designated as 1 for normalization within each experimental set. Cells were treated as indicated with vehicle or BAY 60-6583 (1 μM), preceded by a pre-treatment with CVT-6883 (1 μM) or vehicle as described under Methods. TNF-α level is presented as a ratio to the maximal TNF-α level in vehicle-treated cells (designated as 1). Data are averages +/- SD (n=4). *, $p < 0.05$ for vehicle-treated cells as compared to BAY 60-6583-treated cells, and BAY 60-6583-treated cells as compared to BAY 60-6583 plus CVT-6883-treated cells or just CVT-6883-treated cells. (B) Quantitative PCR analysis of adenosine receptor mRNA levels

in macrophages from WT mice after femoral artery injury as compared to sham injury. Adenosine receptor mRNA levels are shown as a ratio to A2aAR mRNA in each mouse. Data are averages +/- SD (n=5). *, $p < 0.05$ for the A2bAR post injury as compared to sham injury.