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Adenosine Augments IL-10 Production by Microglial Cells through an A_{2B} Adenosine Receptor-Mediated Process

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Microglia are activated by pathogen-associated molecular patterns and produce proinflammatory cytokines, such as TNF- α , IL-6, and IL-12, and the anti-inflammatory cytokine IL-10. Adenosine is an endogenous purine nucleoside and a ligand of four G protein-coupled adenosine receptors (ARs), which are the A₁AR, A_{2A}AR, A_{2B}AR, and A₃AR. ARs have been shown to suppress TNF- α production by microglia, but their role in regulating IL-10 production has not been studied. In this study, we demonstrate that adenosine augments IL-10 production by activated murine microglia while suppressing the production of proinflammatory cytokines. Because the order of potency of selective AR agonists in inducing IL-10 production was NECA > IB-MECA > CCPA \geq CGS21680, and the A_{2B}AR antagonist MRS1754 prevented the effect of NECA, we conclude that the stimulatory effect of adenosine on IL-10 production is mediated by the A_{2B}AR. Mechanistically, adenosine augmented IL-10 mRNA accumulation by a transcriptional process. Using mutant IL-10 promoter constructs we showed that a CREB-binding region in the promoter mediated the augmenting effect of adenosine on IL-10 transcription. Chromatin immunoprecipitation analysis demonstrated that adenosine induced CREB phosphorylation at the IL-10 promoter. Silencing CREB using lentivirally delivered short hairpin RNA blocked the enhancing effect of adenosine on IL-10 production, confirming a role for CREB in mediating the stimulatory effect of adenosine on IL-10 production. In addition, adenosine augmented IL-10 production by stimulating p38 MAPK. Collectively, our results establish that A_{2B}ARs augment IL-10 production by activated murine microglia. *The Journal of Immunology*, 2012, 188: 445–453.

Microglia are the resident macrophages of the CNS parenchyma. They originate from myeloid progenitors that invade the developing brain during the early embryonic period (1). In healthy brain, microglia have a ramified morphology as they continuously monitor the neural tissue. Under conditions of injury, ischemia, or infection, microglia become activated and develop an enlarged soma while retracting their processes (2, 3). As resident innate immune cells of the CNS, microglia form the first line of defense during infections (4). Activated microglia also contribute to inflammatory processes in the CNS during a variety of neurodegenerative diseases, such as

multiple sclerosis (5), Alzheimer's disease (6, 7), and Parkinson's disease (8).

Microglia express TLRs, which are important initiators of innate immune responses and neuroinflammation during infections and other CNS diseases (4, 9). There are 10 functional TLRs in humans and 12 in mice, each of which recognize different pathogen-associated molecular patterns or damage-associated molecular patterns (10). TLR activation induces inflammatory responses, which include secretion of proinflammatory cytokines, chemokines, and reactive oxygen species. For example, peptidoglycan (PGN) or *Staphylococcus aureus* induce proinflammatory cytokine production and elevate the expression of inducible NO synthase and cyclooxygenase-2 in microglia through TLR2 (11–13). Another bacterial product, LPS, activates microglia through TLR4 (12, 14, 15).

The limited regenerative capacity of neuronal tissue makes tight regulation of inflammatory responses in the brain crucial. IL-10 is an anti-inflammatory cytokine that has a pivotal role in limiting and resolving inflammation in the CNS (16, 17). IL-10, a significant source of which is microglia in the brain, inhibits the release of numerous proinflammatory mediators, inhibits Ag presentation, and regulates phagocytosis (18–20). IL-10, expressed by microglia, protects the brain from LPS-induced neurodegeneration (21).

Adenosine is a purine nucleoside with important immunomodulatory functions. Adenosine concentrations in the extracellular space increase in pathophysiological circumstances (22–24), and this increased extracellular adenosine signals to regulate both neural activity and glial function (25–28). Adenosine is recognized by four cell-surface adenosine receptors (ARs), A₁, A_{2A}, A_{2B}, and A₃, all of which are G protein-coupled seven-transmembrane receptors (29–34). AR activation on microglia has been shown to inhibit the production of proinflammatory cytokines; however, the effect of adenosine on IL-10 secretion by microglia

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Abbreviations used in this article: AR, adenosine receptor; CCPA, 2-chloro-N⁶-cyclopropyladenosine; CGS21680, 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethyl-carboxamidoadenosine; ChIP, chromatin immunoprecipitation; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; IB-MECA, N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide; MRS1523, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate; MRS1754, N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide; NECA, 1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl- β -D-ribofuranuronamide; PGN, peptidoglycan; shRNA, short hairpin RNA.

has not been studied. Therefore, the goal of the current study was to determine the effect of adenosine receptor activation on IL-10 production by microglial cells.

Materials and Methods

Drugs and reagents

Adenosine, the selective A₁AR agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA), A_{2A}AR agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethyl-carboxamidoadenosine (CGS21680), A₃AR agonist N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA), nonselective AR agonist 1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuranuronamide (NECA), the selective A₁AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), A_{2A}AR antagonist 4-(2-[7-amino-2-(2-furyl)[1.2.4]triazolo[2.3-a][1.3.5]triazin-5-ylamino] ethyl) phenol (ZM241385), A_{2B}AR antagonist N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide (MRS1754), A₃AR antagonist 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate (MRS1523), β-adrenoceptor agonist isoproterenol, and PGE₂ were purchased from Tocris Cookson (Ellisville, MO). The p38 MAPK pathway inhibitor SB203580 and p42/44 MAPK pathway inhibitor PD98059 were purchased from Calbiochem (San Diego, CA). Protein kinase A inhibitor N-[2-[[3-(4-bromophenyl)-2-propenyl] amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), PI3K inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride (LY294002), and phospholipase C inhibitor 1-[6-[[17β]-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) were

purchased from Tocris. PGN and LPS were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of the various agonists and protein kinase inhibitors were prepared using DMSO.

Experimental animals

C57BL/6 mice were purchased from Charles River Laboratories Hungary (Isaszeg, Hungary). All mice were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the Animal Care Committee of the Hungarian Academy of Sciences.

Cell cultures

BV-2 cells (35) were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Sigma-Aldrich), 50 U/ml penicillin, and 50 g/ml streptomycin (Invitrogen) in a humidified atmosphere of 95% air and 5% CO₂.

Primary microglia were obtained from 1–3-d-old C57BL/6 mice. Cerebral cortices were dissected, carefully stripped of their meninges, and digested with 0.05% trypsin (Sigma-Aldrich) and 0.6 mg/ml DNase (Sigma-Aldrich) dissolved in 2 ml PBS for 10 min. Trypsinization was stopped by adding an equal volume of DMEM. The cells were then placed in cell-culture flasks previously treated with poly-L-lysine (Sigma-Aldrich), and after an overnight incubation, nonadherent cells were removed by washing with culture medium. The remaining adherent mixed astrocyte-microglia culture was then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ and the medium replaced every

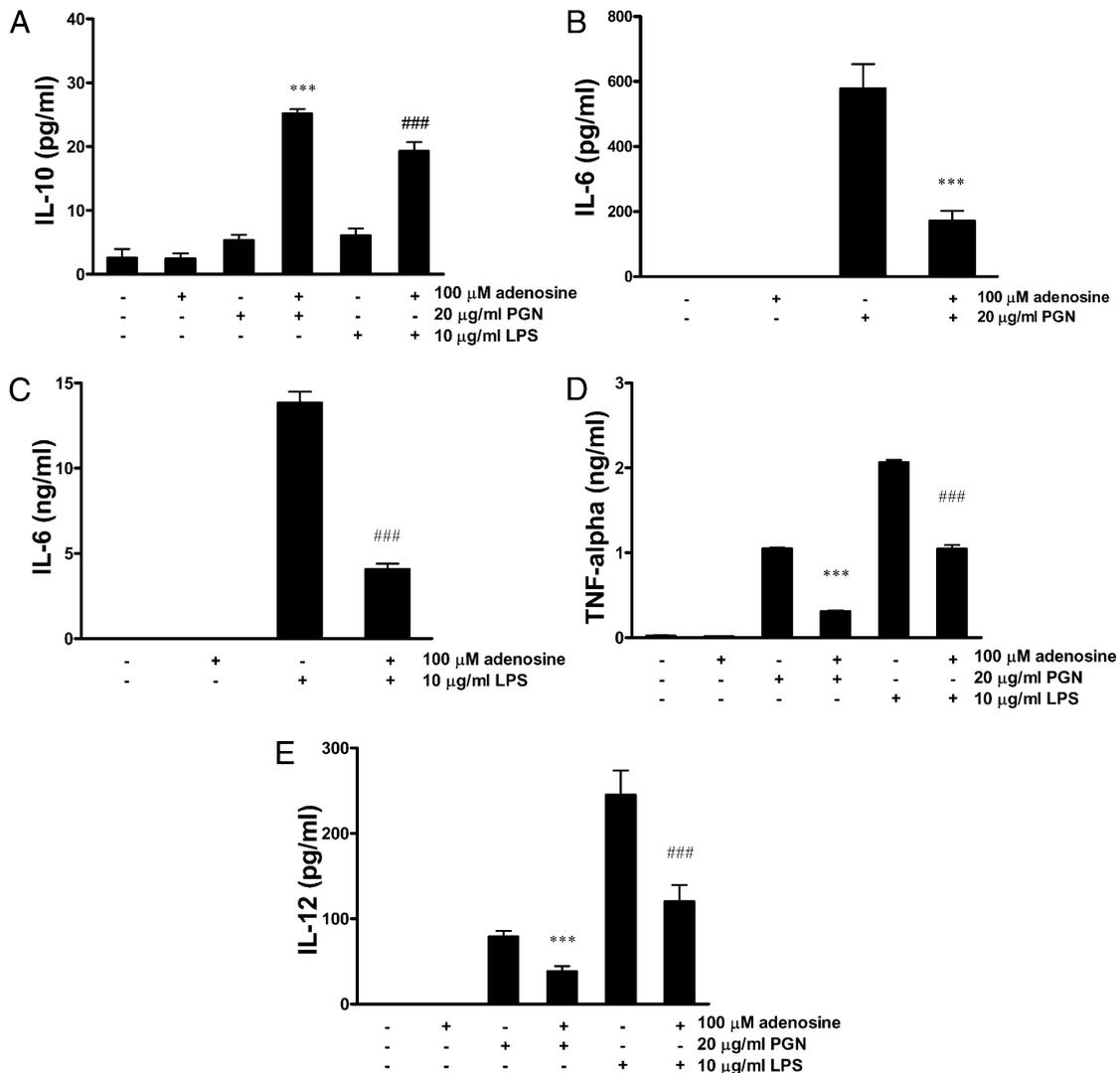


FIGURE 1. Adenosine augments IL-10 production and inhibits IL-6, TNF- α , and IL-12 release by BV-2 microglia activated with PGN or LPS. BV-2 cells were treated with 100 μ M adenosine and/or 20 μ g/ml PGN or 10 μ g/ml LPS for 24 h. IL-10 (A), IL-6 (B, C), TNF- α (D), and IL-12 (E) concentrations were determined from the supernatants that were removed at the end of the 24-h incubation period. All results (mean \pm SEM) are representative of three independent experiments ($n = 4$ in each experiment). *** $p < 0.001$ versus PGN, ### $p < 0.001$ versus LPS.

3 d until the cells reached confluence (14–16 d). Confluent cultures were trypsinized, and microglia were separated using CD11b MicroBeads from Miltenyi Biotec (Auburn, CA).

ELISA for determining cytokine production

BV-2 or primary microglial cells were placed in the wells of 96-well plates (10^5 cells/well). After an overnight incubation, supernatants were replaced with serum-free cell-culture medium, and the cells were incubated for 2 h. The cells were then treated with adenosine or various AR agonists followed immediately by the addition of PGN (20 μ g/ml) or LPS (10 μ g/ml) for 24 h, after which period the supernatants were frozen and stored. AR antagonists or protein kinase inhibitors were administered 30 min prior to NECA and PGN. IL-10, TNF- α , IL-6, and IL-12 levels in cell-culture supernatants were determined using ELISA Duoset kits (R&D Systems, Minneapolis, MN); the detection limit was 7.813 pg/ml.

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was prepared from BV-2 cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen) and from primary microglia using the RNeasy Mini Kit according the manufacturer's protocol (Qiagen, Valencia, CA) and reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). For detection of IL-10 and AR mRNA, real-time PCR commercial kit (Applied Biosystems) was used, and all data were normalized to constitutive rRNA values (18S) with primers that we described previously (36). The Applied Biosystems 7700 sequence detector (Applied Biosystems) was used for amplification of target cDNA, and quantitation of differences between treatment groups was performed according to the manufacturer's instructions.

Transient transfection of BV-2 cells with IL-10 promoter-luciferase and pCRE luciferase constructs and luciferase assay

BV-2 cells were transiently transfected using Polyfect transfection reagent (Qiagen). For transfection, cells were plated at 2.5×10^5 /ml density overnight, and at plating, each well of a 24-well plate contained 0.5 ml cell suspension. The following day, the cells were transfected with 0.4 μ g/well IL-10 reporter plasmids (kind gifts from Stephen T. Smale, University of California, Los Angeles, School of Medicine, Los Angeles, CA) and CREB reporter plasmid (Stratagene, La Jolla, CA). All transfections were performed at 37°C overnight, after which procedure the cells were washed with DMEM and treated with 10 μ M NECA and/or 20 μ g/ml PGN for 8 h. For reporter assays, whole-cell extracts were prepared using 80 μ l $1 \times$ passive lysis buffer from each well (Promega, Madison, WI). Luciferase activity was determined using 20 μ l cell extract with Dual-Luciferase Reporter Assay System (Promega).

Whole-cell protein isolation and Western blotting

BV-2 cells placed in wells of six-well plates were treated with 10 μ M NECA and/or 20 μ g/ml PGN for 15 min. Cells were washed with PBS and pelleted at $800 \times g$ for 5 min at 4°C. The pellet was resuspended in RIPA lysis buffer (0.05 M TRIS-HCl [pH 6.8], 0.25% Na-deoxycholate, 0.15 M NaCl, 1 mM EDTA [pH 7.4], 1 mM Na_3VO_4 , 1 mM NaF, 1% Nonidet P-40, 1 mM PMSF, and 100 \times diluted protease inhibitor mixture mix) and incubated on ice for 15 min. The lysates were centrifuged at $15,000 \times g$ for 15 min at 4°C, and the supernatant was recovered. Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Whole-cell lysates containing 30 μ g protein were separated on 10% Tris-glycine gel (Invitrogen). After electrophoresis, the gel was electroblotted in $1 \times$ Transfer buffer (Invitrogen) containing 20% methanol onto a nitrocellulose membrane (Invitrogen). The membranes were probed with monoclonal rabbit anti-mouse primary Abs raised against p38, phospho-p38, p42/44, phospho-p42/44, or CREB (Cell Signaling Technology, Danvers, MA). Thereafter, the membranes were incubated with a secondary HRP-conjugated goat anti-rabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated polyclonal goat anti- β -actin Ab to assess equal loading was used from Santa Cruz Biotechnology. Bands were detected using Chemiluminescent HRP Detection Reagent (Denville Scientific, South Plainfield, NJ). X-ray films were exposed for 1–15 min.

Silencing CREB using lentivirally delivered short hairpin RNA

BV-2 cells placed in 12-well plates (5×10^4 cells/well) were transduced with Mission Lentiviral particles containing CREB-specific short hairpin RNA (shRNA) or nontargeting shRNA (Sigma-Aldrich) in the presence of 8 μ g/ml hexadimethrine bromide. The sequences of the shRNA oligos were: 5'-CCG-GACTGATGGACAGCAGATTCTACTCGAGTAGAATCTGCTGTCCATC-

AGTTTTTTG-3', CREB; and 5'-CCGGCAACAAGATGAAGGCACCAAC-TCGAGTTGGTGTCTTTCATCTTGTGTTTT-3', nontargeting. The multiplicity of infection was 4 for both transductions. After overnight incubation, the supernatants were replaced with complete cell-culture medium, and the cells were incubated for a further 48 h. Transduced cells were selected by culturing in the presence of 2 μ g/ml puromycin. The efficiency of silencing was tested by Western blotting using CREB-specific mAbs from Cell Signaling Technology.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the ChIP assay kit from Millipore (Billerica, MA) according to the manufacturer's protocol. Phospho-CREB-specific mAb and normal IgG Ab provided with the kit for control were used. PCR reaction was performed using DNA purified from ChIP samples using primers specific for the region between -376 and -158 relative to the transcription start site of the IL-10 gene. Primers were designed by using Primer3 software and synthesized by the Molecular Research Facility of University of Medicine and Dentistry of New Jersey. The sequences of the primers used were: 5'-AGCCCAT-TATCCACGTCAT-3', pIL10 forward; and 5'-TTGTATTTCCTGAGGCA-GACAG-3', pIL10 reverse. The PCR was performed using the Taq PCR Polymerase kit from Qiagen.

Statistical analysis

Values in the figures are expressed as mean \pm SEM of the indicated number of observations. Statistical analyses of the data were performed using Student *t* test or one-way ANOVA followed by Dunnett's test, as appropriate.

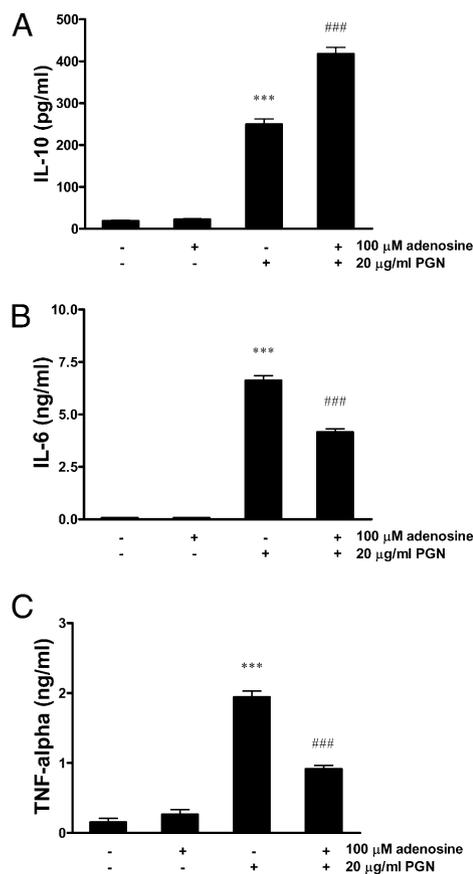


FIGURE 2. Adenosine augments IL-10 production and inhibits IL-6 and TNF- α release by primary microglia activated with PGN. Primary microglia isolated from C57BL/6 mice were activated with 100 μ M adenosine and/or 20 μ g/ml PGN for 24 h. IL-10 (A), IL-6 (B), and TNF- α (C) concentrations were determined from the supernatants using ELISA. All results (mean \pm SEM) are representative of three independent experiments ($n = 4$ in each experiment). *** $p < 0.001$ versus adenosine- and PGN-untreated, ### $p < 0.001$ versus PGN.

Results

Adenosine augments IL-10 production by activated microglia through A_{2B} receptors

To examine the effect of adenosine on IL-10 production by activated microglia, we first treated BV-2 cells with adenosine and PGN for 24 h. The combination of adenosine and PGN synergistically induced IL-10 production, whereas neither adenosine nor PGN alone was able to elicit IL-10 release by BV-2 cells (Fig. 1A). To test whether the effect of adenosine is present in cells activated through TLR4, we treated cells with adenosine and LPS. We found that adenosine and LPS synergistically induced IL-10 production in LPS-activated BV-2 cells as well (Fig. 1A).

To investigate whether adenosine has any effect on proinflammatory cytokine production by microglia, we determined TNF- α , IL-6, and IL-12 concentrations from the supernatants of BV-2 cells treated with adenosine and PGN or LPS. We found that PGN or LPS alone induced the production of TNF- α , IL-6, and IL-12, and adenosine inhibited the production of each proinflammatory cytokine (Fig. 1B–E).

In primary microglia, PGN induced the release of IL-10, which was further augmented by adenosine (Fig. 2A). In addition, adenosine inhibited IL-6 (Fig. 2B) and TNF- α (Fig. 2C) production by PGN-activated primary microglia.

To identify the adenosine receptor subtype that is responsible for the effect of adenosine in enhancing IL-10 production, we next challenged PGN-activated BV-2 cells with different AR ago-

nists and antagonists. The nonselective agonist NECA turned out to be the most potent IL-10 inducer, whereas the A_1 AR agonist CCPA and A_3 AR agonist IB-MECA were less potent, and the A_{2A} AR agonist CGS21680 failed to induce IL-10 production (Fig. 3A). We found a similar order of potency of agonists in triggering IL-10 production in cells activated with LPS (Fig. 3B). NECA in the absence of PGN did not have any effect on IL-10 production by BV-2 cells (Fig. 3C).

The stimulatory effect of NECA on IL-10 production was inhibited by pretreatment with the selective A_{2B} AR antagonist MRS1754, but not with DPCPX, ZM241385, and MRS1523, which are selective A_1 AR, A_{2A} AR, and A_3 AR antagonists, respectively (Fig. 3D, 3E).

Finally, we examined the expression levels of adenosine receptors in BV-2 cells using real-time PCR, and we found that all receptors were expressed, with A_{2B} AR and A_3 AR having the highest expression levels. Furthermore, PGN treatment augmented the expression of A_{2B} ARs and reduced the expression of A_3 ARs (Fig. 4A). LPS treatment elevated the expression of all receptors but A_3 AR. PGN or LPS treatment also augmented A_{2B} AR expression in primary microglia, but had no effect on the expression of the other AR subtypes (Fig. 4B).

We conclude that the stimulatory effect of adenosine on IL-10 production by BV-2 cells is A_{2B} AR dependent. Because NECA was the most potent agonist, we used NECA in all subsequent experiments.

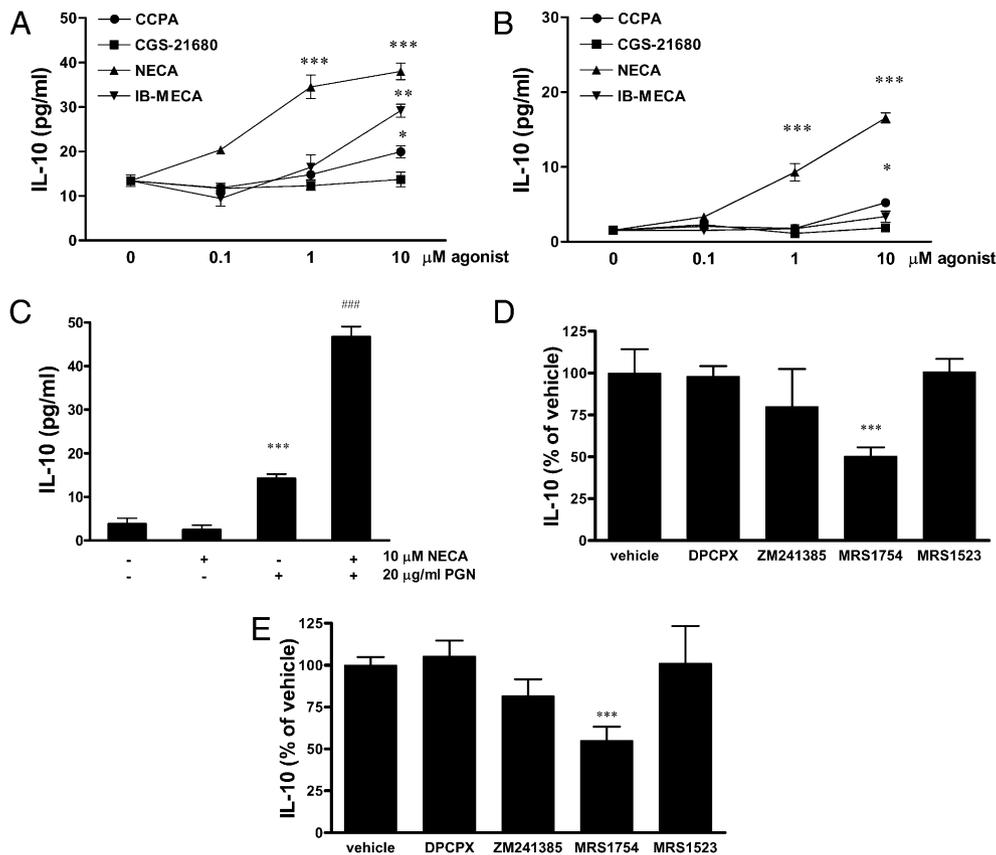


FIGURE 3. Adenosine increases IL-10 production via A_{2B} ARs. BV-2 microglia were treated with increasing concentrations of CCPA, CGS21680, NECA, or IB-MECA and 20 μ g/ml PGN (A) or 10 μ g/ml LPS (B) for 24 h, and IL-10 concentrations were determined from the supernatants obtained after the 24-h incubation period. * p < 0.05, ** p < 0.01, *** p < 0.001 versus vehicle. C, BV-2 cells were treated with NECA or its vehicle and PGN for 24 h, and IL-10 concentrations were determined from the supernatants with ELISA. *** p < 0.001 versus vehicle, ### p < 0.001 versus PGN. BV-2 cells were pretreated with 0.1 μ M AR antagonists DPCPX (A_1 AR), ZM241385 (A_{2A} AR), MRS1754 (A_{2B} AR), or MRS1523 (A_3 AR) 30 min prior to treatment with 10 μ M NECA and 20 μ g/ml PGN (D) or 10 μ g/ml LPS (E). After 24 h, supernatants were removed, and IL-10 concentrations were determined from the supernatants using ELISA. Results are expressed as percent of vehicle (group treated with NECA and PGN). *** p < 0.001 versus vehicle.

NECA and PGN trigger IL-10 production by a transcriptional mechanism

To examine whether the synergistic stimulatory effect of NECA and PGN on IL-10 production was transcriptional, we first measured the accumulation of IL-10 mRNA in NECA- and PGN-treated BV-2 cells. Our results showed that NECA augmented IL-10 mRNA levels in BV-2 cells exposed to PGN (Fig. 5A), indicating a pretranslational effect. We then pretreated the cells with actinomycin D, a widely used transcriptional inhibitor (37) before administering NECA and PGN. The results confirmed that NECA and PGN augmented IL-10 transcription, because actinomycin D completely abolished the stimulatory effect of NECA and PGN on IL-10 mRNA accumulation (Fig. 5A).

The IL-10-inducing effect of NECA is CREB dependent

We next aimed to determine which region of the IL-10 promoter was responsible for the increased mRNA transcription after NECA and PGN treatment. We transfected BV-2 cells with constructs in which luciferase activity was driven by IL-10 promoter mutants containing successive deletions from the 5' end (38). We treated the cells with NECA and/or PGN and measured the luciferase activity from cell lysates. NECA augmented luciferase activity in cells transfected with the full promoter as well as constructs containing deletions between -1538 and -376 relative to the transcription start site (Fig. 5B). In contrast, NECA failed to augment luciferase activity in cells transfected with constructs containing deletion between -376 and -78 (Fig. 5B). Using the Searching Transcription Factor Binding Sites program, we found that there was a binding site for CREB in this region (Fig. 5C). We then transfected the BV-2 cells with a construct in which luciferase activity was driven by CREB binding sequences (pCRE). NECA treatment induced luciferase activity in cells transfected with pCRE but not in cells transfected with pCIS, the control plasmid (Fig. 5D).

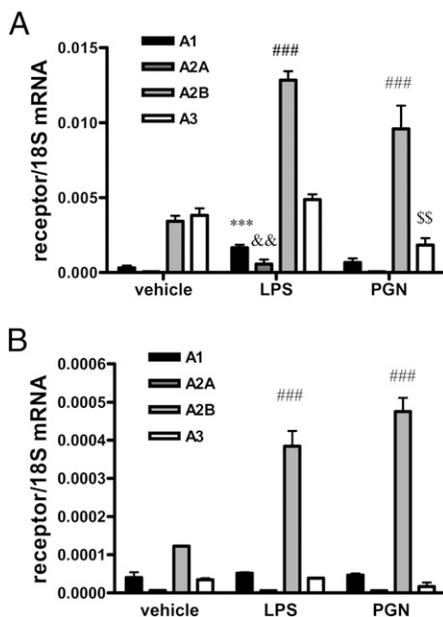


FIGURE 4. PGN or LPS treatment augments A_{2B}AR mRNA expression BV-2 cells (A), or primary microglia (B) were treated with 20 μ g/ml PGN or 10 μ g/ml LPS for 4 h, after which period RNA was isolated from the cells. RNA was then reverse transcribed to cDNA. Real-time PCR was performed using the cDNA samples and primers specific for A₁AR, A_{2A}AR, A_{2B}AR, A₃AR, or 18S. Results were normalized to 18S mRNA content. Results (mean \pm SEM) shown are representative of three separate experiments ($n = 4$ in each experiment). *** $p < 0.001$ versus vehicle, && $p < 0.01$ versus vehicle, ### $p < 0.001$ versus vehicle, \$\$ $p < 0.01$ versus vehicle.

To further elucidate the role of CREB, we next evaluated the effect of NECA/PGN on CREB phosphorylation at the IL-10 promoter using ChIP assay. Immunoprecipitation with a phospho-CREB-specific Ab followed by PCR with primers specific for the region between -376 and -158 of the IL-10 promoter showed that CREB phosphorylation at this site occurred after NECA and PGN treatment (Fig. 6A).

We then studied whether CREB was required for the stimulatory effect of NECA/PGN on IL-10 production using a gene silencing approach. We silenced CREB in BV-2 cells using specific shRNA, which was delivered in lentiviral particles. We first confirmed that in cells transduced with CREB-specific shRNA containing lentiviral particles, the expression of CREB was downregulated in comparison with cells transduced with control particles as well as untransduced BV-2 cells (Fig. 6B). We then treated nontargeting and CREB-specific shRNA-expressing BV-2 cells with NECA and/or PGN and determined IL-10 concentrations from the supernatants. The results showed that NECA/PGN induced significantly less IL-10 production in CREB shRNA-expressing cells than in nontargeting shRNA expressing cells (Fig. 6C). Next, we studied whether other agents capable of activating CREB would also induce IL-10 production in our system. To that end, we treated BV-2 cells with the β adrenoceptor agonist isoproterenol or PGE₂ and PGN and then determined IL-10 concentrations from the supernatants. Our data showed that both isoproterenol and PGE₂ augmented IL-10 production by PGN-activated microglia (Fig. 6D). In conclusion CREB is necessary for the IL-10-inducing effect of NECA/PGN.

p38 MAPK phosphorylation is crucially required for the induction of IL-10 release

To further characterize the signaling pathways that participate in the effect of NECA and PGN in inducing IL-10 production by microglia, we investigated the role of MAPKs in the process. Using Western blotting that employed Abs against the active, doubly phosphorylated forms of p38, p42/44, and JNK, we found that PGN augmented the phosphorylation/activation of all three MAPKs, and NECA increased JNK phosphorylation (Fig. 7A, 7C, 7E). In addition, NECA further increased the PGN-induced phosphorylation/activation of p38 (Fig. 7A, 7C, 7E). To study whether MAPK activation was necessary for NECA/PGN to trigger IL-10 production, we pretreated cells with selective MAPK inhibitors prior to administering NECA/PGN. Pretreatment with the p38 inhibitor SB203580 blocked the IL-10-inducing effect of NECA/PGN (Fig. 7B). In contrast, the p42/44 inhibitor PD98059 and JNK inhibitor Sp600125 failed to reverse the induction of IL-10 production by NECA/PGN (Fig. 7D, 7F).

NECA and PGN treatment also augmented the phosphorylation of Akt (Fig. 8A). The PI3K inhibitor LY294002 decreased the IL-10 production induced by NECA and PGN treatment (Fig. 8B). These observations suggest a role of the PI3K–Akt signaling pathway in triggering IL-10.

Next, we treated BV-2 cells that had been transfected with pCRE construct with SB203580 or LY294002 30 min prior to NECA/PGN to determine the effect of p38 and PI3K inhibition on CREB activation. We found that the SB203580 inhibited the NECA/PGN-induced elevation of luciferase activity. LY294002, however, failed to reverse the NECA/PGN augmentation of CREB activation (Fig. 8C). These results suggest that p38 activation is crucial for CREB induction by NECA/PGN.

Discussion

In this study, we have provided evidence, for the first time to our knowledge, that adenosine in conjunction with TLR ligands augments the release of IL-10 by murine microglial cells. Our results thus extend previous observations in model systems, in

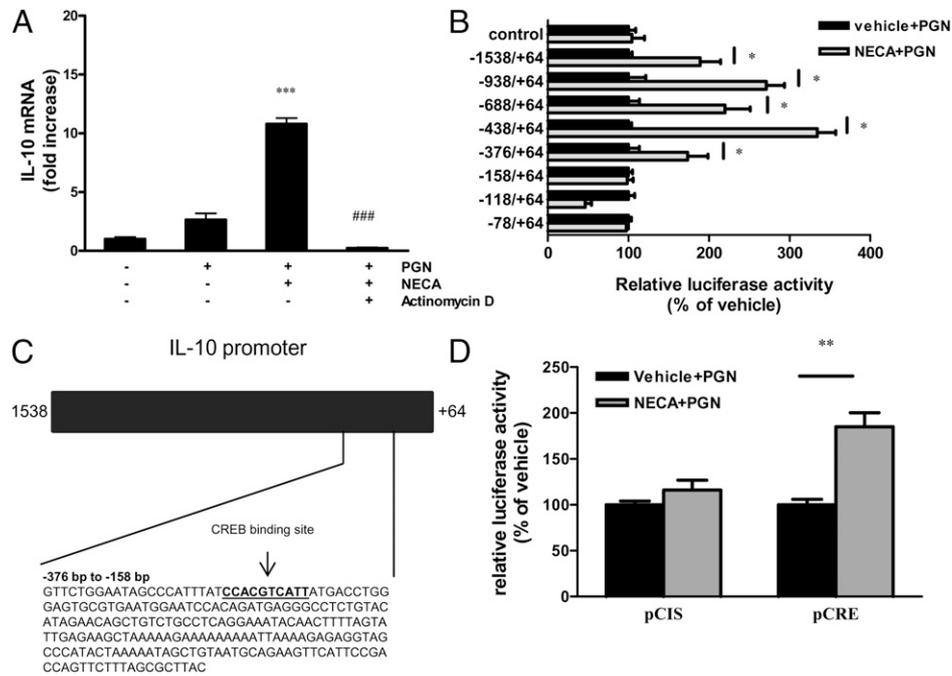


FIGURE 5. $A_{2B}AR$ activation augments IL-10 transcription. **A**, BV-2 cells were treated with 5 $\mu\text{g/ml}$ actinomycin D or its vehicle 2 h prior to treatment with 10 μM NECA and 20 $\mu\text{g/ml}$ PGN. After 6 h of incubation, RNA was isolated from the cells and reverse transcribed to cDNA. Real-time PCR was performed using the cDNA samples using IL-10- and 18S-specific primers. Results were normalized to 18S mRNA content and shown as fold increase relative to untreated samples. **B**, BV-2 cells transfected with IL-10 promoter-luciferase constructs and control plasmid were treated with 10 μM NECA and 20 $\mu\text{g/ml}$ PGN for 8 h. Luciferase activity was determined from whole-cell extracts and normalized to protein content. Data are shown as percent of vehicle (treated only with PGN). **C**, The figure represents the IL-10 promoter region. The numbers show the positions relative to the transcription start site. The highlighted area shows the nucleotide sequence of the region between -376 and -158, which harbors a binding site for CREB. **D**, BV-2 cells transfected with pCRE-luciferase construct and control plasmid (pCIS) were treated with 10 μM NECA and 20 $\mu\text{g/ml}$ PGN for 8 h. Luciferase activity was determined from whole-cell extracts and normalized to protein content. Data shown are percent of vehicle (groups treated only with PGN). Results (mean \pm SEM) shown are representative of three separate experiments ($n = 4$ in each). * $p < 0.05$ versus vehicle, ** $p < 0.01$ versus vehicle, *** $p < 0.001$ versus PGN, #### $p < 0.001$ versus NECA+PGN.

which the effect of adenosine was studied in cells from the periphery (39–42). Using AR agonists and antagonist, we demonstrate that the $A_{2B}AR$ is primarily responsible for the stimulatory

effect of adenosine on IL-10 production. Specifically, we found that the order of potency of agonists was NECA > IB-MECA > CCPA \geq CGS21680, which indicates a predominant role for A_{2B}

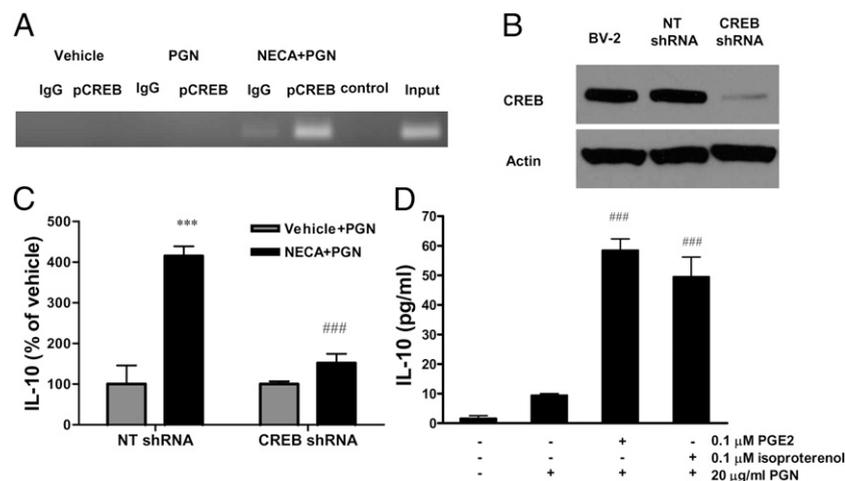


FIGURE 6. The stimulatory effect of $A_{2B}AR$ activation on IL-10 production by microglia is CREB dependent. **A**, BV-2 cells were treated with 10 μM NECA and 20 $\mu\text{g/ml}$ PGN for 30 min, and ChIP assay was performed on cell extracts using phospho-CREB-specific and control Abs. PCR reaction was performed using the DNA purified from ChIP samples using primers specific for the IL-10 promoter. PCR products were separated on a 3% agarose gel stained with ethidium bromide and visualized under UV light. **B**, BV-2 cells were transfected with lentiviral vectors containing CREB-specific or non-targeting (NT) shRNA. Protein was then isolated from transfected and untransfected BV-2 cells, and CREB expression was tested by Western blotting using Abs specific for CREB or for actin as loading control. **C**, BV-2 cells transfected with NT or CREB-specific shRNA were treated with 10 μM NECA and 20 $\mu\text{g/ml}$ PGN and incubated for 24 h. IL-10 concentrations were determined from the supernatants that were taken at the end of the incubation period using ELISA. Data are expressed as percent of vehicle (treated only with PGN). *** $p < 0.001$ versus vehicle, #### $p < 0.001$ versus NECA/NT shRNA-transfected group. **D**, BV-2 cells were treated with 0.1 μM isoproterenol or PGE₂ and 20 $\mu\text{g/ml}$ PGN for 24 h, and IL-10 concentrations were determined from the supernatants. Results (mean \pm SEM) shown are representative of three separate experiments ($n = 4$ in each experiment). #### $p < 0.001$ versus PGN.

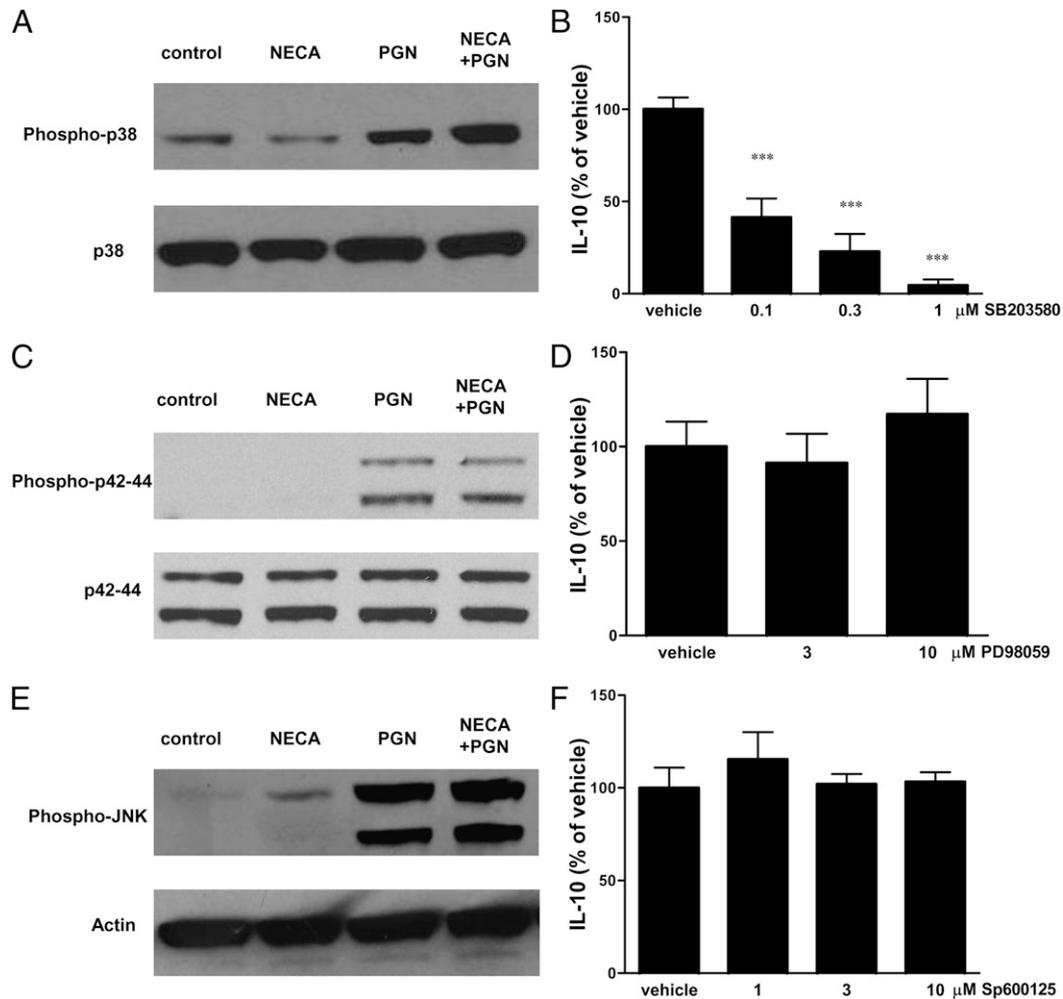


FIGURE 7. The stimulatory effect of A_{2B} AR activation on IL-10 production by microglia is p38 but not p42/44 or JNK dependent. BV-2 cells were treated with 10 μ M NECA and 20 μ g/ml PGN, and 15 min later, protein was isolated from the cells. Western blotting was performed using Abs specific for p38 and phospho-p38 (A), p42/44 and phospho-p42/44 (C), and JNK and phospho-JNK (E). BV-2 cells were pretreated with increasing concentrations of SB203580 (B), PD98059 (D), or Sp600125 (F) 30 min prior to treatment with 10 μ M NECA and 20 μ g/ml PGN for 24 h. IL-10 concentrations were determined from supernatants using ELISA. Data are shown as percentage of vehicle for inhibitor (treated with NECA and PGN). Results (mean \pm SEM) are representative of three independent experiments ($n = 4$ /experiment). *** $p < 0.001$ versus vehicle.

receptors in triggering IL-10 production (43). In addition, the fact that the A_{2B} AR antagonist MRS1754 but not antagonists of the other ARs reversed the stimulatory effect of both adenosine and NECA on IL-10 production lends further credence to the proposal that A_{2B} ARs are the most important ARs in augmenting IL-10 production by activated microglia. In agreement with a primary role for A_{2B} ARs, mRNA levels of A_{2B} ARs were highest after PGN and LPS treatment of BV-2 cells. Interestingly, although we found that the expression level of A_{2B} AR and A_3 AR mRNAs was comparable in unstimulated BV-2 cells, a previous study by Haselkorn et al. (44) showed that the expression of A_{2B} AR mRNA was highest of all the ARs in unstimulated BV-2 cells.

We also confirmed the previously described inhibitory effect of adenosine on the LPS-induced production of TNF- α and IL-12 by microglia (45, 46). In addition, we showed for the first time, to our knowledge, that adenosine also diminishes IL-6 production by microglial cells activated with different TLR agonists. Although we did not investigate the role of the various ARs in suppressing the production of proinflammatory cytokines in the current study, it appears that different ARs regulate the production of pro- versus anti-inflammatory cytokines by microglia. That is because, although the inhibitory effect of adenosine on IL-12 and TNF- α has been found to be A_{2A} AR or A_3 AR dependent (45, 46), our data

show that A_{2B} receptors mediate the stimulatory effect of adenosine on IL-10 production.

Our data revealed that A_{2B} AR stimulation increased IL-10 mRNA levels in BV-2 cells via a transcriptional mechanism, as this effect could be completely prevented when transcription was blocked using actinomycin D. This result was unexpected in microglia, because we previously showed that A_{2B} AR stimulation augmented IL-10 production by a translational mechanism in TLR-activated macrophages (47). These observations indicate that A_{2B} AR activation differentially regulates IL-10 production by microglia versus macrophages. Using a number of approaches, we have pinpointed CREB as the transcription factor that mediated the stimulatory effect of A_{2B} AR stimulation on IL-10 transcription. Firstly, employing mutant IL-10 promoter constructs, we showed that a CREB-harboring region of the IL-10 promoter was necessary for the stimulatory effect of A_{2B} AR stimulation. Secondly, we showed that CREB phosphorylation occurs at the IL-10 promoter in response to A_{2B} AR stimulation in BV-2 cells. Thirdly, we demonstrated that silencing CREB prevents the effect of NECA on IL-10 release. Although previous studies have linked the induction of IL-10 transcription to CREB in different cell types and in response to different stimuli (48–51), our study is the first one, to our knowledge, to show that IL-10 induction relies on CREB in microglia.

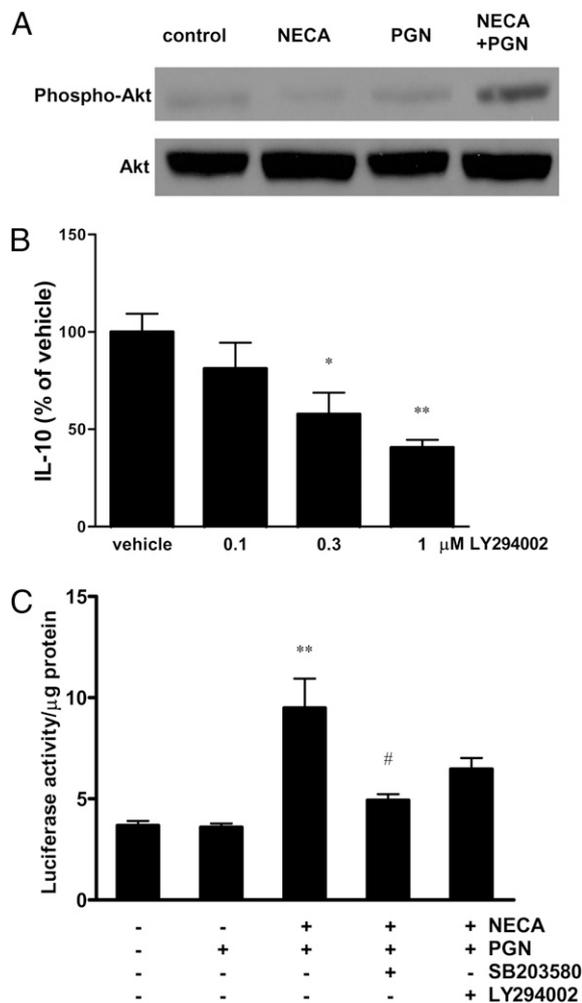


FIGURE 8. The effect of $A_{2B}AR$ activation on IL-10 production by microglia is PI3K/Akt dependent. **A**, BV-2 cells were treated with 10 μM NECA and 20 $\mu g/ml$ PGN, and 15 min later, protein was isolated from the cells. Western blotting was then performed using Abs specific for Akt and phospho-Akt. **B**, BV-2 cells were pretreated with LY294002 30 min prior to treatment with 10 μM NECA and 20 $\mu g/ml$ PGN for 24 h. IL-10 concentrations were determined from supernatants using ELISA. Data are shown as percentage of vehicle (NECA plus PGN). * $p < 0.05$ versus vehicle, ** $p < 0.01$ versus vehicle. **C**, BV-2 cells transfected with pCRE-luciferase construct were treated with SB203580 or LY294002 30 min prior to treatment with 10 μM NECA and 20 $\mu g/ml$ PGN for 8 h. Luciferase activity was determined from whole-cell extracts and normalized to protein content. Results (mean \pm SEM) shown are representative of three independent experiments ($n = 4$ /experiment). ** $p < 0.01$ versus vehicle and PGN, # $p < 0.05$ versus NECA+PGN.

We have previously noted that AR stimulation enhances CREB phosphorylation and transcriptional activity in a p38-dependent manner in macrophages (52). The results of the current study with microglia also suggest a role of p38 in stimulating both CREB activation and IL-10 release, as $A_{2B}AR$ signaling augmented p38 phosphorylation and pharmacological inhibition of p38 blocked both the CREB and the IL-10 responses to $A_{2B}AR$ activation. Another member of the MAPK family, p42/44, has also been linked to CREB activation and IL-10 transcription (48). However, in our system, $A_{2B}AR$ activation failed to increase p42/44 phosphorylation/activation, and inhibiting p42/44 failed to prevent the effect of $A_{2B}AR$ activation.

The participation of the PI3K/Akt pathway in AR signaling has been proposed by several previous studies (51, 53–55). Our results show that $A_{2B}AR$ activation augments IL-10 production by activating

the PI3K/Akt pathway, but this pathway is independent of CREB. Our findings thus extend previous observations, which showed that PI3K/Akt activation augments IL-10 production by macrophages and dendritic cells (51, 56–58).

Previous studies have shown that A_1AR , $A_{2A}AR$, and A_3AR regulate CNS inflammation, and it appears that some of these regulatory effects are mediated by AR on microglia (44, 59–63). Our data showing that $A_{2B}AR$ activation augments IL-10 production by microglia point to an anti-inflammatory role of $A_{2B}AR$ activation in the brain. We propose that targeting $A_{2B}AR$ s may be a new approach for the treatment of neuroinflammatory diseases.

Disclosures

The authors have no financial conflicts of interest.

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