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A_{2A} adenosine receptors and C/EBPβ are crucially required for IL-10 production by macrophages exposed to *Escherichia coli*

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We recently showed that A_{2A} adenosine receptor activation by endogenous adenosine contributes to interleukin-10 (IL-10) production in polymicrobial sepsis. Here we investigated the molecular mechanisms underpinning this interaction between adenosine receptor signaling and infection by exposing macrophages to *Escherichia coli*. We demonstrated using receptor knockout mice that A_{2A} receptor activation is critically required for the stimulatory effect of adenosine on IL-10 production by *E coli*–challenged macrophages, whereas A_{2B} receptors have a minor role. The stimulatory effect of adenosine on *E coli*–induced IL-10 production did not require toll-like receptor 4 (TLR4) or MyD88, but was blocked by p38 inhibition. Using shRNA we demonstrated that TRAF6 impairs the potentiating effect of adenosine. Measuring IL-10 mRNA abundance and transfection with an IL-10 promoter-luciferase construct indicated that *E coli* and adenosine synergistically activate IL-10 transcription. Sequential deletion analysis and site-directed mutagenesis of the IL-10 promoter revealed that a region harboring C/EBP binding elements was responsible for the stimulatory effect of adenosine on *E coli*–induced IL-10 promoter activity. Adenosine augmented *E coli*–induced nuclear accumulation and DNA binding of C/EBPβ. C/EBPβ-deficient macrophages failed to produce IL-10 in response to adenosine and *E coli*. Our results suggest that the A_{2A} receptor–C/EBPβ axis is critical for IL-10 production after bacterial infection. (Blood. 2007;110:2685-2695)

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Introduction

The innate immune system is responsible for identifying infectious pathogens and eliciting specific immune responses to eliminate the pathogens. Pathogen recognition can be mediated by a set of pattern recognition receptors. One class of pattern recognition receptors involves the Toll-like receptors (TLRs), which consist of a family of transmembrane molecules found in a broad range of organisms from *Drosophila* to higher mammals.[1-5] TLR4 was first recognized as the receptor mediating the macrophage stimulatory effect of endotoxins (lipopolysaccharide, LPS) of Gram-negative bacteria, such as *Escherichia coli*. Other Gram-negative bacterial components can also induce macrophage activation via various other pattern recognition receptors, which include TLR2 (lipopeptide receptor), TLR5 (flagellin receptor), and TLR9 (CpG DNA receptor), as well as nucleotide-binding oligomerization domain (nucleotide-binding oligomerization domain 1, peptidoglycan receptor) and nucleotide-binding oligomerization domain 2 (muramyl dipeptide receptor).[4,5] Lipoteichoid acid, a component of Gram-positive bacteria, activates cellular responses via TLR2.[4,5]

The engagement of pattern recognition receptors by microbial components triggers the activation of signaling cascades leading to the induction of genes involved in antimicrobial host defense such as cytokines and chemokines.[6-8] Myeloid differentiation factor 88 (MyD88) has been shown to be a critical adaptor protein linking TLRs to several downstream intracellular pathways.[4,5] Many of the activating signals originating from TLRs and MyD88 converge on tumor necrosis factor receptor–associated factor 6 (TRAF6), which transduces the signals toward the nuclear factor-κB system and mitogen-activated protein kinases (MAPKs), resulting in induction of genes involved in pro-inflammatory responses.[4,5]

Much less is known about the regulation of anti-inflammatory cytokines and their genes, the most prominent of which is IL-10.[9,10] IL-10 is an inducible gene and several transcriptional factors have been implicated in its regulation, which include Sp1,[11-13] Sp3,[12] C/EBPδ and C/EBPβ,[14] STAT3,[15] and c-Maf.[16] IL-10 gene expression also requires transient remodeling of the IL-10 promoter that occurs as a result of MAPK activation.[17] In addition, we and others[18,19] have recently shown that IL-10 production is also regulated at various posttranscriptional levels, including alterations in mRNA stability and translation efficacy.

Adenosine, an endogenous purine nucleoside, is a biologically active extracellular signaling molecule that is formed at sites of metabolic stress associated with hypoxia, ischemia, trauma, or inflammation. Adenosine mediates its effects via engaging A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors,[20] which are all expressed abundantly on monocytes and macrophages.[21] Stimulation of adenosine receptors has been shown to convert the phenotype of

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TLR4 (LPS)-stimulated macrophages from a pro-inflammatory to an anti-inflammatory one.[21-27] Most notably, adenosine receptor activation has been documented to upregulate the TLR4-induced production of IL-10.[28-30] In addition to TLR4-stimulated macrophages, adenosine enhanced IL-10 production by tumor necrosis factor-α–stimulated or hydrogen peroxide–stimulated human monocytes.[31]

We recently demonstrated that genetic inactivation of the A_{2A} receptor almost abolished IL-10 production in the cecal ligation and puncture model of murine polymicrobial sepsis, which suggested that A_{2A} receptor activation is required for IL-10 production after bacterial stimuli.[32] Because during sepsis the host is challenged with whole bacteria that possess multiple components capable of triggering IL-10 production via various pattern recognition receptors, the relevance of findings with limited stimuli such as LPS, tumor necrosis factor-α, or hydrogen peroxide to bacterial infections is not clear. In addition, the cellular mechanisms by which A_{2A} receptor activation regulates IL-10 production in response to clinically relevant stimuli, such as whole bacteria, are unknown. Here we show that A_{2A} receptor activation and bacteria synergistically induce IL-10 production by macrophages, a process that is critically dependent on the transcription factor C/EBPβ.

Materials and methods

Drugs and reagents

Adenosine, the selective A_1 receptor agonist CCPA (2-chloro-N^6-cyclopentyladenosine), A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethyl-carboxamidoadenosine (CGS21680), A_3 receptor agonist IB-MECA [N^6-(3-iodobenzyl)adenosine-5′-N-methyluronamide], nonselective agonist 5′-N-ethylcarboxamidoadenosine (NECA), and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO). The selective A_{2A} receptor antagonist 4-(2-[7-amino-2-(2-furyl)[1.2.4]triazolo[2.3-a][1.3.5]-triazin-5-ylamino] ethyl) phenol (ZM241385) was purchased from Tocris Cookson (Ellisville, MO). The p38 pathway inhibitor SB203580 and p42/44 pathway inhibitor PD98059 were purchased from Calbiochem (San Diego, CA). Stock solutions of the various agonists and protein kinase inhibitors were prepared using dimethylsulphoxide. Lipoteichoic acid prepared from *Staphylococcus aureus* was from Invitrogen (Carlsbad, CA).

Experimental animals and cell cultures

Male CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). A_{2A} receptor knockout (KO) mice and their wild-type (WT) littermates[32,33] and MyD88 KO and WT mice on the C57Bl/6J background,[34] kindly provided by Dr Shizuo Akira (Osaka University, Osaka, Japan) were bred in a specific pathogen-free facility using founder heterozygous male and female mice. Male TLR4 KO (C57BL/10ScNJ) and WT (C57BL/10ScSnJ) littermates mice were obtained from the Jackson Laboratory (Bar Harbor, ME). A_{2B} receptor KO mice were acquired from Deltagen Inc. (San Mateo, CA). Mice were backcrossed 10 generations onto a C57Blk/6J background. A_{2B}-null mice were generated by the insertion of a lacZNeo cassette into exon 1 of an A_{2B} receptor WT allele (Figure S1A, on the *Blood* website via the Supplemental Data link at the top of the online article, shows genotyping details). Measurement of A_{2B} receptor transcripts in large intestine and bladder revealed an absence of A_{2B} receptor transcripts in mice homozygous for the targeted A_{2B} receptor allele (Figure S1B). A normal Mendelian ratio of inheritance of the mutant allele was seen after heterozygous matings. A_{2B} receptor KO mice survive a normal lifespan with no obvious external defects and reproduce normally (data not shown). 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 New Jersey Medical School Animal Care Committee. Thioglycolate-elicited mouse peritoneal macrophages,[18] C/EBPβ-deficient macrophages, control macrophages (kind gifts from Valeria Poli, Department of Genetics, Biology, and Biochemistry, University of Turin, Turin, Italy),[35,36] and RAW 264.7 macrophages (ATCC) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 1.5 mg/mL sodium bicarbonate in humidified atmosphere of 95% air and 5% CO_2.

Preparation of heat-killed *E coli*

E coli strain K12 was grown in Luria Bertani (LB) medium overnight at 37°C with shaking at 220 rpm. Cells were centrifuged at 3000*g* for 15 minutes at 4°C and resuspended in phosphate-buffered saline at a density of 10^8/mL. Thereafter, bacteria were heat-inactivated by incubation at 65°C for 90 minutes. The killed cells were collected by centrifugation at 3000*g* for 15 minutes at 4°C and resuspended in phosphate-buffered saline at a density of 6×10^9/mL.

RNA extraction, cDNA synthesis, and real-time polymerase chain reaction

Total RNA was prepared from macrophages and reverse-transcribed, as previously described.[32] For detection of IL-10 and TRAF6 mRNA, real-time polymerase chain reaction commercial kits (Applied Biosystems, Foster City, CA) were used, and all data were normalized to constitutive rRNA values. The Applied Biosystems 7700 sequence detector was used for amplification of target mRNA, and quantitation of differences between treatment groups was calculated according to the manufacturer's instructions.

Generation of C/EBP consensus mutant IL-10 promoter luciferase construct

The mutated IL-10 promoter was prepared by gene synthesis by GenScript (Piscataway, NJ). The following nucleotides were changed in the C/EBP consensus sequences of the 5′-flanking region of the IL-10 gene between −410/−390 relative to the transcription start site: −410-TGGA*GGAAA*CAATTA-*TTT*CTC-390, The mutated construct had the following sequence: −410 TGGA*ctgtg*CAATTA*gta*CTC-390. The mutated DNA fragment was inserted into pGL2-Basic vector. The mutation sites were confirmed by DNA sequencing.

Transient transfection of RAW 264.7 cells with IL-10 promoter-luciferase constructs, C/EBP luciferase construct and A_{2A} receptor overexpressing construct, and luciferase assay

RAW 264.7 cells were transiently transfected using FUGENE 6.0 transfection reagent (Roche, Indianapolis, IN). For transfection, 0.5×10^6/mL cells were plated in a 24-well plate. The next day, the cells were transfected with 0.4 μg of IL-10 reporter plasmids (kind gifts from Stephen T. Smale, University of California, Los Angeles, School of Medicine, Los Angeles, CA), and an A_{2A} adenosine receptor overexpressing pA_{2A}–cytomegalovirus construct (Origene) or C/EBP reporter plasmid (Stratagene). All transfections were performed at 37°C overnight, after which procedure the cells were washed with Dulbecco modified Eagle medium and treated with heat-killed *E coli* and/or adenosine for 8 hours. For reporter assays, whole-cell extracts were prepared using 80 μL 1× passive lysis buffer (Promega, Madison, WI). Luciferase activity was determined from 20 μL cell extract.

Preparation of pSilencer plasmids with hairpin siRNA constructs and stable transfection

Specific hairpin siRNA (shRNA) oligonucleotide constructs for murine TRAF6 silencing were designed using the Ambion siRNA design algorithm, synthesized in the New Jersey Medical School Biotechnology Central facility, and cloned into the pSilencer3.1-H1neo expression vector (Ambion, Austin, TX), following the manufacturer's protocol. The shRNA constructs were designed targeting both the 5′ end and the

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3′ of the gene. As controls, nonspecific shRNA with the same nucleotide composition as the specific inserts, but lacking significant homology with any sequence in the murine genome database, were also prepared. RAW 264.7 cells were transfected with shRNA-containing plasmids using Superfect transfection reagent (Qiagen, Valencia, CA), following the manufacturer's protocol. G418-resistant colonies were selected after approximately 2 weeks of growth. Six single clones of each shRNA transfection were selected and grown. G418 was maintained in the medium for the growth of the clones.

Nuclear protein extraction and C/EBP electrophoretic mobility shift assay

RAW 264.7 cells were stimulated with adenosine and *E coli* for various periods and nuclear protein extracts were prepared as described previously.[37] The C/EBP consensus oligonucleotide probe used for electrophoretic mobility shift assay was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Electrophoretic mobility shift assays were performed as described previously for nuclear factor-κB[37] or CREB.[38] For supershift studies, samples were preincubated with a C/EBPβ or C/EBPδ antibody (Santa Cruz Biotechnology).

Western blot analysis of C/EBPβ, C/EBPδ, phospho-p38, and phospho-p42/44

C/EBPβ and C/EBPδ protein levels were analyzed using 10 μg of nuclear extracts using antibodies raised against C/EBPβ and C/EBPδ, respectively. Western blotting to detect p38 and p42/44 phosphorylation was performed as we have described previously[38] using rabbit anti-phospho-p42/44 and monoclonal mouse anti-phospho-p38 (both from Cell Signaling, Danvers, MA).

IL-10 enzyme-linked immunosorbent assay

Peritoneal macrophages in 96-well plates (2×10^5/mL) were treated with adenosine or various adenosine receptor agonists, followed immediately by addition of *E coli*. IL-10 levels in cell supernatants were determined by enzyme-linked immunosorbent assay.[32]

Statistical analysis

Values in the figures are expressed as mean plus or minus the SEM of the indicated number of observations. Statistical analyses of the data were performed by Student *t* test or one-way analysis of variance followed by the Dunnett test, as appropriate.

Results

A_{2A} adenosine receptor activation and *E coli* synergistically induce IL-10 release by macrophages

We have shown recently that genetic inactivation of A_{2A} receptors almost completely abolishes IL-10 production induced by a polymicrobial challenge in mice, suggesting that A_{2A} receptor activation is required for IL-10 production after bacterial stimuli.[32] To begin to examine the nature of the interaction of bacterial stimuli and A_{2A} receptor signaling in vitro, peritoneal macrophages were obtained from A_{2A} adenosine receptor KO and WT mice and treated with *E coli* and adenosine. Macrophages from A_{2A} receptor WT mice produced low levels of IL-10 after exposure to *E coli* but not adenosine (Figure 1A). Challenging *E coli*–treated WT cells with adenosine dramatically increased IL-10 levels (Figure 1A). However, neither adenosine (Figure 1A) nor *E coli* (Figure 1A) was capable of eliciting IL-10 release by A_{2A} KO mouse macrophages. Moreover, the combination of these 2 agents was also ineffective in triggering IL-10 release by A_{2A} KO macrophages (Figure 1A). We next treated macrophages with *E coli* in the presence or absence of various adenosine receptor agonists. Both the selective A_1 receptor agonist CCPA and A_3 receptor agonist IB-MECA failed to mimic the stimulatory effect of adenosine on IL-10 release. However, both the selective A_{2A} receptor agonist CGS21680 and nonselective agonist NECA increased IL-10 release by *E coli*–challenged macrophages, with CGS21680 being the most potent (Figure 1B).

We then investigated the role of A_{2B} receptors using A_{2B} receptor KO and WT mice, because previous studies have implicated A_{2B} receptors in regulating IL-10 release.[18,22] *E coli* induced the release of IL-10 by macrophages from both A_{2B} WT and KO mice to the same extent. Adenosine enhanced substantially this *E coli*–induced IL-10 release in A_{2B} WT mice, which was slightly (approximately 10%) but consistently decreased in A_{2B} KO mice (Figure 1C).

Adenosine and *E coli* synergistically upregulate IL-10 release by a partially TLR4-independent mechanism

Recent studies have illustrated that bacteria contain a number of components that are able to elicit IL-10 secretion independently of LPS and TLR4.[39] To determine whether bacterial components that are not TLR4 ligands could be involved in the synergistic upregulation of IL-10 release after combined administration of

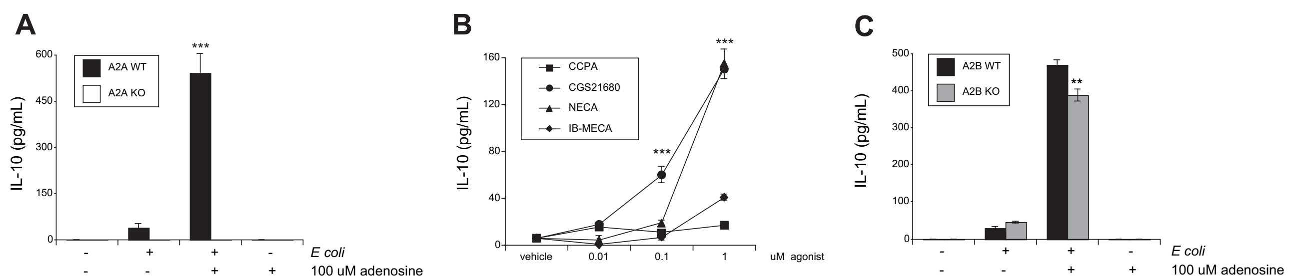

Figure 1. A_{2A} receptors are critical for IL-10 production by *E coli*–challenged macrophages. (A) *E coli* and adenosine synergize in inducing IL-10 production by A_{2A} receptor WT peritoneal macrophages. *E coli* both in the absence and presence of adenosine fails to induce IL-10 production by A_{2A} KO macrophages. A_{2A} KO and WT peritoneal macrophages were treated with heat-killed *E coli* (at a macrophage:bacterium ratio of 1:15) or cotreated with 100 μM adenosine and heat-killed *E coli* for 5 hours, and then IL-10 was measured from the supernatant using enzyme-linked immunosorbent assay. ***$P < .001$ vs. *E coli* A_{2A} WT group. (B) Effect of adenosine receptor agonists on IL-10 production. The agonists were added to macrophages before stimulating with *E coli*. IL-10 concentrations were measured from the supernatants taken 5 hours after *E coli* stimulation. ***$P < .001$ vs. vehicle. (C) *E coli* and adenosine synergize in inducing IL-10 production by A_{2B} receptor WT and, to a lesser extent, A_{2B} KO peritoneal macrophages. A_{2B} KO and WT peritoneal macrophages were treated with heat-killed *E coli* or cotreated with 100 μM adenosine and heat-killed *E coli* for 5 hours, and then IL-10 was measured from the supernatant using enzyme-linked immunosorbent assay. **$P < .01$ vs. *E coli* A_{2B} WT group. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 6 in each experiment.

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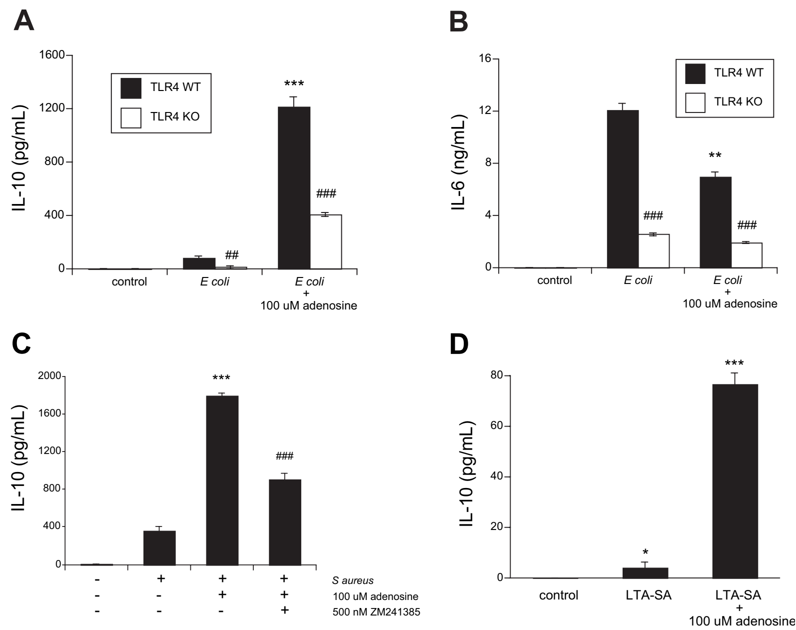


Figure 2. Effects of *E coli* and adenosine on IL-10 and IL-6 production by TLR4 WT and KO macrophages. (A) Adenosine synergizes with heat-killed *E coli* to up-regulate IL-10 production in both TLR4 WT and KO macrophages. Peritoneal macrophages were obtained from TLR4 KO and WT mice and were treated with heat-killed *E coli* (at a macrophage:bacterium ratio of 1:15) or *E coli* plus 100 μM adenosine for 5 hours, after which procedure IL-10 production was determined from the supernatant. ***$P < .001$ vs. corresponding *E coli*–treated group. ###$P < .001$ vs. corresponding *E coli*-treated groups. ##$P < .01$ vs. TLR4 WT group. (B) Effect of adenosine and *E coli* on IL-6 production. IL-6 levels were determined from the same supernatant that was used for measuring IL-10 production. ###$P < .001$ vs. corresponding TLR4 WT groups, **$P < .01$ vs. *E coli*-stimulated group. (C) *S aureus* (at a macrophage:bacterium ratio of 1:15) stimulates IL-10 levels, which is enhanced by adenosine. The enhancing effect of adenosine on *S aureus*–stimulated IL-10 production is reversed by the A_{2A} receptor antagonist ZM241385. Peritoneal macrophages were obtained from CD-1 mice. IL-10 production was measured after 5 hours of stimulation with *S aureus* and/or 100 μM adenosine. ***$P < .001$ vs. *S aureus* alone. ###$P < .001$ vs. 100 μM adenosine. (D) Lipoteichoic acid prepared from *S aureus* stimulates IL-10 release by CD-1 mouse peritoneal macrophages, which is enhanced by adenosine. Peritoneal macrophages were obtained from CD-1 mice and treated with 1 μg/mL lipoteichoic acid prepared from *S aureus* or lipoteichoic acid prepared from *S aureus* plus 100 μM adenosine for 5 hours, after which IL-10 release was determined from the supernatant. *$P < .05$ vs. control and ***$P < .001$ vs. control. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 6 in each experiment.

E coli and adenosine, we treated peritoneal macrophages obtained from TLR4 KO and WT mice with adenosine and heat-killed *E coli*. We found that *E coli* was capable of inducing low levels of IL-10 in TLR4 WT macrophages, which was decreased in TLR4 KO cells (Figure 2A). Exogenous adenosine synergistically upregulated (> 10-fold) *E coli*–induced IL-10 release in TLR4 WT cells. Although the combined exposure of adenosine and *E coli* induced lower levels of IL-10 in TLR4 KO macrophages than the same treatment in TLR4 WT cells, adenosine upregulated *E coli* induced IL-10 release to the same degree (> 10-fold) in TLR4 WT and TLR4 KO cells (Figure 2A). We next examined whether adenosine affected the release of IL-6, a pro-inflammatory cytokine, independently of TLR4. *E coli* triggered IL-6 release in TLR4 WT and to a lesser extent in TLR4 KO macrophages, and adenosine decreased this IL-6 release in both WT and KO cells approximately to the same degree (by approximately 30%; Figure 2B).

To confirm that adenosine can upregulate bacteria-induced IL-10 release independently of TLR4, we challenged peritoneal macrophages with heat-killed *S aureus*, a Gram-positive bacterium, which, unlike Gram-negative *E coli*, is thought to act in a TLR4-independent fashion to elicit macrophage activation. We observed that *S aureus* increased IL-10 levels, which were further enhanced by adenosine (Figure 2C). We then investigated the effect of adenosine on IL-10 release that was induced by the specific TLR2 agonist lipoteichoic acid prepared from *S aureus*. This TLR2 agonist elicited the release of IL-10 by macrophages, and adenosine boosted the TLR2 agonist-induced IL-10 level (Figure 2D).

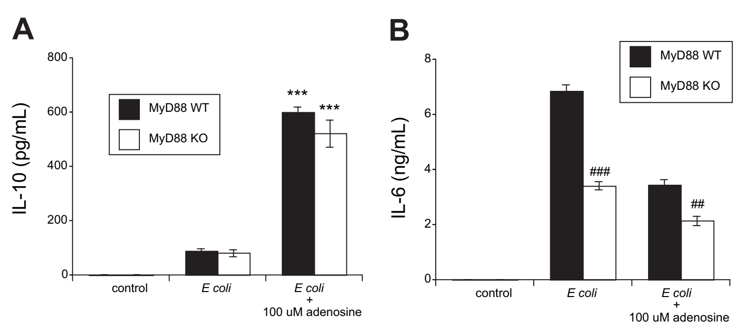
Collectively, our results demonstrate that *E coli* increases IL-10 release in macrophages, and this effect is partially dependent on TLR4. Moreover, adenosine upregulates bacteria-induced IL-10 secretion and down-regulates IL-6 release by a mechanism that does not require TLR4.

E coli–induced and adenosine-induced IL-10 release is MyD88-independent

MyD88 has been documented to contribute to intracellular signaling from all TLRs except TLR3. In addition, recent evidence indicates that TLR signaling consists of both MyD88-dependent and a MyD88-independent pathways.[40] Because of the central role of MyD88 in many bacteria-induced macrophage responses,[41,42] we first studied whether MyD88 regulated IL-10 release in macrophages challenged with *E coli,* adenosine, or the combination of *E coli* and adenosine. There was no difference in IL-10 release by peritoneal macrophages obtained from MyD88 WT and KO mice after *E coli* treatment and the IL-10 level was upregulated to the same degree in WT and KO macrophages after adenosine treatment (Figure 3A).

Previous work has shown that in the absence of MyD88 the production of IL-6 is decreased or completely abolished depending on which TLR activates IL-6 gene expression.[34,42] In addition,

Figure 3. Role of MyD88 in regulating IL-10 and IL-6 production by *E coli*–treated and adenosine-stimulated macrophages. (A) MyD88 is not necessary for the synergistic effect of adenosine and *E coli* on IL-10 production. Peritoneal macrophages were taken from MyD88 KO and WT mice. After 5 hours of stimulation with heat-killed *E coli* or *E coli* plus adenosine IL-10 enzyme-linked immunosorbent assay was performed. ***$P < .001$ vs. corresponding adenosine-untreated, *E coli*–stimulated groups. (B) MyD88 is partially required for *E coli*–induced IL-6 production. IL-6 cytokine levels were determined from the same supernatants that were used for IL-10 detection. ###$P < .001$ vs. *E coli*–treated MyD88 WT group, ##$P < .01$ vs. adenosine/*E coli*–treated MyD88 WT group. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 6 in each experiment.



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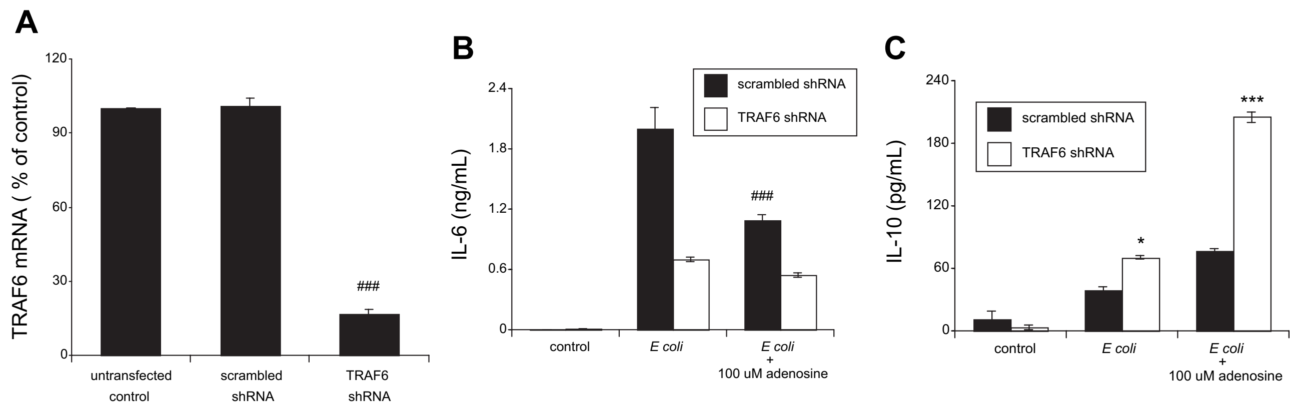

Figure 4. Effect of TRAF6 gene silencing on cytokine production by *E coli*–treated and adenosine-stimulated macrophages. (A) TRAF6 mRNA level was decreased in RAW 264.7 macrophages stably transfected with TRAF6 shRNA compared with scrambled shRNA. TRAF6 mRNA level was measured using real-time reverse-transcription polymerase chain reaction using total RNA isolated from untransfected, scrambled shRNA-transfected and TRAF6 shRNA-transfected RAW 264.7 macrophages. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 4 in each experiment. ###$P < .001$. (B) IL-6 production was decreased by TRAF6 gene silencing and the suppressive effect of adenosine is TRAF6-dependent in *E coli*-stimulated macrophages. RAW 264.7 cells were transfected with a plasmid generating TRAF6 shRNAs for silencing of TRAF6. The transfected macrophages were treated with heat-killed *E coli* or *E coli* plus 100 μM adenosine for 5 hours, and IL-6 levels were measured using enzyme-linked immunosorbent assay from the supernatants taken at the end of the incubation period. ###$P < .001$ vs. scrambled. (C) Effect of TRAF6 silencing on IL-10 production by macrophages exposed to *E coli* or *E coli* plus adenosine. IL-10 production was determined from the supernatants that were obtained from stably transfected RAW 264.7 cells after 5 hours of exposure to *E coli* or *E coli* plus adenosine. *$P < .05$ vs. scrambled. ***$P < .001$ vs. scrambled. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 4 in each experiment.

contrary to its stimulatory effect on IL-10 production, adenosine has been shown to suppress IL-6 production by TLR4-stimulated macrophages.[43] IL-6 release in *E coli*–stimulated MyD88 KO macrophages was decreased compared with WT macrophages, and adenosine decreased the IL-6 level in both MyD88 KO and WT peritoneal macrophages (Figure 3B). These results indicate that the release of the anti-inflammatory cytokine IL-10 and pro-inflammatory IL-6 were regulated differentially by adenosine, and that while IL-10 release is completely MyD88-independent, IL-6 release is partially MyD88-dependent.

TRAF6 negatively impacts the effect of *E coli* and adenosine on IL-10 release

TRAF6 has been shown to be a crucial intracellular protein for the induction of pro-inflammatory genes in macrophages in response to bacteria or TLR agonists.[44-46] Therefore, we assessed using an shRNA approach whether TRAF6 would be required for the effect of *E coli* and/or adenosine also on IL-10 release. First, we confirmed that TRAF6 shRNA–expressing cells expressed 83% less TRAF6 mRNA than cells stably transfected with a control vector expressing scrambled TRAF6 sequences (Figure 4A). Then, we treated cells with heat-killed *E coli* and/or adenosine and measured cytokine levels. In agreement with previous data,[44] IL-6 release by *E coli*–induced macrophages was decreased in TRAF6 shRNA–expressing cells compared with controls (scrambled shRNA-expressing cells; Figure 4B), indicating that IL-6 release after inflammatory stimuli requires TRAF6. Moreover, adenosine decreased IL-6 release in control cells, but did not reduce it in TRAF6 shRNA–expressing cells (Figure 4B), indicating that the suppressive effect of adenosine was TRAF6-dependent. In contrast, IL-10 levels were markedly higher in TRAF6 shRNA–expressing cells than in control cells after both *E coli* and *E coli*/adenosine treatment (Figure 4C). The fact that adenosine was less efficacious in potentiating the stimulatory effect of *E coli* on IL-10 release in RAW 264.7 cells (Figure 4) than in peritoneal macrophages (Figures 1-3) is probably a reflection of the low expression of A_{2A} receptors on RAW cells.[37] In sum, these findings indicate that TRAF6 is not required for the effect of *E coli* and adenosine in inducing IL-10, but TRAF6 negatively modulates this synergistic interaction.

The stimulatory effect of adenosine on *E coli*–induced IL-10 release is associated with increased IL-10 mRNA accumulation and promoter activity

To investigate the mechanisms that are responsible for the increased release of IL-10 by bacteria-activated macrophages both in the absence and presence of adenosine, we determined IL-10 mRNA levels from CD-1 peritoneal macrophages using real-time polymerase chain reaction. We found that *E coli* increased IL-10 mRNA levels by approximately 4-fold, and adenosine further augmented by approximately 8-fold the *E coli*–induced accumulation of IL-10 mRNA (Figure 5A). In addition, adenosine had no effect on IL-10 mRNA levels in the absence of *E coli* (Figure 5A). The transcriptional inhibitor actinomycin D prevented the synergistic effect of *E coli* and adenosine in inducing IL-10 mRNA and protein accumulation arguing for the transcriptional nature of this synergism (Figure 5A,B).

We also studied the effect of adenosine on *E coli*–induced IL-10 promoter activity by transfecting RAW 264.7 cells with a construct in which luciferase expression was driven by the full-length IL-10 promoter.[11] We found that *E coli* increased IL-10 promoter activity, and adenosine enhanced IL-10 promoter activity approximately 2-fold in *E coli*-induced but not control macrophages that were not exposed to *E coli* (Figure 5C). Because RAW 264.7 macrophages express low levels of the A_{2A} receptor endogenously,[37] we transfected these cells with an A_{2A} receptor-expressing construct (Figure 5C). This enforced expression of the A_{2A} receptor resulted in a more pronounced increase in IL-10 promoter activity both after *E coli* and the combination of *E coli* plus adenosine, underscoring the importance of A_{2A} receptors in augmenting IL-10 production.

The sequence encompassing −438 to −376 of the 5′ flanking region of the IL-10 gene is required for the stimulatory effect of adenosine on *E coli*–induced IL-10 promoter activity

To identify the DNA sequences that are necessary for adenosine to increase IL-10 promoter activity in *E coli*–challenged cells, a series

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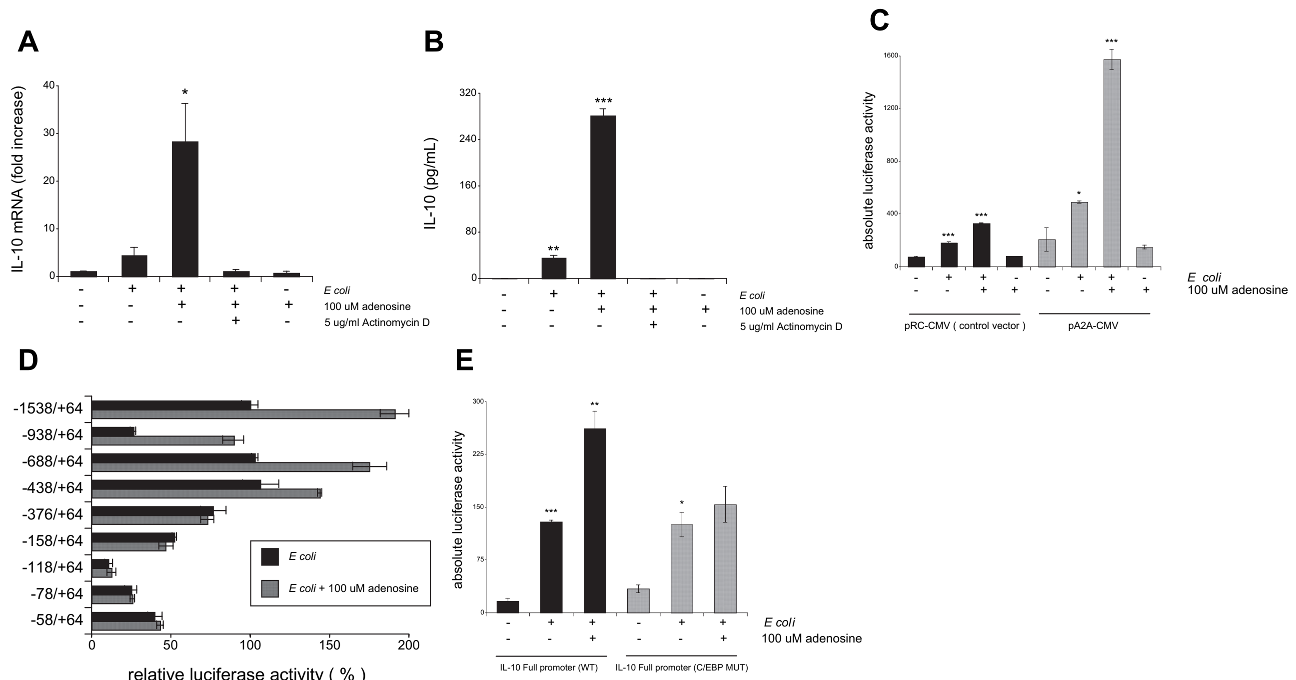

Figure 5. The stimulatory effect of adenosine on IL-10 production is transcriptional. (A) Adenosine enhances *E coli*–induced IL-10 mRNA accumulation. Peritoneal macrophages were pretreated for 2 hours with 5 μg/mL Actinomycin D before adding 100 μM adenosine and heat-killed *E coli*. IL-10 mRNA concentrations were measured by real-time polymerase chain reaction using RNA isolated 5 hours after stimulating with *E coli* and adenosine. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 4 in each experiment. *$P < .05$ vs. *E coli* alone. (B) Actinomycin D inhibits the stimulatory effect of *E coli* and adenosine on IL-10 protein release. Supernatants were harvested from these treatments and IL-10 levels were measured using enzyme-linked immunosorbent assay. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 4 in each experiment. **$P < .005$ vs. unstimulated control. ***$P < .001$ vs. *E coli* alone. (C) Adenosine up-regulates IL-10 promoter luciferase activity in RAW 264.7 macrophages exposed to *E coli*. To measure IL-10 promoter activity, cells were transiently transfected with an IL-10 promoter-luciferase reporter construct and an A_{2A} receptor-expressing (pA2A-cytomegalovirus) or control (pRC-cytomegalovirus) plasmid. Cells were treated with *E coli* in the presence or absence of adenosine for 8 hours. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 3 in each experiment. *$P < .05$ and ***$P < .001$ vs. control. (D) Sequences between −438 and −376 from the transcription start site in the IL-10 promoter are necessary for the stimulatory effect of adenosine on IL-10 promoter activity. RAW 264.7 cells were transfected with a series of IL-10 promoter deletion mutants that were inserted in the pGL2B luciferase reporter vector. Transfected cells were stimulated with *E coli* or *E coli* plus adenosine for 8 hours. Luciferase activities are expressed as the mean activity and SEM relative to the activity of the full-length promoter (−1538/ + 64; 100%) after *E coli* stimulation followed by normalization to protein concentration. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 3 in each experiment. (E) C/EBP consensus sequences in the 5′ flanking region between −410/−385 of the IL-10 promoter are crucial for the stimulatory effect of adenosine. RAW 264.7 cells were transfected with a C/EBP consensus mutant of the IL-10 promoter that was inserted in the pGL2B luciferase reporter vector. Transfected cells were treated with *E coli* in the presence or absence of adenosine for 8 hours. Luciferase activities were normalized to protein concentration. C/EBP MUT: mutant. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 3 in each experiment. *$P < .05$, **$P < .005$ vs *E coli* alone, and ***$P < .001$ vs. control.

of promoter mutants that contain successive deletions from the 5′ end were inserted upstream of the luciferase reporter gene[11] (Figure 5D). After transfection of RAW 264.7 cells with these constructs, luciferase activity was detected after adenosine/*E coli* treatment. Analysis of luciferase activity from the 5′ deletion mutants revealed that deletion of sequences between −1538 and −438 from the transcription start site did not affect the effect of adenosine in stimulating promoter activity. In contrast, the effect of adenosine was completely abolished by deletion of sequences between −438 and −376 (Figure 5D). These results show that DNA sequences in the IL-10 promoter between −438 and −376 are sufficient for the enhancing effect of adenosine on IL-10 promoter activity in *E coli*–treated macrophages. Using Searching Transcription Factor Binding Sites program we found that there were 2 potential binding sites for C/EBP (−410/−399 and −398/−385) in this promoter region.

To confirm that these 2 potential C/EBP binding sites are necessary for the stimulatory effect of adenosine on IL-10 promoter activity, we mutated these sites and this modified sequence was inserted upstream of the luciferase reporter gene into pGL2. Analysis of luciferase activity from this construct revealed that mutating the C/EBP consensus sites prevented the stimulatory effect of adenosine on *E coli*–induced promoter activity (Figure 5E).

C/EBPβ is required for the stimulatory effect of adenosine on IL-10 release by *E coli*–challenged macrophages

Because the IL-10 promoter sequence between −438 and −376 contains binding sites for C/EBP transcription factors, we first determined the effect of *E coli* as well as *E coli* plus adenosine on C/EBP transcriptional activity. To this end, RAW 264.7 macrophages were transfected with a construct in which luciferase expression is driven by C/EBP. The transfected cells were treated with adenosine and *E. coli,* and luciferase activity was measured after 8 hours of treatment. We found that *E coli* stimulated C/EBP luciferase activity by approximately 2-fold and adenosine upregulated this activity by another approximately 2-and-one-half-fold (Figure 6A). Adenosine alone had no effect on C/EBP luciferase activity (Figure 6A). We then determined the effect of *E coli* and adenosine on C/EBP DNA binding. Using nuclear extracts from *E coli*–stimulated macrophages, we observed a significant increase in protein binding to a C/EBP consensus sequence at 30 and 60 minutes compared with untreated cells, and adenosine further enhanced this induction of C/EBP DNA binding (Figure 6B). In competition binding

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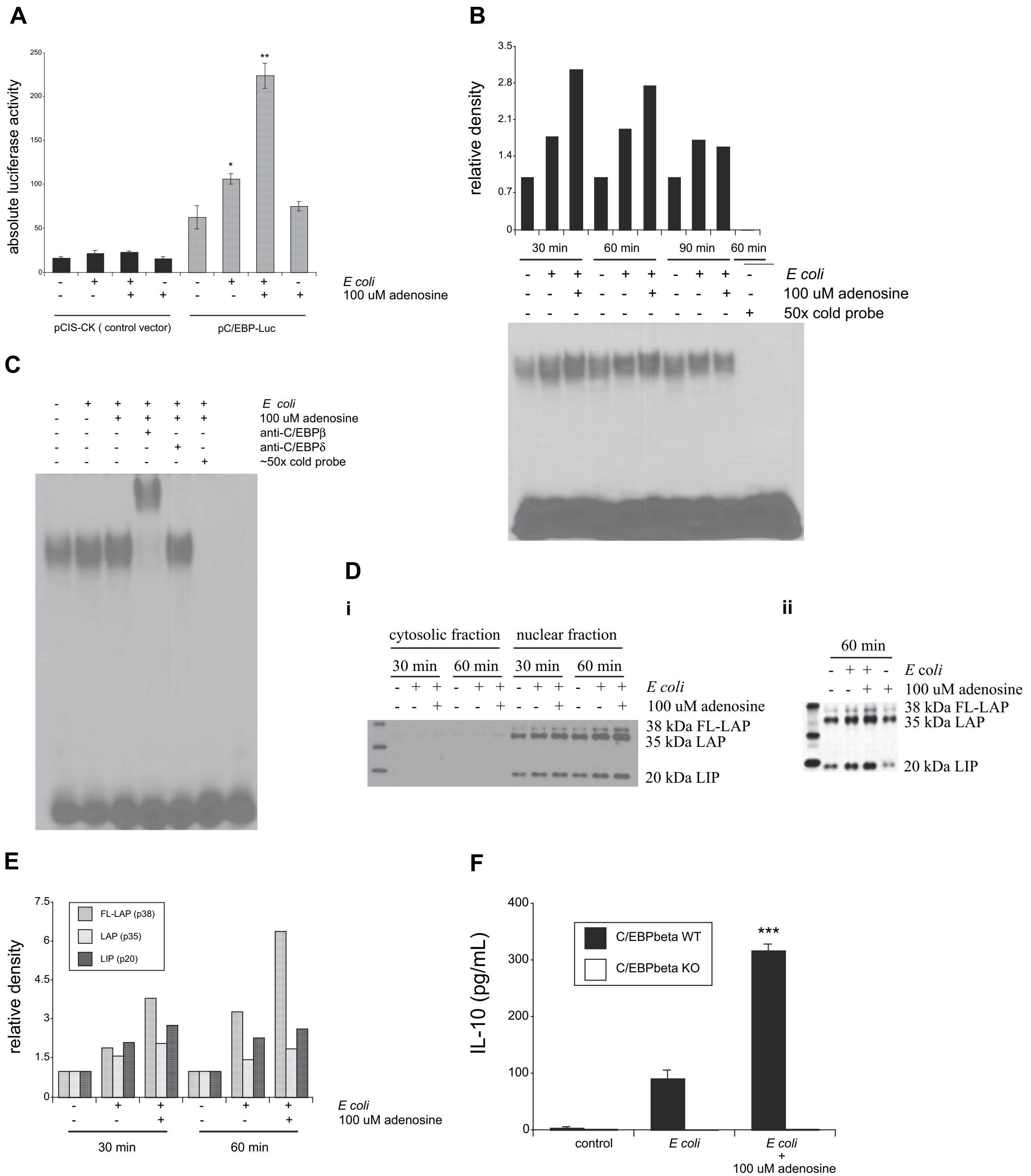

Figure 6. Adenosine enhances C/EBPβ activation in *E coli*–stimulated macrophages. (A) Adenosine up-regulates C/EBP–luciferase activity in macrophages exposed to *E coli*. RAW 264.7 macrophages were transfected with a luciferase reporter vector driven by C/EBP (pC/EBP-luc) or a control vector (pCIS-CK). Cells were treated with *E coli* and adenosine (100 μM) for 8 hours, after which period the cells were lysed and luciferase activity determined. Luciferase reporter activities were normalized to protein concentration. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 3 in each experiment. $^*P < .05$ and $^{**}P < .01$ vs. control. (B) Adenosine enhances *E coli*–induced C/EBPβ DNA binding. RAW cells were treated with heat-killed *E coli* or simultaneously with 100 μM adenosine and heat-killed *E. coli,* and nuclear proteins were extracted at 30, 60, and 90 minutes thereafter. C/EBPβ DNA binding of nuclear proteins was measured using electrophoretic mobility shift assay. The upper panel shows densitometric analysis of intensities of complexes (fold increase vs. control) observed on the gel (lower panel). (C) An antibody against C/EBPβ shifts the adenosine/*E coli*–induced (for 60 minutes) DNA–protein complex. For supershift studies nuclear extracts were preincubated with C/EBPβ and C/EBPδ antibodies before the binding reaction, and the complexes were separated by electrophoretic mobility shift assay and visualized using autoradiography. The figure shown is representative of 3 separate experiments. (Di) Adenosine increases *E coli*–induced C/EBPβ protein accumulation in the nuclear fraction of RAW 264.7 cells. Cytosolic and nuclear protein extracts were taken at 30 and 60 minutes after exposure to *E coli* or *E coli* plus adenosine (100 μM) and C/EBPβ expression was determined by Western blotting and autoradiography. (Dii)Adenosine alone fails to affect C/EBPβ protein accumulation in the nuclear fraction of RAW 264.7 cells. The figures shown are representative of 3 separate experiments. (E) Densitometric analysis of the 3 C/EBPβ isoforms detected by autoradiography on the blot shown in (Di; fold increase vs. control). The figure is representative of 3 separate experiments. (F) *E coli* both in the absence and presence of adenosine fails to increase IL-10 production by C/EBPβ-deficient macrophages. C/EBPβ WT and KO macrophages were challenged with *E coli* or *E coli* plus adenosine and IL-10 production was determined from the supernatants after a 5-hour stimulation. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 6 in each experiment. $^{***}P < .001$ vs. *E coli.*

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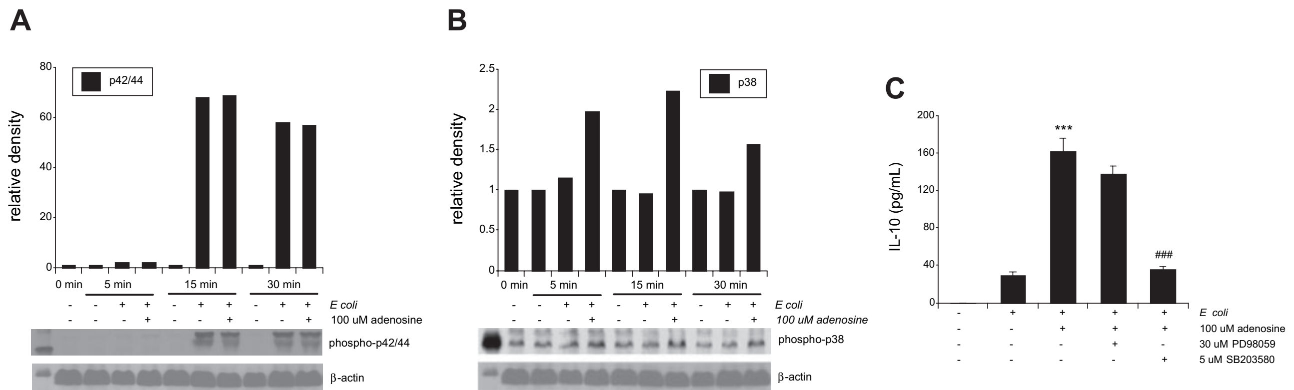

Figure 7. Effect of adenosine on MAPK activation in *E coli*-stimulated macrophages. (A) *E coli* but not adenosine increases p42/44 activation. Peritoneal macrophages were challenged with *E coli* in the presence or absence of adenosine for 5, 15, and 30 minutes. p42/44 MAPK activation was determined from cytosolic extracts taken at the end of the 5-, 15-, and 30-minute incubation periods using Western blotting with antibodies raised against the active, doubly phosphorylated form of p42/44. Bands were detected using autoradiography. Relative densities are fold increase vs. control. This figure is representative of 3 separate experiments. (B) Adenosine but not *E coli* increases p38 activation. Peritoneal macrophages were challenged with *E coli* in the presence or absence of adenosine for 5, 15 and 30 minutes. p38 MAPK activation was determined from cytosolic extracts taken at the end of the 5-, 15-, and 30-minute incubation periods using Western blotting with antibodies raised against the active, doubly phosphorylated form of p38. Bands were detected using autoradiography. Relative densities are fold increase vs. control. This figure is representative of 3 separate experiments. (C) Inhibition p38 but not p42/44 MAPK prevents the stimulatory effect of adenosine on IL-10 production in *E coli*–treated macrophages. Peritoneal macrophages were pretreated for 30 minutes with 5 μM SB203580 (p38 inhibitor) or 30 μM PD98059 (p42/44 inhibitor) before adding 100 μM adenosine and heat-killed *E coli*. After 5 hours, supernatants were harvested and IL-10 levels measured using enzyme-linked immunosorbent assay. Results (mean ± SEM) shown are representative of at least 3 experiments with n = 6 in each experiment. $^{***}P < .001$ vs. *E coli* alone. $^{\#\#\#}P < .001$ vs. 100 μM adenosine plus *E coli*.

assay, a 50-fold molar excess of cold C/EBP consensus probe completely abrogated the binding of the radiolabeled C/EBP probe (Figure 6B). The C/EBP family contains several members and within this family C/EBPβ and C/EBPδ are both expressed in macrophages.[47] Therefore, we determined the nature of the different C/EBP isoforms binding to the C/EBP consensus oligo using antibodies specific for C/EBPβ or δ in supershift studies using nuclear extracts from *E coli*/adenosine–treated RAW 264.7 macrophages. Figure 6C shows that only the C/EBPβ antibody shifted the DNA–protein complex. To obtain additional evidence for the role of C/EBP, we performed immunodetection of C/EBPβ and C/EBPδ from nuclear and cytosolic fractions of macrophages after adenosine and/or *E coli* treatment using antibodies raised against C/EBPβ and C/EBPδ. This analysis revealed that C/EBPδ did not accumulate in the nuclear fraction after either *E coli* or adenosine treatment (data not shown). However, 3 C/EBPβ isoforms corresponding to the previously described[31,48,49] liver-enriched inhibitory protein, liver-enriched activating protein and full-length liver-enriched activating protein accumulated in nuclear fractions obtained from *E coli*-stimulated macrophages (Figure 6D,E). Furthermore, adenosine enhanced the nuclear accumulation of all 3 isoforms in the presence but not absence of *E coli* (Figure 6D,E). To provide further insight into the role of C/EBPβ in regulating IL-10 production, C/EBPβ WT and KO immortalized macrophage cell lines[31] were stimulated with heat-killed *E coli* and adenosine for 5 hours, and IL-10 release was measured by enzyme-linked immunosorbent assay. We found that *E coli* or the combination of *E coli* and adenosine failed to induce IL-10 release by C/EBPβ KO cells, whereas these stimuli efficiently triggered IL-10 production by C/EBPβ WT macrophages (Figure 6F). These data identify C/EBPβ as the major transcription factor mediating the stimulatory effect of adenosine on IL-10 release in *E coli*–challenged cells.

p38 MAPK activation is required for the synergistic effect of adenosine and *E coli* on IL-10 release

Both TLR ligands and adenosine have been reported to be able to activate both p38 and p42/44 in macrophages.[38,50,51] Both p38 and p42/44 have been implicated in the regulation of IL-10 production in response to various TLR ligands.[52,53] Therefore, we tested the possibility that either p38 or p42/44 MAPK was involved in mediating the stimulatory effect of *E coli* or the combination of *E coli* and adenosine on IL-10 release. We first examined whether *E coli* or the combination of *E coli* and adenosine triggered the activation of p38 and p42/44 MAPKs. The results of these experiments showed that *E coli* increased p42/44 MAPK activation (Figure 7A) but not that of p38 (Figure 7B). When macrophages were treated with *E coli* and adenosine together, p38 activation was increased (Figure 7B), but the activation of p42/44 was not changed (Figure 7A), compared with *E coli* treatment alone. To examine whether this activation of p38 caused by adenosine contributed to the stimulatory effect of adenosine on *E coli*–induced IL-10 release, we investigated whether MAPK inhibition decreased the adenosine-stimulated IL-10 cytokine level. Treatment of peritoneal macrophages with the selective p38 MAPK pathway inhibitor SB203580 but not p42/44 MAPK inhibitor PD98059 completely abolished the IL-10 response to adenosine (Figure 7C).

Discussion

The most striking finding of this work is that endogenous adenosine acting at A_{2A} receptors represents a crucial second signal in inducing the production of IL-10. In addition, our results unequivocally demonstrate that C/EBPβ is a central intracellular mediator on which signals from both *E coli* and adenosine receptors converge to induce IL-10 gene expression.

As we and others demonstrated, the most potent anti-inflammatory effects of adenosine are mediated by A_{2A} or A_{2B} receptor stimulation on monocytes/macrophages,[18,54] lymphocytes,[55,56] neutrophils,[57,58] dendritic cells,[59] and endothelial cells.[22,60] However, the majority of these studies on the immunomodulatory effects of adenosine were confined to investigating TLR4-agonist (LPS)–induced inflammation. Ramakers et al[39] have recently shown that the modulatory effects of adenosine receptor stimulation on cytokine production critically depend on

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the specific TLR ligand used to activate the monocyte inflammatory response. Our group has recently demonstrated that genetic inactivation of A_{2A} receptors almost abolished (by $> 95\%$) cecal ligation and puncture-induced IL-10 production, which suggested that A_{2A} receptor activation by endogenous adenosine is required for IL-10 production after bacterial stimuli in vivo. By showing that *E coli* is unable to trigger IL-10 production by macrophages isolated from A_{2A} KO mice, we provide in vitro evidence for the first time that A_{2A} receptors have a crucial role in inducing IL-10 production in conjunction with a physiologic stimulus, such as *E coli*. In addition, we provide indirect proof that macrophages can serve as a source of endogenous adenosine at concentrations that are sufficient to engage A_{2A} receptors. Although macrophages have been documented to release adenosine even under resting conditions as well as in response to inflammatory stimuli, such as LPS,[61,62] the current study establishes that this endogenously released adenosine has immunomodulatory effects. In addition, the current results demonstrate that although A_{2B} receptors have a minor regulatory role in mediating the stimulatory effect of exogenous adenosine on IL-10 production, A_{2A} receptors are crucially required for the effect of both exogenous and endogenous adenosine.

Recent data have shown that IL-10 production by macrophages was induced via TLR-mediated MyD88-dependent or TRIF-dependent pathways, as well as via non-TLR signals.[63] For example, LPS induction of IL-10 via TLR4 was dependent on MyD88 and TRIF. Triggering through TLR9 by CpG showed that the induction of high levels of IL-10 was completely MyD88-dependent in macrophages. Unlike with LPS and CpG, our results clearly indicate that IL-10 production in *E coli*–stimulated or *E coli*/adenosine stimulated macrophages is completely MyD88-independent. Because of the complexity of *E coli*, it is unclear at this juncture what *E coli* components trigger IL-10 production by macrophages. It is interesting to note that a nucleotide-binding oligomerization domain 2 ligand had synergistic effect on the induction of IL-10 on costimulation with a TLR agonist,[64] indicating that nucleotide-binding oligomerization domain 2, a TLR-independent protein, may have also contributed to the effect of *E coli*. It is also worth noting that because *S aureus* also elicited IL-10 secretion (Figure 2C), ligands common to both Gram-negative and Gram-positive bacteria, such as peptidoglycan and muramyl dipeptide, may have had a role in triggering IL-10 production. However, because adenosine synergistically augmented IL-10 release induced by lipoteichoic acid prepared from *S aureus*, a true TLR2 agonist, it is also possible that the adenosine–*S aureus* interaction was mediated by the specific Gram-positive bacterial product lipoteichoic acid prepared from *S aureus*.

We have shown recently that adenosine through A_{2B} receptors enhanced LPS-induced IL-10 production, and this effect was posttranscriptional, because adenosine had no effect either on LPS-induced IL-10 mRNA accumulation or on IL-10 promoter activity.[18] In contrast, our current results indicate that the mechanism of action of adenosine in upregulating IL-10 production by *E coli*–treated macrophages is transcriptional. Specifically, adenosine increased *E coli*–induced IL-10 mRNA accumulation and upregulated IL-10 promoter activity, and the effect of adenosine was blocked by actinomycin D. Our data have clearly demonstrated that C/EBPβ is critical for the stimulatory effect of adenosine on IL-10 production in *E coli*–treated murine macrophages. First, sequential deletion analysis of the IL-10 promoter as well as site-directed mutagenesis showed that a region containing C/EBPβ binding elements was responsible for the potentiating effect of adenosine in stimulating IL-10 promoter activity. Second, adenosine stimulated *E coli*–induced nuclear accumulation and DNA binding of C/EBPβ. Third, C/EBPβ-deficient macrophages failed to produce IL-10 in response to adenosine and *E coli*. Interestingly, C/EBPβ was also important for the stimulatory effect *E coli* even in the absence of exogenous adenosine, because C/EBPβ-deficient macrophages failed to respond to *E coli* alone, and *E coli* alone induced increases in the nuclear levels and DNA binding of C/EBPβ.

From studies performed with different cell types a number of different transcriptional, translational and posttranslational mechanisms have been proposed to regulate C/EBPβ activity. The most frequently reported mechanism is the increased induction of C/EBPβ gene transcription.[65,66] Regulation by nuclear translocation[67] and alternative translation initiation,[68] phosphorylation,[69] and acetylation[49,70] have also been described. Our data suggest that C/EBPβ activity is not regulated by nuclear translocation, because C/EBPβ was undetectable in cytoplasmic fractions both before and after adenosine and/or *E coli* treatment.

The fact that adenosine alone, unlike *E coli*, was not able to induce IL-10 production, mRNA accumulation, and C/EBPβ activation suggests that adenosine and *E coli* act by different mechanisms on the C/EBPβ transcription factor system. Previous studies demonstrated that C/EBPβ promoter activity is induced during monocyte activation, and binding of CREB to C/EBPβ promoter elements is critical for the activation of C/EBPβ transcription.[71] Moreover, we have recently found that adenosine enhances CREB transcriptional activity in macrophages.[38] Thus, it is plausible that adenosine up-regulates *E coli*-induced C/EBPβ accumulation via CREB activation. This explanation is supported by our findings that adenosine stimulates both CREB activation[38] and IL-10 production (Figure 7C,D) in a p38-dependent manner. The fact that in contrast to the slow (4 hours) accumulation of C/EBPβ in response to LPS reported previously,[72] we observed a rapid (30 minutes) increase in C/EBPβ abundance after *E coli* exposure of macrophages suggests that the mechanism by which *E coli* activates C/EBPβ is fundamentally different from not only that of adenosine but also that of LPS. Because adenosine can stimulate the activation of CREB[38] but not C/EBPβ in the absence *E coli*, it is plausible that this CREB activation by exogenous or endogenous adenosine plays a permissive role in activating C/EBPβ in response to *E. coli*. Further studies are warranted to investigate the mechanisms by which *E coli* and adenosine activate the C/EBPβ system.

In summary, we propose that A_{2A} receptor activation provides a crucial permissive and synergistic signal for IL-10 production in response to infectious agents.

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Authorship

Contribution: B.C., E.A.D., E.S.V., P.G., L.V., and G.H. designed the research and analyzed data. S.J.L., M.R.B., and C.-X.S. contributed vital new reagents. B.C., Z.H.N., S.J.L., P.P., and G.H. performed research. B.C. and G.H. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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