S-Adenosylmethionine Decreases Lipopolysaccharide-Induced Phosphodiesterase 4B2 and Attenuates Tumor Necrosis Factor Expression via cAMP/Protein Kinase A Pathway^[S]

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ABSTRACT

S-Adenosylmethionine (SAM) treatment has anti-inflammatory, cytoprotective effects against endotoxin-induced organ injury. An important component of the anti-inflammatory action of SAM involves down-regulation of the lipopolysaccharide (LPS)-induced transcriptional induction of tumor necrosis factor- α (TNF) expression by monocytes/macrophages. We examined the effect of SAM on expression and activity of LPS-induced up-regulation of phosphodiesterase 4 (PDE4), which regulates cellular cAMP levels and TNF expression. LPS treatment of RAW 264.7, a mouse macrophage cell line, led to the induction of Pde4b2 mRNA expression with no effect on Pde4a or Pde4d. SAM pretreatment led to a significant decrease in LPS-induced up-regulation of Pde4b2 expression in both RAW 264.7 cells and primary human CD14⁺ monocytes. Of note, the decreased Pde4b2 mRNA expression correlated with the SAM-dependent increase in the transcriptionally repressive histone H3 lysine 9

trimethylation on the *Pde4b2* intronic promoter region. The SAM-mediated decrease in LPS-inducible Pde4b2 up-regulation resulted in an increase in cellular cAMP levels and activation of cAMP-dependent protein kinase A (PKA), which plays an inhibitory role in LPS-induced TNF production. In addition, SAM did not affect LPS-inducible inhibitor of nuclear factor- κ B degradation or nuclear factor- κ B (NF- κ B)-p65 translocation into the nucleus but rather inhibited NF- κ B transcriptional activity. These results demonstrate for the first time that inhibition of LPS-induced PDE4B2 up-regulation and increased cAMP-dependent PKA activation are significant mechanisms contributing to the anti-TNF effect of SAM. Moreover, these data also suggest that SAM may be used as an effective PDE4B inhibitor in the treatment of chronic inflammatory disorders in which TNF expression plays a significant pathogenic role.

Introduction

Lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, functions as the

prototypical endotoxin causing the systemic inflammatory response syndrome, endotoxic shock, disseminated intravascular coagulation, and multiple organ failure. LPS-induced tissue injury and lethality are caused mainly by tumor necrosis factor- α (TNF), which is produced primarily by cells of the monocyte/macrophage lineage. Increased production of inflammatory cytokines such as TNF has been shown to play a dominant pathogenic role in multiple inflammatory disorders including arthritis, heart disease, and liver injury (Young et al., 2002; Tilg et al., 2006; Meshkani and Adeli, 2009; Gonzalez-Gay et al., 2010; Schaible et al., 2010). Hence,

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ABBREVIATIONS: LPS, lipopolysaccharide; TNF, tumor necrosis factor- α ; SAM, S-adenosylmethionine; PDE, phosphodiesterase; TLR, Toll-like receptor; PKA, cAMP-dependent protein kinase A; NF- κ B, nuclear factor κ B; CREB, cAMP response element-binding protein; ELISA, enzyme-linked immunosorbent assay; H-89, *N*-[2-*p*-bromocinnamylamino]ethyl]-5-isoquinolinesulfonamide; I κ B, inhibitor of κ B; UT, untreated; ChIP, chromatin immunoprecipitation; bp, base pairs; PCR, polymerase chain reaction; H3K9, histone H3 lysine 9; H3K9Me3, histone H3 lysine trimethylation.

appropriate regulation of LPS-inducible TNF production may play an important role in the development of clinical interventions aimed to reduce the many pathological conditions linked to TNF and is a target of various immunopharmacological strategies.

Work done by our group and others has shown that *S*-adenosylmethionine (SAM) supplementation can significantly attenuate LPS-induced TNF expression in monocytes/macrophages and Kupffer cells (Watson et al., 1999; Veal et al., 2004; Song et al., 2005), and this constitutes a major component of the ability of SAM to attenuate inflammation-related organ injury (Lieber et al., 1990; Barve et al., 2006). SAM is found in all living cells, where it functions as a methyl group donor in more than 100 different reactions catalyzed by methyltransferase enzymes (Dryden, 1999). As a principal methyl donor, SAM supports trans-methylation reactions and the synthesis and modification of several key cellular components including proteins, lipids, RNA, and DNA (Mato et al., 1997). Moreover, SAM controls essential metabolic pathways by regulating several important enzymatic reactions including those involved in polyamine biosynthesis and trans-sulfuration leading to GSH synthesis. Although the mechanisms of action of SAM have not been fully elucidated, our earlier work indicates that the ability of SAM to decrease TNF production in LPS-stimulated monocytes/macrophages probably involves increased cellular cAMP (Song et al., 2005). Increasing the cellular cAMP level with different types of cAMP enhancers has invariably resulted in the suppression of TNF production either in vitro or in vivo in LPS-stimulated monocytes/macrophages of both human and murine origin (Zidek, 1999; Jin and Conti, 2002; Jin et al., 2005; Ouagued et al., 2005; Gobejishvili et al., 2008).

Intracellular levels of cAMP as well as cGMP are precisely regulated by the coordinated control of its rate of synthesis via adenylyl cyclase activity and its rate of degradation via a large family of phosphodiesterases (PDEs). There are 11 different members of the mammalian class I PDE super family, (PDE1–PDE11), which through the complexity formed by multiple genes within the families and a large number of PDE splicing variants serve to fine-tune cyclic nucleotide signals and contribute to specificity in the functional responses of cells to a variety of extracellular stimuli (Conti and Beavo, 2007; Houslay, 2010). Distinct kinetic and functional properties are attributed to PDE families with some specifically hydrolyzing cAMP (PDE4, 7, and 8), some both cAMP and GMP (PDE1, 2, 3, 10, and 11), and others only cGMP (PDE5, 6, and 9) (Conti and Beavo, 2007; Houslay, 2010). Of the cAMP-specific PDEs, the PDE4 family is widely expressed and is the current therapeutic target of selective inhibitors for the treatment of inflammatory diseases, such as asthma and chronic obstructive pulmonary disease, as well as depression and cognitive deficits (Spina, 2008; Houslay, 2010). PDE4 represents a large complex family, with four genes (PDE4A/B/C/D) encoding more than 20 distinct PDE4 isoforms as a consequence of distinct promoter usage and mRNA splicing; of note, these isoforms have similar catalytic activities but distinct cellular functions. The distinct cellular functions exist because of differences in specific intracellular targeting and signaling complex formation with various binding partners, which generate the temporal and compart-

mentalized dynamics of cAMP levels (Conti and Beavo, 2007; Houslay, 2010).

In monocytes/macrophages, LPS-mediated activation of TLR-4, a transmembrane LPS receptor, leads to downstream signaling, which plays a critical role in the production of TNF (Beutler and Rietschel, 2003). Of importance, in monocytes/macrophages that predominantly express isoforms of PDE4 A, B, and D, it has been established that PDE4B is involved in LPS-induced signaling mediated by TLR-4 and is essential for LPS-induced TNF expression (Jin and Conti, 2002; Jin et al., 2005). Several studies including ours have shown that LPS-inducible TNF production by monocytes is markedly decreased when PDE activity is blocked by PDE4-specific inhibitors (Kwak et al., 2005; Ouagued et al., 2005; Gobejishvili et al., 2008). Peritoneal and lung macrophages, as well as peripheral leukocytes from *Pde4b* knockout mice, showed significantly attenuated production of TNF in response to LPS, whereas the responses of *Pde4d* (–/–) mice were similar to those of wild-type mice (Jin and Conti, 2002). Moreover, LPS stimulation of mouse peritoneal macrophages resulted in up-regulation of only *Pde4b*, and *Pde4b* (–/–) mice were protected from LPS-induced shock (Jin et al., 2005). Hence, in the present work we examined the effects of SAM on LPS-induced PDE4B expression and activity as a potential mechanism underpinning the anti-TNF activity of SAM. The results obtained demonstrate for the first time that SAM can significantly suppress LPS-induced up-regulation of PDE4B expression/activity, increase cellular cAMP levels, and decrease LPS-induced TNF expression. Furthermore, in conjunction with increasing cellular cAMP levels, SAM increases PKA activity and decreases NF- κ B transcriptional activity, both of which play a significant role in down-regulating LPS-induced TNF expression.

Materials and Methods

Materials. The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA). LPS (*Escherichia coli* 0111:B4) was purchased from Difco (Detroit, MI). Before use, LPS was dissolved in sterile, pyrogen-free water, sonicated, and diluted with sterilized phosphate-buffered saline. Stabilized SAM salt [*S*-(5'-adenosyl)-L-methionine *p*-toluenesulfonate salt], phospho-CREB (Ser133), CREB antibodies, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin, streptomycin, Dulbecco's modified Eagle's medium, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA); the murine TNF- α ELISA kit was from BioSource International (Camarillo, CA). *N*-[2-*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) and p65 antibody were from Enzo Life Sciences (Farmingdale, NY), and β -actin antibody was from Cell Signaling Technology (Danvers, MA). I κ B- α and phospho-PKA II α reg (Ser96) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antisera specific for mouse PDE4B2 was a generous gift from Dr. Marco Conti (University of California, San Francisco, CA).

Isolation of Human CD14⁺ Monocytes from Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells from healthy volunteers were isolated by Ficoll-Paque PREMIUM (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient centrifugation. CD14⁺ monocytes were further purified by positive selection using magnetic cell sorting CD14⁺ microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) as described by the manufacturer. Purity of CD14⁺ cells was determined using a FACSCanto II flow cytometry system (BD, Franklin Lakes, NJ).

Cell Culture and Treatments. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium and CD14⁺ monocytes were cultured in RPMI 1640 medium, both containing 10% (v/v) fetal bovine serum, 10 U/ml penicillin, and 10 µg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. For RNA extraction and PDE assay, cells were plated at 2×10^6 per well in 2 ml of medium in six-well plates and treated for 2 or 4 h after LPS stimulation, respectively. For Western blot analysis and chromatin immunoprecipitation assay, cells were plated in 100-mm dishes at a density of 8×10^6 per dish in 10 ml of medium. For TNF ELISA, 24-well plates were used, in which cells were plated at 0.6×10^6 per well in 1 ml of medium and treated for 8 h with LPS. In all experiments, cells were pretreated with SAM for 16 h, and LPS was used at a concentration of 100 ng/ml.

RNA Isolation and Real-Time PCR Analysis. Total RNA was isolated from RAW 264.7 cells using TRIzol reagent (Invitrogen) and treated with DNase I to remove any contaminating genomic DNA (RQ1 RNase-Free DNase; Promega, Madison, WI). For real-time PCR, the first-strand cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD). The reverse transcription was performed using 10 ng of total RNA. The reverse transcription conditions were 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR was performed in triplicate with an ABI Prism 7500 sequence detection system and PerfeCTa SYBR Green FastMix, Low ROX reagents (Quanta Biosciences, Inc.). Mouse 18S rRNA and TNF-specific primers were purchased from Superarray Bioscience Corporation (Frederick, MD). Additional primer pairs were designed with Primer3 (<http://frodo.wi.mit.edu/primer3/>). The REFSEQ (Pruitt et al., 2005) mRNA sequences of mouse *Pde4a* (NM_019798.5, U97586) (Sullivan et al., 1998) and *Pde4d* (NM_011056.2, M94541) (Repaske et al., 1992) and rodent *Pde4b1* (AF202732) (Shepherd et al., 2003), *Pde4b2* (NM_001177980.1, L27058) (Bolger et al., 1994; Monaco et al., 1994), *Pde4b3* (NM_019840.2, U95748) (Huston et al., 1997), *Pde4b4* (AF202733) (Shepherd et al., 2003), and *Pde4b5* (NM_001177981.1, EF595686.1) (Cheung et al., 2007) were retrieved and aligned to the mouse genome with the BLAT utility (Kent, 2002) at the Genome Browser Gateway (<http://genome.ucsc.edu/>) (Kent et al., 2002). Primer pairs preferentially amplifying mRNA, not genomic DNA, of each mouse subfamily gene, *Pde4a*, *Pde4b*, and *Pde4d* were designed from the sequence regions common to all isoforms of each to measure "pan-specific" expression. In addition, primer pairs specific for *Pde4b2* and *Pde4b5* were designed with a forward primer from the unique portion of the 5' exon of each isoform. A primer pair amplifying *Pde4b1*, *Pde4b3*, and *Pde4b4* mRNAs but not *Pde4b2* or *Pde4b5* mRNA was designed with a forward primer in exon sequences common to those three mRNA forms but absent from *Pde4b2* and *Pde4b5*. Mouse oligonucleotide sequences for the primers are presented in Table 1. For mRNA quantification of human PDE4B2, a primer pair specifically amplifying human isoform PDE4B2 was similarly designed (Table 1). The forward primer is from a sequence specific to the unique coding portion of this isoform (NM_001037339.1, L20971, and M97515) (Bolger et al., 1993; McLaughlin et al., 1993).

The parameter C_t (threshold cycle) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene expression of treated versus untreated was analyzed using $2^{-\Delta\Delta C_t}$ method by normalizing with 18S gene expression in all the experiments and is presented as fold change over UT, which is set at 1.

Chromatin Immunoprecipitation Assay. The status of histone modifications in the mouse *Pde4b2* promoter region was detected using a chromatin immunoprecipitation (ChIP) assay kit (Millipore Corporation, Billerica, MA) following the protocol provided by the manufacturer. In brief, 10×10^6 cells were cross-linked with 1% formaldehyde treatment and $1 \times$ glycine neutralization. Cells were lysed in lysis buffer containing a protease inhibitor cocktail. Enzymatic digestion of DNA was performed using an EZ-Zyme Magna ChIP Kit (Millipore Corporation) to produce a one nucleosome size chromatin [100–166 base pairs (bp)]. ChIP antibodies directed either

TABLE 1

Primers for quantitative reverse transcriptase-PCR of *pde4* genes and *pde4b* isoforms

	Primer
Mouse <i>Pde4a</i> pan-specific	
Pde4a F	5'-CACAGCCTCTGTGGAGAAGTC-3'
Pde4a R	5'-GTGATACCAATCCCGTTGTC-3'
Mouse <i>Pde4b</i> pan-specific	
Pde4b F	5'-GACCGGATACAGGTTCTTCG-3'
Pde4b R	5'-CAGTGGATGGACAATGTAGTCA-3'
Mouse <i>Pde4d</i> pan-specific	
Pde4d F	5'-TGTCCACAGTCAACGCCGGGAG-3'
Pde4d R	5'-CCAAGACCTGAGCAAACGGGGTCA-3'
Mouse <i>Pde4b1</i> , 3, 4 limited isoform-specific	
Pde4b1, 3, 4 F	5'-GCCAGCTTGCAGAGTGAAG-3'
Pde4b1, 3, 4 R	5'-TAGTTCCTCCAGCGTCTCCA-3'
Mouse <i>Pde4b2</i> isoform-specific	
Pde4b2 F	5'-AGGTGGAGGAGACTCGGCTA-3'
Pde4b2 R	5'-CGGTTCAGCATCCTTTTGA-3'
Mouse <i>Pde4b5</i> isoform-specific	
Pde4b5 F	5'-AGAGCTTCCAAAATGCCTGA-3'
Pde4b5 R	5'-GGATTCCACATCGTTCTGC-3'
Human <i>PDE4B2</i> isoform-specific	
PDE4B2 F	5'-GCGGTGACTCTGCTATGGAC-3'
PDE4B2 R	5'-CCTCCAGCGTTTCCATGCTAA-3'

F, forward; R, reverse.

against trimethyl-histone H3 (Lys9) (Millipore Corporation) or an unspecific control rabbit IgG (Cell Signaling Technology) were used for immunoprecipitation. The lysates were incubated overnight with the respective antibody followed by immunoprecipitation of antibody-protein complexes with magnetic beads and one wash each with low-salt, high-salt, LiCl and Tris-EDTA buffers. After reverse cross-linking and purification, pure DNA was eluted in 1% SDS and 0.1 M NaHCO₃. ChIP-quantitative PCR was performed using the ABI Prism 7500 Sequence Detection System. The four primer pairs tested (Supplemental Table 1) spanned regions –692 to –585, –265 to –146, +3 to +122, and +283 to +451 with respect to the transcription start site of REFSEQ NM_001177980.1.

ChIP-quantitative PCR results were calculated by the $\Delta\Delta C_t$ method, where the C_t value of each ChIP DNA fraction was normalized to the input DNA fraction C_t value using

$$\Delta C_t[\text{normalized ChIP}] = C_t[\text{ChIP}] - (C_t[\text{Input}]$$

$$- \log_2(\text{input dilution factor}))$$

The data are presented as fold difference between the treated sample (S2) and the untreated sample (S1) calculated as fold change = $2^{-\Delta\Delta C_t[S2-S1]}$, where $\Delta\Delta C_t[S2-S1] = \Delta C_t[S2: \text{normalized ChIP}] - \Delta C_t[S1: \text{normalized ChIP}]$.

Cytoplasmic and Nuclear Protein Extraction. Cells were washed twice with phosphate-buffered saline and lysed by hypotonic shock in cytoplasmic extraction buffer (10 mM HEPES, pH 7.8, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, protease inhibitor cocktail, and phosphatase inhibitors). The cells were collected by scraping and the nuclei were separated from the cytosolic proteins by low-speed centrifugation at 1500g for 5 min at 4°C. The supernatant (cytoplasmic extract) was stored at –70°C. The nuclear pellet was resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.8, 520 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.2% Nonidet P-40, protease inhibitor cocktail, and phosphatase inhibitors) and incubated on ice for 60 min. The samples were centrifuged at 100,000g for 15 min at 4°C, and the supernatant containing nuclear proteins was stored in small aliquots at –70°C until further use. The protein concentration in extracts was measured using Bio-Rad Dye Reagent (Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer's protocol.

Western Blot Analysis. Proteins (30–50 µg) were analyzed by SDS-polyacrylamide gel electrophoresis using a Bio-Rad Laborato-

ries electrophoresis system, followed by immunoblotting according to the manufacturer's instructions. Immunoreactive bands were visualized using enhanced chemiluminescence light detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Detection of β -actin served as a loading control. Quantification was performed with Scion Image analysis software (Scion Corporation, Frederick, MD).

PDE4 Enzymatic Assay. PDE4-specific enzymatic activity was determined using a PDE4 assay kit (FabGennix International Inc., Frisco, TX) as described previously (Gobejishvili et al., 2008). In brief, cells were lysed after treatment using SolOBuffer (FabGennix International Inc.), and 25 μ g of protein was used per assay. Assays were performed in duplicate; the final concentration of cAMP and rolipram was 2 and 10 μ M, respectively. PDE4 activity was estimated from the difference between total and rolipram-resistant PDE activity. Linearity of the enzyme activity was assessed over the range from 500 pg to 5 ng of cAMP hydrolyzed/min/mg protein, achieved by adding 2 to 7 μ g of partially purified PDE4A5. Rolipram at 100 μ M inhibited 95 to 98% of the PDE4 activity of partially purified PDE4 enzymes.

Plasmids and Transfections. An NF- κ B-luciferase reporter construct was obtained from Clontech (Mountain View, CA). RAW 264.7 cells were transfected with NF- κ B-luciferase reporter plasmids using Lipofectamine LTX reagent (Invitrogen). Cells were seeded in 24-well plates for 24 h before transfection. For transfection, 0.3 μ g of total DNA was used per well. Cells were cotransfected with a β -galactosidase expression vector as a control. After transfection, cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C for 24 h. After 24 h, the transfected cells were treated with SAM (0.75 mM) overnight and further stimulated with LPS (100 ng/ml for 6 h). Total cell lysates were then used for the luciferase assays.

Luciferase and β -Galactosidase Assay. Luciferase activity was measured using Luciferase Assay Reagent (Promega). After treatments, total cell lysates were prepared in reporter lysis buffer (Promega). Luciferase enzymatic activity was measured in an Orion L Microplate Luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) using a specific substrate provided by Promega. β -Galactosidase activity was measured using a β -Galactosidase Enzyme Assay System (Promega) and was used to normalize the measurement of luciferase expression in all subsequent experiments.

Statistical Analysis. All experiments were repeated at least three times. Data are presented as mean $\pm$ S.D. for the indicated number of independently performed experiments. Student's *t* test and linear mixed-effects models were used for the determination of statistical significance. $p < 0.05$ was considered significant. To analyze the effect of SAM supplementation on LPS-induced TNF and PDE4B2 mRNA expression, we fit a linear mixed-effects model with

SAM dosage as the fixed effect and experiment number as the random effect. The model specification is then

$$y_{ij} = \alpha + u_{0i} + (\beta + u_{1i})x_{\text{SAM}} + \varepsilon_{ij}$$

where y_{ij} is the relative level of mRNA expression for experiment *i* and replication *j*, measured relative to LPS-induced expression without any SAM suppression. The α term represents the intercept and the β term the slope for the effect of level of SAM suppression (given by x_{SAM}) on mRNA expression. The u_{0i} and u_{1i} terms represent random effects for the intercept and slope associated with experiment *i*, respectively, with $u_{0i} \sim N(0, \sigma_{u_0}^2)$ and $u_{1i} \sim N(0, \sigma_{u_1}^2)$. Finally, $\varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2)$ is the residual error term associated with the *j*th replication within the *i*th experiment.

Results

SAM Pretreatment Decreases LPS-Induced TNF Expression in a Dose-Dependent Manner. The effect of SAM supplementation on TNF mRNA expression in RAW 264.7 cells was determined by pretreating cells with different doses of SAM (0.25–1 mM) for 16 h and further stimulating them with LPS (100 ng/ml) for 2 h. After treatment, total RNA was extracted and analyzed for TNF mRNA expression by real-time PCR. As expected, LPS strongly induced TNF (Fig. 1A). A linear mixed model fitted to the $2^{-\Delta\Delta C_t}$ TNF expression values from the two experiments showed significant differential expression between LPS-induced and untreated cells ($p < 0.001$) and between LPS-induced and 0.5 ($p = 0.03$), 0.75 ($p < 0.001$), and 1.0 ($p < 0.001$) mM SAM-pretreated cells (Supplemental Table 2). To better visualize the effect of SAM pretreatment on LPS-induced TNF expression, expression values were presented as percentages relative to LPS-induced expression without any SAM suppression. A linear mixed-effects model (see *Materials and Methods*) was fitted to the relative expression values, with fitted regression curves plotted in Fig. 1B, which showed that SAM pretreatment significantly attenuated LPS-induced TNF expression in a dose-dependent manner. The estimated slope for the effect of SAM pretreatment on relative expression of LPS-induced TNF expression was -64.71 (95% confidence interval -46.66 to -82.75), suggesting an $\sim 50\%$ decrease in LPS-induced TNF mRNA per 0.77 mM SAM. In most of the subsequent experiments SAM was used at a concentration of 0.75 mM.

Fig. 1. Effect of SAM pretreatment on LPS-induced TNF expression in RAW cells. A, TNF mRNA in UT and LPS-treated cells 2 h after LPS stimulation was quantified using real-time PCR. ***, $p < 0.001$ compared with UT. B, cells were pretreated with increasing concentrations of SAM (0.25–1 mM) overnight (16 h) and stimulated with LPS for 2 h before RNA extraction. TNF mRNA was quantified using real-time PCR. Expression values are presented as percentages relative to LPS-induced expression (which was set to 100%) without SAM treatment. Each experiment is plotted with a different symbol, and the fitted regression line is shown.

TABLE 2
Effect of SAM on PDE4 activity in RAW 264.7 cells

Treatments	PDE4 Activity		
	Experiment 1	Experiment 2	Experiment 3
	<i>pmol cAMP hydrolyzed/min/mg protein</i> (% relative to UT)		
UT	13.76 (100)	10.49 (100)	9.14 (100)
LPS (100 ng/ml)	24.11 (175.2)	16.13 (153.8)	17.88 (195.6)
SAM (0.75 mM)	9.38 (68.2)	8.81 (84)	5.31 (58.1)
SAM (0.75 mM) + LPS	17.92 (130.3)	12.87 (122.7)	10.02 (109.6)

SAM Pretreatment Attenuates PDE4 Activity. Suppression of LPS-induced TNF expression in macrophages can be achieved by inhibition of PDE4, which is critical for LPS signaling and TNF expression (Jin and Conti, 2002; Jin et al., 2005). Hence, we examined whether the SAM effect on TNF was a result of decreased PDE4 activity. Cells were pretreated with 0.75 mM SAM for 16 h followed by stimulation with LPS (100 ng/ml) for 4 h. Cellular extracts were assayed for PDE4-specific activity. The time point for PDE4 activity was selected on the basis of previously established expression optima (Gobejishvili et al., 2008). As expected, LPS stimulation led to the induction of PDE4 activity. Of note, SAM pretreatment markedly attenuated both LPS-induced up-regulation and basal PDE4 activity (Table 2).

Effect of SAM on LPS-Inducible PDE4 Isoforms and cAMP Levels in Murine Macrophages. Previous studies in primary mouse macrophages have shown that PDE4B is the predominant LPS-inducible PDE (Jin and Conti, 2002; Jin et al., 2005). To assess whether the same PDE4 subfamily is induced in RAW 264.7 macrophages upon LPS stimulation, cells were treated with LPS for 2 h, total RNA was extracted, and *Pde4a*, *Pde4b*, and *Pde4d* mRNAs were quantified by real-time PCR. In agreement with earlier observations, we found that only *Pde4b* was induced by LPS (Fig. 2A). Subsequently we screened for the expression of isoforms *Pde4b1* to *Pde4b5* in response to LPS and found that *Pde4b2* was the predominant LPS-induced *Pde4b* isoform (Fig. 2B). No expression was detected for *Pde4b* isoforms 1, 3, or 4 and LPS-inducible *Pde4b5* mRNA was <1% of the *Pde4b2* level.

We then examined the effect of SAM on LPS-induced expression of PDE4B2 mRNA and protein. PDE4B2 mRNA and protein were quantified in cells pretreated with SAM and stimulated with LPS. In agreement with its effects on the LPS-induced PDE4 activity, SAM decreased the up-regula-

tion of both PDE4B2 mRNA and protein expression (Fig. 3, A and B) and resulted in a significant increase in LPS-induced intracellular cAMP levels (Fig. 3C). Significant differences in *Pde4b2* mRNA were found between LPS-induced and untreated cells ($p < 0.001$) and between LPS-induced cells and cells pretreated with SAM at 0.25 mM ($p = 0.03$), 0.5 mM ($p = 0.001$), 0.75 mM ($p < 0.001$), and 1.0 mM ($p < 0.001$) (linear mixed model) (Supplemental Table 3). Figure 3A plots the fitted regression curves from the linear mixed-effects model, for expression values relative to LPS-induced expression. The estimated slope for the effect of SAM pretreatment on relative expression of LPS-induced *Pde4b2* expression was -37.43 (95% confidence interval -22.24 to -52.62) (Supplemental Table 3).

Effect of SAM on LPS-Induced Up-Regulation of TNF and PDE4B2 in Human CD14⁺ Monocytes. The effect of SAM on LPS-induced TNF and PDE4B2 expression was also examined in primary human monocytes. CD14⁺ monocytes were purified from human peripheral blood mononuclear cells as described under *Materials and Methods* and treated with different concentrations of SAM and subsequent stimulation with LPS. SAM was able to significantly blunt LPS induction of TNF mRNA (Fig. 4A; Supplemental Table 4), PDE4B2 mRNA (Fig. 4B; Supplemental Table 4), and PDE4 activity (Table 3). The effect of SAM pretreatment on LPS-induced up-regulation of TNF and PDE4B2 mRNA expression was even more marked in CD14⁺ cells compared with that in RAW cells, with levels of 0.25 mM resulting in >50% reduction of mRNA (Fig. 4, A and B).

Effect of SAM on the Methylation Status of Histone H3 Associated with the *Pde4b2* Promoter Region. Histone lysine methylation is an important posttranslational modification that specifies the transcriptional state of a gene (Kouzarides, 2002). In relation to the inhibition of gene transcription, a distinct link between histone H3 lysine 9 (H3K9) trimethylation (H3K9Me3) and transcriptional repression has been established (Kouzarides, 2002; Hublitz et al., 2009). Furthermore, relevant to the present work, it has been demonstrated that the promoter H3K9 trimethylation causing transcriptional repression occurs in a SAM-dependent manner (Wang et al., 2003). Hence, we examined the effect of SAM treatment on the H3K9 trimethylation of the mouse *Pde4b2* intronic promoter region as a potential mechanism underlying its repressive effect on the *Pde4b2* mRNA expression. The mouse *Pde4b2* promoter location was defined by

Fig. 2. LPS induces only the *Pde4b2* isoform in RAW cells. Cells were stimulated with LPS for 2 h before RNA extraction. mRNA was quantified using real-time PCR and is represented as fold change over UT as described under *Materials and Methods*. A, induction of *Pde4a*, *Pde4b*, and *Pde4d* mRNA in response to LPS. B, induction of *Pde4b2* mRNA in response to LPS. Data are presented as mean $\pm$ S.D. ($n = 3$). ***, $p < 0.001$ compared with UT.

Fig. 3. Effect of SAM on LPS-induced Pde4b2 up-regulation and cAMP levels. A, cells were pretreated with SAM (0.25–0.75 mM) and stimulated with LPS for 2 h before RNA extraction. Pde4b2 mRNA was quantified using real-time PCR. Expression values are presented as percentages relative to LPS-induced expression (which was set to 100%) without SAM treatment. Each experiment is plotted with a different symbol, and the fitted regression line is shown. B, cells were pretreated with SAM (0.25–0.75 mM) and stimulated with LPS for 4 h. PDE4B2 protein levels were analyzed by Western blotting (a representative Western blot is shown). β -Actin was used as a loading control. C, cells were pretreated with SAM (0.75 mM) and stimulated with LPS. After 3 h, cells were collected and lysed, and intracellular cAMP levels were measured. Data are presented as mean $\pm$ S.D. **, $p < 0.01$ compared with LPS.

Fig. 4. Effect of SAM on LPS-induced TNF and PDE4B2 up-regulation in human CD14⁺ cells. Purified human CD14⁺ cells were pretreated with the indicated concentrations of SAM and stimulated with LPS (100 ng/ml) for 3 h. TNF (A) and Pde4b2 (B) mRNA expression was quantified using real-time PCR and are represented as fold change over UT as described under *Materials and Methods*. Data are presented as mean $\pm$ S.D. ***, $p < 0.001$ (mixed-effects model), LPS alone compared with SAM pretreated.

TABLE 3
Effect of SAM on PDE4 activity in human CD14⁺ cells

Treatments	PDE4 Activity	
	Experiment 1	Experiment 2
	pmol cAMP hydrolyzed / min / mg protein (% relative to UT)	
UT	5.78 (100)	5.96 (100)
LPS, 100 ng/ml	7.23 (125)	7.81 (131.2)
SAM (0.25 mM) + LPS	4.51 (78)	5.86 (98.4)
SAM (0.5 mM) + LPS	3.46 (60)	4.95 (83.1)
SAM (0.25 mM)	N.D.	4.78 (80.2)
SAM (0.5 mM)	4.23 (73.2)	N.D.

N.D., not done.

sequence similarity to the rat *Pde4b2* promoter (Monaco et al., 1994) and the 5' terminus of the mouse REFSEQ mRNA NM_001177980.1. The effect of SAM treatment on H3K9 methylation at the *Pde4b2* promoter was examined by ChIP

analysis using an H3K9Me3-specific antibody and the promoter region primer pairs shown in Fig. 5A. Regions I and II interrogated for H3K9Me3 status are located within –700 bp upstream of the transcriptional start site (TSS), whereas regions III and IV are located within +400 bp downstream of the TSS in the 5' untranslated region. In comparison with the SAM untreated cells, a distinct increase in the trimethylation of H3K9 in all four regions of the *Pde4b2* intronic promoter was observed in SAM-treated cells, under both basal and LPS-stimulated conditions (Fig. 5B). Of note, the greatest increases in the SAM-mediated H3K9Me3 levels were observed in region III, which overlaps the transcriptional start site. Commensurate with the LPS-inducible transcriptional activation of the *Pde4b2* gene, in SAM untreated cells, there was a gradual decrease in H3K9Me3 levels at 30 and 60 min upon LPS stimulation. In comparison, in SAM-treated cells, the H3K9Me3 levels remained unchanged at 30 min

Fig. 5. A, locations of PCR primer pairs for analysis of epigenetic modifications in the vicinity of the mouse *Pde4b2* promoter. The coordinate locations shown are with respect to the transcription start site in REFSEQ NM_001177980.1. The functionality of this region as a promoter in mouse is supported by representation in GenBank of more than 20 mRNAs and spliced expressed sequence tags with 5'-terminal sequences located downstream of the indicated TSS. These include cDNAs originating from bone marrow, brain, and visual cortex (<http://genome.ucsc.edu/>). B, SAM (S) pretreatment resulted in increased histone H3K9 trimethylation in the *Pde4b2* promoter. Chromatin was immunoprecipitated with anti-H3K9Me3 and ChIP-quantitative PCR was performed. Differences are expressed as fold change between treated and untreated samples as described under *Materials and Methods*.

and showed a decline only at 60 min after LPS stimulation. It should be noted that, because the basal H3K9Me3 levels were higher in SAM-treated cells, the net levels of H3K9Me3 after LPS stimulation remained significantly higher than those in SAM-untreated cells. These data strongly suggest that the increase in SAM-dependent histone H3K9 trimethylation associated with the *Pde4b2* intronic promoter region leads to the transcriptional repression and consequent decrease in the LPS-inducible *Pde4b2* mRNA expression.

Role of PKA on SAM-Mediated Decrease in LPS-Induced TNF Expression. Because PDE4 inhibition and the consequent increase in cAMP levels leads to activation of cAMP-dependent PKA, we examined the effect of SAM supplementation on PKA activation. Cells pretreated with SAM were stimulated with 100 ng/ml LPS, and cytoplasmic and nuclear lysates were obtained for Western blot analysis. SAM pretreatment resulted in an increase in basal as well as an LPS-induced (30 min after LPS) PKA activation as shown by the enhanced phosphorylation of PKA type II- α regulatory subunit at Ser96 (Fig. 6A). Furthermore, the increased PKA activation by SAM led to an enhanced and sustained CREB phosphorylation at serine 133 (Fig. 6B). The role of increased activation of PKA by SAM in attenuation of LPS-induced TNF expression was further examined using the specific PKA inhibitor H-89. LPS-induced TNF protein and TNF mRNA expression were quantified in cells pretreated with SAM in the presence and absence of H-89. H-89 treatment reversed the effect of SAM on TNF expression at both mRNA (Fig. 6C) and protein levels (Fig. 6D).

SAM Does Not Alter LPS-Induced I κ B α Degradation and NF- κ B Nuclear Translocation. Under unstimulated conditions, cytoplasmic inhibitory proteins, collectively referred to as I κ B, bind to NF- κ B and sequester it in the cytoplasm. A variety of stimuli including LPS can initiate signaling that leads to the degradation of specific I κ B proteins, resulting in the release and nuclear translocation of NF- κ B proteins (Karin and Ben-Neriah, 2000). To examine the effect of SAM on I κ B α degradation, Western blot analysis

was performed with cytoplasmic extracts obtained from cells stimulated with LPS with and without SAM pretreatment. As expected, I κ B α degradation was observed at 15 and 30 min in cells treated with LPS (Fig. 7A, lanes 2 and 3), and this effect was not altered by SAM pretreatment (Fig. 7A, compare lanes 2, 3 and 6, 7). After degradation, I κ B α returned to the basal level in 60 min in both SAM-treated and untreated cells (Fig. 7A, lanes 4 and 8). These data show that SAM pretreatment does not affect LPS-induced I κ B α proteolysis. The effect of SAM on LPS-induced NF- κ B activation, as indicated by the nuclear translocation of p65, was also examined. Nuclear proteins extracted from cells pretreated with 0.75 mM SAM followed by stimulation with LPS (100 ng/ml) for 30 min were examined by Western blot analysis. SAM pretreatment did not alter the nuclear levels of LPS-induced p65, demonstrating that there was no effect on NF- κ B activation (Fig. 7B).

SAM Down-Regulates LPS-Stimulated NF- κ B Transcriptional Activity. Because LPS-induced NF- κ B activation was not altered, the effect of SAM on LPS-stimulated NF- κ B transcriptional activity was examined. Cells were transiently transfected with a reporter construct carrying a luciferase gene under the control of an NF- κ B promoter containing three tandem repeats of the κ B sequence (κ B-luc). LPS stimulation for 6 h increased NF- κ B-dependent transcription approximately 4-fold. Of note, in contrast to its effect on NF- κ B activation, SAM pretreatment significantly attenuated LPS-stimulated NF- κ B activity (Fig. 8). These results are in agreement with our earlier findings (Gobejishvili et al., 2006, 2008), showing that increased cellular cAMP levels achieved by PDE4 inhibition have no effect on LPS-inducible NF- κ B activation but can significantly decrease transcriptional stimulation by NF- κ B.

Discussion

An important characteristic of SAM is its ability to reduce the production of the inflammatory cytokine TNF, which

Fig. 6. Inhibition of PKA reverses the SAM effect on LPS-induced TNF expression. A, cells were pretreated with SAM (0.75 mM) and stimulated with LPS for 30 min. Cytoplasmic lysates were analyzed for phospho-PKA (pPKA, S96) and (B) nuclear phospho-CREB (pCREB, S133) levels by Western blotting (a representative Western blot is shown). β-Actin and total CREB were used as loading controls. C, cells were pretreated with SAM (0.75 mM) in the presence and absence of PKA inhibitor H-89 (10 μM) and stimulated with LPS for 2 h before RNA extraction. TNF mRNA was quantified using real-time PCR and is represented as fold change over UT. Data are presented as mean ± S.D. (*n* = 3). *, *p* < 0.05 compared with LPS; #, *p* < 0.05 compared with S+LPS. D, cells were pretreated with SAM (0.75 mM) in the presence and absence of PKA inhibitor H-89 (10 μM) and stimulated with LPS for 8 h. Cell-free supernatants were assayed for TNF using an ELISA kit. Data are presented as mean ± S.D. (*n* = 3). *, *p* < 0.05 compared with LPS; #, *p* < 0.05 compared with S+LPS. S, SAM.

Fig. 7. SAM does not affect LPS-induced IkBα degradation and p65 translocation into nucleus. A, RAW cells were treated with SAM (0.75 mM) and stimulated with LPS for the indicated treatments and times. Cytoplasmic extracts were prepared, and Western blot analysis was performed by using anti-IkBα antibody. β-Actin was used as a loading control (a representative Western blot is shown). B, cells pretreated with SAM were stimulated with LPS for 30 min. Nuclear lysates were collected, and p65 levels were analyzed by Western blotting (a representative Western blot is shown); β-actin was used as a loading control.

plays a critical pathogenic role in multiple clinical disorders mediated by inflammation. Work from our research group and others has demonstrated that exogenous SAM supplementation can attenuate endotoxin-stimulated TNF produc-

tion both in vitro and in vivo and can also attenuate inflammation-related organ injury in vivo (Lieber et al., 1990; Watson et al., 1999; Song et al., 2005; Barve et al., 2006). In particular, SAM supplementation has been shown to signif-

Fig. 8. Down-regulation of LPS-induced NF- κ B transcriptional activity by SAM (S). Cells were transfected with a luciferase reporter construct containing the NF- κ B-responsive I κ B promoter and a β -galactosidase control plasmid as described under *Materials and Methods*. The transfected cells pretreated with SAM (0.75 mM) were stimulated with LPS for 6 h. Cytoplasmic extracts were prepared and assayed for luciferase activity. A β -galactosidase enzyme assay was performed on the same lysates to normalize for transfection efficiency. Data are presented as mean $\pm$ S.D. ($n = 3$). **, $p < 0.01$ compared with UT; ***, $p < 0.001$ compared with LPS-stimulated.

icantly attenuate LPS-induced TNF expression in monocytes/macrophages and Kupffer cells (Veal et al., 2004). Our previous research showed that SAM leads to an increase in cellular cAMP levels in monocytes, which correlates with the decrease in LPS-inducible TNF expression (Song et al., 2005). In addition, we have shown that decreased cellular cAMP levels caused by increased PDE4B expression play a causal role in LPS-induced TNF expression in ethanol-mediated priming of monocytes/macrophages (Gobejishvili et al., 2006, 2008). On the basis of these observations, it was important to examine the impact of SAM on the LPS-inducible PDE4 expression and activity and resultant cellular cAMP levels, as a potential mechanism for its anti-inflammatory effect.

As observed previously, SAM supplementation resulted in a dose-dependent decrease in LPS-induced TNF expression (Figs. 1B and 4A). Examination of the potential involvement of cellular cAMP levels and PDE4B expression as a mechanism of SAM anti-inflammatory function showed that SAM effectively decreased LPS-induced up-regulation of PDE4B expression; in particular, SAM decreased PDE4B2 mRNA and protein expression and increased cellular cAMP levels (Figs. 3 and 4B). SAM is the principal biological methyl donor and mediates the methylation of histones and DNA, which can have a tremendous affect on the epigenetic regulation of regional and global gene activities. Histone methylation plays a distinctive role in the regulation of both transcriptional activation and repression of genes (Kouzarides, 2002). In the context of transcriptional repression and site-specific methylation events, a causal link between promoter-associated H3K9Me3 and transcriptional repression has been established (Hublitz et al., 2009). Of note, this H3K9 trimethylation and transcriptional repression occurs via specific histone methyl transferases in a SAM-dependent manner (Wang et al., 2003). Hence, alterations in the transcriptionally repressive histone methylation modifications on the *Pde4b2* intronic promoter region were examined as a poten-

tial mechanism underlying SAM-mediated suppression of *Pde4b2* mRNA expression (Fig. 5). The data obtained in Fig. 5 strongly suggest that SAM treatment could be affecting H3K9-specific histone methyl transferases, causing the observed increase in the promoter-associated H3K9 trimethylation and leading to the suppression of LPS-induced up-regulation of PDE4B2 mRNA expression.

The observed anti-inflammatory mechanism of SAM is supported by findings that demonstrated the critical role of PDE4B2 in LPS-induced TNF expression in mouse macrophages (Jin et al., 2005). In addition, these observations are in agreement with studies demonstrating that decreasing PDE4 activity with specific inhibitors causes a significant down-regulation in LPS-induced TNF expression and an inflammatory response in vitro and in vivo (Kwak et al., 2005; Ouagued et al., 2005; Hertz et al., 2009). Taken together, these observations strongly suggest that inhibition of LPS-induced up-regulation of PDE4B2 plays a major role in the ability of SAM to attenuate LPS-induced TNF production and related organ injury.

Suppression of TNF production mediated by inhibition of PDE4B is cAMP-dependent and requires PKA (Jin et al., 2005). Work examining the role of PKA-anchoring proteins has further emphasized the critical role of PKA in the anti-inflammatory effects of cAMP and down-regulation of LPS-induced TNF expression (Wall et al., 2009). We show here that SAM-mediated inhibition of LPS-induced up-regulation of PDE4B2 led to an increase in cellular cAMP and activation of PKA (Figs. 3C and 6, A and B). The role for PKA in the SAM-mediated decrease in LPS-inducible TNF expression was further supported by the observation that PKA inhibition reverses the SAM-inhibitory effect on TNF expression (Fig. 6, C and D). These data strongly suggest that cAMP-dependent PKA activation plays a significant role in attenuating LPS-induced TNF expression and the anti-inflammatory mechanism of SAM. Although increased cellular cAMP levels and downstream signaling decrease LPS-inducible TNF expression, it is significant to note that work done by Beavo's group (Hertz et al., 2009) shows that during differentiation of monocytes/macrophages, in the absence of LPS, enhanced cellular cAMP levels caused by agonists can lead to a large increase in the expression of several proinflammatory chemokines.

Numerous studies have demonstrated the critical role of NF- κ B in the LPS-inducible transcriptional induction of the *TNF* gene (Haas et al., 1990; Müller et al., 1993; Barnes and Karin, 1997). LPS mediates its effects on NF- κ B nuclear translocation by the targeted phosphorylation and subsequent degradation of I κ B α , which results in the release of p65 from the I κ B-p65 complex and its translocation into the nucleus, DNA binding, and transactivation of target genes such as TNF. Examination of the effect of SAM on LPS-induced I κ B α proteolysis and NF- κ B nuclear translocation showed that none of these events is affected by SAM (Fig. 7). Furthermore, in agreement with previous work (Veal et al., 2004), we showed that SAM inhibited NF- κ B transcriptional activity, as demonstrated by the suppression of the LPS-inducible NF- κ B promoter reporter activity (Fig. 8). Taken together, these data suggest that SAM does not affect LPS-inducible TLR-4-mediated signaling events involved in NF- κ B activation and nuclear translocation but rather

influences the nuclear events leading to NF- κ B-dependent transcription.

It is likely that the SAM-mediated increase in cellular cAMP caused by inhibition of LPS-induced up-regulation of PDE4B2 contributes to its anti-NF- κ B effects. Our earlier work along with several other observations shows that cAMP represses NF- κ B-mediated transcription and TNF mRNA expression without affecting NF- κ B activation/DNA binding activity (Ollivier et al., 1996; Newman et al., 1998; Shames et al., 2001; Gobejishvili et al., 2006, 2008). Work examining the anti-TNF effects of cAMP showed that LPS-induced *c-fos* is stabilized by cAMP, leading to the accumulation of the c-Fos protein, which directly interacts with p65 and impedes its recruitment to the *TNF* promoter and decreases transcription (Koga et al., 2009). The possibility that LPS/cAMP-mediated effects on *c-fos* play a role in decreasing TNF expression in SAM-treated cells is currently being investigated.

The results of this study demonstrate for the first time that the anti-inflammatory mechanism of SAM can be attributed primarily to its ability to inhibit LPS-induced PDE4B2 activity and expression, increase cellular cAMP levels, and modulate PKA and NF- κ B activity. Interest in inhibition of PDE4 activity as a potential therapeutic approach has been increasing in the last decade because of its significant role in regulating inflammatory responses (Spina, 2008). Indeed, PDE4 inhibitors have been demonstrated to possess both immunomodulatory and anti-inflammatory actions; however, despite reports of the efficacy of PDE4 inhibitors, their therapeutic application is hampered by the presence of significant dose-limiting side effects, including the gastrointestinal side effects of nausea, vomiting, and diarrhea and the development of vascular injury (Spina, 2008). In contrast, SAM has been used in several clinical studies and has a very favorable safety profile, comparable to that of placebo (Bottiglieri, 2002). Hence, the present findings showing that SAM effectively decreases LPS-induced PDE4 gene expression and activity and TNF production, along with its safe tolerability profile, strongly support its therapeutic potential in the treatment of inflammatory disorders.

Authorship Contributions

Participated in research design: Gobejishvili, McClain, and Barve.
Conducted experiments: Gobejishvili, Avila, Barker, Ghare, and Kirpich.

Contributed new reagents or analytic tools: Barker and Henderson.
Performed data analysis: Gobejishvili, Barker, and Brock.

Wrote or contributed to the writing of the manuscript: Gobejishvili, Barker, Brock, Joshi-Barve, Mokshagundam, McClain, and Barve.

References

Barnes PJ and Karin M (1997) Nuclear factor- κ B: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* **336**:1066–1071.
 Barve S, Joshi-Barve S, Song Z, Hill D, Hote P, Deaciu I, and McClain C (2006) Interactions of cytokines, S-adenosylmethionine, and S-adenosylhomocysteine in alcohol-induced liver disease and immune suppression. *J Gastroenterol Hepatol* **21**:S38–S42.
 Beutler B and Rietschel ET (2003) Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol* **3**:169–176.
 Bolger G, Michaeli T, Martins T, St John T, Steiner B, Rodgers L, Riggs M, Wigler M, and Ferguson K (1993) A family of human phosphodiesterases homologous to the dunce learning and memory gene product of *Drosophila melanogaster* are potential targets for antidepressant drugs. *Mol Cell Biol* **13**:6558–6571.
 Bolger GB, Rodgers L, and Riggs M (1994) Differential CNS expression of alternative mRNA isoforms of the mammalian genes encoding cAMP-specific phosphodiesterases. *Gene* **149**:237–244.
 Bottiglieri T (2002) S-Adenosyl-L-Methionine (SAME): from the bench to the bedside—molecular basis of a pleiotropic molecule. *Am J Clin Nutr* **76**:S1151–S1157.
 Cheung YF, Kan Z, Garrett-Engle P, Gall I, Murdoch H, Baillie GS, Camargo LM,

Johnson JM, Houslay MD, and Castle JC (2007) PDE4B5, a novel, super-short, brain-specific cAMP phosphodiesterase-4 variant whose isoform-specifying N-terminal region is identical to that of cAMP phosphodiesterase-4D6 (PDE4D6). *J Pharmacol Exp Ther* **322**:600–609.
 Conti M and Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* **76**:481–511.
 Dryden DTF (1999) Bacterial DNA methyltransferases. In *S-Adenosylmethionine-Dependent Methyltransferases: Structures and Functions* (Cheng X and Blumen-thal RM eds) pp 283–340, World Scientific Publishing, River Edge, NJ.
 Gobejishvili L, Barve S, Joshi-Barve S, and McClain C (2008) Enhanced PDE4B expression augments LPS-inducible TNF expression in ethanol-primed monocytes: relevance to alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol* **295**:G718–G724.
 Gobejishvili L, Barve S, Joshi-Barve S, Uriarte S, Song Z, and McClain C (2006) Chronic ethanol-mediated decrease in cAMP primes macrophages to enhanced LPS-inducible NF- κ B activity and TNF expression: relevance to alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol* **291**:G681–G688.
 Gonzalez-Gay MA, Gonzalez-Juanatey C, Vazquez-Rodriguez TR, Miranda-Filloo JA, and Llorca J (2010) Insulin resistance in rheumatoid arthritis: the impact of the anti-TNF- α therapy. *Ann NY Acad Sci* **1193**:153–159.
 Haas JG, Baeuerle PA, Riethmüller G, and Ziegler-Heitbrock HW (1990) Molecular mechanisms in down-regulation of tumor necrosis factor expression. *Proc Natl Acad Sci USA* **87**:9563–9567.
 Hertz AL, Bender AT, Smith KC, Gilchrist M, Amieux PS, Aderem A, and Beavo JA (2009) Elevated cyclic AMP and PDE4 inhibition induce chemokine expression in human monocyte-derived macrophages. *Proc Natl Acad Sci USA* **106**:21978–21983.
 Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* **35**:91–100.
 Hublitz P, Albert M, and Peters AH (2009) Mechanisms of transcriptional repression by histone lysine methylation. *Int J Dev Biol* **53**:335–354.
 Huston E, Lumb S, Russell A, Catterall C, Ross AH, Steele MR, Bolger GB, Perry MJ, Owens RJ, and Houslay MD (1997) Molecular cloning and transient expression in COS7 cells of a novel human PDE4B cAMP-specific phosphodiesterase, *HSPDE4B3*. *Biochem J* **328**:549–558.
 Jin SL and Conti M (2002) Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF- α responses. *Proc Natl Acad Sci USA* **99**:7628–7633.
 Jin SL, Lan L, Zoudilova M, and Conti M (2005) Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. *J Immunol* **175**:1523–1531.
 Karin M and Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: the control of NF- κ B activity. *Annu Rev Immunol* **18**:621–663.
 Kent WJ (2002) BLAT—the BLAST-like alignment tool. *Genome Res* **12**:656–664.
 Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, and Haussler D (2002) The human genome browser at UCSC. *Genome Res* **12**:996–1006.
 Koga K, Takaesu G, Yoshida R, Nakaya M, Kobayashi T, Kinjo I, and Yoshimura A (2009) Cyclic adenosine monophosphate suppresses the transcription of proinflammatory cytokines via the phosphorylated c-Fos protein. *Immunity* **30**:372–383.
 Kouzarides T (2002) Histone methylation in transcriptional control. *Curr Opin Genet Dev* **12**:198–209.
 Kwak HJ, Song JS, No ZS, Song JH, Yang SD, and Cheon HG (2005) The inhibitory effects of roflumilast on lipopolysaccharide-induced nitric oxide production in RAW264.7 cells are mediated by heme oxygenase-1 and its product carbon monoxide. *Inflamm Res* **54**:508–513.
 Lieber CS, Casini A, DeCarli LM, Kim CI, Lowe N, Sasaki R, and Leo MA (1990) S-Adenosyl-L-methionine attenuates alcohol-induced liver injury in the baboon. *Hepatology* **11**:165–172.
 Mato JM, Alvarez L, Ortiz P, and Pajares MA (1997) S-Adenosylmethionine synthesis: molecular mechanisms and clinical implications. *Pharmacol Ther* **73**:265–280.
 McLaughlin MM, Cieslinski LB, Burman M, Torphy TJ, and Livi GP (1993) A low- K_m , rolipram-sensitive, cAMP-specific phosphodiesterase from human brain. Cloning and expression of cDNA, biochemical characterization of recombinant protein, and tissue distribution of mRNA. *J Biol Chem* **268**:6470–6476.
 Meshkani R and Adeli K (2009) Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. *Clin Biochem* **42**:1331–1346.
 Monaco L, Vicini E, and Conti M (1994) Structure of two rat genes coding for closely related rolipram-sensitive cAMP phosphodiesterases. Multiple mRNA variants originate from alternative splicing and multiple start sites. *J Biol Chem* **269**:347–357.
 Müller JM, Ziegler-Heitbrock HW, and Baeuerle PA (1993) Nuclear factor κ B, a mediator of lipopolysaccharide effects. *Immunobiology* **187**:233–256.
 Newman WH, Zhang LM, Lee DH, Dalton LM, Warejcka DJ, Castresana MR, and Leeper-Woodford SK (1998) Release of tumor necrosis factor- α from coronary smooth muscle: activation of NF- κ B and inhibition by elevated cyclic AMP. *J Surg Res* **80**:129–135.
 Ollivier V, Parry GC, Cobb RR, de Prost D, and Mackman N (1996) Elevated cyclic AMP inhibits NF- κ B-mediated transcription in human monocytic cells and endothelial cells. *J Biol Chem* **271**:20828–20835.
 Ouagued M, Martin-Chouly CA, Brinchault G, Lepointier-Comoy C, Depincé A, Bertrand C, Lagente V, Belleguic C, and Pruniaux MP (2005) The novel phosphodiesterase 4 inhibitor, CI-1044, inhibits LPS-induced TNF- α production in whole blood from COPD patients. *Pulm Pharmacol Ther* **18**:49–54.
 Pruitt KD, Tatusova T, and Maglott DR (2005) NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* **33** (Database issue): D501–D594.
 Repaske DR, Swinnen JV, Jin SL, Van Wyk JJ, and Conti M (1992) A polymerase chain reaction strategy to identify and clone cyclic nucleotide phosphodiesterase

- cDNAs. Molecular cloning of the cDNA encoding the 63-kDa calmodulin-dependent phosphodiesterase *J Biol Chem* **267**:18683–18688.
- Schaible HG, von Banchet GS, Boettger MK, Bräuer R, Gajda M, Richter F, Hensellek S, Brenn D, and Natura G (2010) The role of proinflammatory cytokines in the generation and maintenance of joint pain. *Ann NY Acad Sci* **1193**:60–69.
- Shames BD, McIntyre RC Jr, Bensard DD, Pulido EJ, Selzman CH, Reznikov LL, Harken AH, and Meng X (2001) Suppression of tumor necrosis factor α production by cAMP in human monocytes: dissociation with mRNA level and independent of interleukin-10. *J Surg Res* **99**:187–193.
- Shepherd M, McSorley T, Olsen AE, Johnston LA, Thomson NC, Baillie GS, Houslay MD, and Bolger GB (2003) Molecular cloning and subcellular distribution of the novel PDE4B4 cAMP-specific phosphodiesterase isoform. *Biochem J* **370** (Pt 2): 429–438.
- Song Z, Uriarte S, Sahoo R, Chen T, Barve S, Hill D, and McClain C (2005) S-Adenosylmethionine (SAME) modulates interleukin-10 and interleukin-6, but not TNF, production via the adenosine (A2) receptor. *Biochim Biophys Acta* **1743**: 205–213.
- Spina D (2008) PDE4 inhibitors: current status. *Br J Pharmacol* **155**:308–315.
- Sullivan M, Rena G, Begg F, Gordon L, Olsen AS, and Houslay MD (1998) Identification and characterization of the human homologue of the short PDE4A cAMP-specific phosphodiesterase RD1 (PDE4A1) by analysis of the human HSPDE4A gene locus located at chromosome 19p13.2. *Biochem J* **333** (Pt 3):693–703.
- Tilg H, Kaser A, and Moschen AR (2006) How to modulate inflammatory cytokines in liver diseases. *Liver Int* **26**:1029–1039.
- Veal N, Hsieh CL, Xiong S, Mato JM, Lu S, and Tsukamoto H (2004) Inhibition of lipopolysaccharide-stimulated TNF- α promoter activity by S-adenosylmethionine and 5'-methylthioadenosine. *Am J Physiol Gastrointest Liver Physiol* **287**:G352–G362.
- Wall EA, Zavzavadjian JR, Chang MS, Randhawa B, Zhu X, Hsueh RC, Liu J, Driver A, Bao XR, Sternweis PC, et al. (2009) Suppression of LPS-inducible TNF- α production in macrophages by cAMP is mediated by PKA-AKAP95–p105. *Sci Signal* **2**:ra28.
- Wang H, An W, Cao R, Xia L, Erdjument-Bromage H, Chatton B, Tempst P, Roeder RG, and Zhang Y (2003) mAM facilitates conversion by ESET of dimethyl to trimethyl lysine 9 of histone H3 to cause transcriptional repression. *Mol Cell* **12**:475–487.
- Watson WH, Zhao Y, and Chawla RK (1999) S-Adenosylmethionine attenuates the lipopolysaccharide-induced expression of the gene for tumour necrosis factor α . *Biochem J* **342**:21–25.
- Young JL, Libby P, and Schönbeck U (2002) Cytokines in the pathogenesis of atherosclerosis. *Thromb Haemostasis* **88**:554–567.
- Zidek Z (1999) Adenosine - cyclic AMP pathways and cytokine expression. *Eur Cytokine Netw* **10**:319–328.

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