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Inducible Cyclooxygenase Released Prostaglandin E₂ Modulates the Severity of Infection Caused by *Streptococcus pyogenes*

Oliver Goldmann,* Erika Hertzén,† Alexander Hecht,* Heike Schmidt,* Sabine Lehne,* Anna Norrby-Teglund,‡ and Eva Medina*

*Streptococcus pyogenes* is a significant human pathogen that can cause life-threatening invasive infections. Understanding the mechanism of disease is crucial to the development of more effective therapies. In this report, we explored the role of PGE₂, an arachidonic acid metabolite, and its rate-limiting enzyme cyclooxygenase 2 (COX-2) in the pathogenesis of severe *S. pyogenes* infections. We found that the COX-2 expression levels in tissue biopsies from *S. pyogenes*-infected patients, as well as in tissue of experimentally infected mice, strongly correlated with the severity of infection. This harmful effect was attributed to PGE₂-mediated suppression of the bactericidal activity of macrophages through interaction with the G₂-coupled E prostanoid receptor.

The suppressive effect of PGE₂ was associated with enhanced intracellular cAMP production and was mimicked by the cAMP-mediating suppression of the bactericidal activity of macrophages through interaction with the G₂-coupled E prostanoid receptor. The inhibitory effect exerted by PKA in the generation of antimicrobial oxygen radical species seems to be the ultimate effector mechanism responsible for the PGE₂-mediated downregulation of the macrophage bactericidal activity. Importantly, either genetic ablation of COX-2, pharmacological inhibition of COX-2 or treatment with the G₂-coupled E prostanoid antagonist, AH6809, significantly improved the disease outcome in *S. pyogenes* infected mice. Therefore, the results of this study open up new perspectives on potential molecular pathways that are prone to pharmacological manipulation during severe streptococcal infections. *The Journal of Immunology*, 2010, 185: 2372–2381.

*S. pyogenes* is a significant human pathogen that normally causes mild infections of the skin and throat, but it can also cause severe invasive diseases, such as necrotizing fasciitis and toxic shock (1). The morbidity and mortality associated with severe *S. pyogenes* infections emphasize the urgency in understanding the disease mechanisms to develop safe and more effective therapies. In this regard, the identification of cellular functions and molecular pathways that influence the ability of the host to mount an appropriate immune response to *S. pyogenes* and that are prone to pharmacological manipulation will facilitate the development of more efficient therapeutic approaches.

Recently, Abdeltawab and coworkers (2) have shown that the genes involved in the synthesis of the lipid inflammatory mediator PGE₂ were strongly upregulated upon infection in mouse strains that were susceptible to *S. pyogenes*. In contrast, these genes were unchanged or exhibited slightly decreased expression levels in resistant mouse strains after bacterial inoculation (2). Although these observations imply a potential contribution of PGE₂ to the severity and outcome of *S. pyogenes* infection, no studies have addressed this issue to date.

PGE₂, an arachidonic acid metabolite produced by various types of cells, participates in a wide range of biological responses, including the modulation of inflammation and immune responses (3). Thus, PGE₂ can suppress cytokine and chemokine production by activated macrophages (4, 5) and influence Th1/Th2 differentiation of T cells (6). PGE₂ can also exert multiple effects on dendritic cells, including promoting survival (7), enhancing maturation and migration (8), and regulating cytokine production (9). In contrast, overproduction of PGs has been shown to have damaging consequences to the host (10, 11).

PGE₂ synthesis starts with the liberation of arachidonic acid from cell membranes, and then the rate-limiting enzyme cyclooxygenase (COX) converts the arachidonic acid into PGs (12). Two different isoforms of COX have been identified: COX-1 and COX-2. Although COX-1 is constitutively expressed in most tissues and thought to be involved in regulation of physiological functions, COX-2 is mainly an inducible enzyme that is expressed by a variety of cells upon stimulation with bacterial products, such as LPS and lipoteiochoic acid, growth factors, or cytokines (12, 13). The COX-2 isoform has been reported to have a significant impact in the outcome of several infections (14–17). The purpose of this study was to establish the influence of PGE₂ and its rate-limiting enzyme COX-2 in the host response to *S. pyogenes*.

**Materials and Methods**

*Bacteria*

*S. pyogenes* A20 (M23) strain is a human isolate obtained from the German Culture Collection (DSM 2071), other streptococcal strains used in
the study are *S. pyogenes* S448 (M1), *S. pyogenes* A60 (M3), *S. pyogenes* NZ131 (M49), and *S. pyogenes* A115 (M18). Stocks were maintained at –70°C and were routinely cultured at 37°C in Todd-Hewitt broth (Oxoid, Basingstoke, U.K.), supplemented with 1% yeast extract. Bacteria were collected in midlog-phase, washed twice with sterile PBS, diluted to the required inoculum, and the number of viable bacteria determined by counting CFU after diluting and plating in blood agar plates (Life Technologies, Paisley, U.K.) containing 5% sheep blood.

**Specific reagents**

PGE₂, AH-6809, AH23848, butaprost free acid, and sulprostone were purchased from Cayman Chemicals (Ann Arbor, MI). Forskolin and rolipram were obtained from Calbiochem (San Diego, CA). NS-398, PKI₁₄–₂₂, and (5)-p-8-(4-chlorophenylthio) asenosine-3’, 5’-cAMP were purchased from Sigma-Aldrich (St. Louis, MO). Stock solution of PGE₂ was prepared by dissolving the compound in ethanol. Stock solution of NS-398, AH23848, AH6809, butaprost, sulprostone, forskolin, and rolipram were prepared by dissolving the compounds in DMSO. PHI₁₄–₂₂ and (5)-p-8-(4-chlorophenylthio) asenosine-3’, 5’-cAMP were dissolved in PBS. Further dilutions of the stock solutions into PBS were made prior to their use in experiments. Similar dilutions were prepared for only the vehicle solution for the treatment of control groups.

**Human biopsies**

Snap-frozen tissue biopsies collected from the epicentre of infection from three patients (one patient with cellulitis and two with necrotizing fasciitis plus toxic shock syndrome) infected with *S. pyogenes* serotype M1T1. Biopsies were cryosectioned, fixed, and mounted as previously described (18). The study was conducted in accordance with the Declaration of Helsinki, and ethical approval to obtain the biopsies was granted by the human subjects review committee at the University of Toronto (Toronto, Ontario, Canada). Staining for COX-2 was achieved by incubation with a polyclonal rabbit Ab from Sigma-Aldrich. *S. pyogenes* was stained using a polyclonal rabbit antiserum specific for the Lancefield group A carbohydrate (diluted 1:10,000; Difco, Detroit, MI). Biotinylated secondary Abs included goat anti-rabbit IgG (diluted 1:500; Vector Laboratories, Burlingame, CA). Preimun rabbit sera at appropriate dilutions were used to control for nonspecific staining reactions. The immunostaining were evaluated in a RXM Leica Microscope (Leica, Wetzlar, Germany) with a 40 x/0.55 NA objective lens. The whole section of each biopsy was analyzed using acquired computerized image analysis (ACIA), yielding an analyzed cell area defined by the blue H&E counterstaining. The results are presented as ACIA values, which equal percentage positively stained area × mean intensity of positive staining.

**Mice**

C3H/HeN female mice were purchased from Harlan-Winkelmann (Borchen, Germany). COX-2 mice and corresponding COX+/+ littermates were used in this study (19). Mice were inoculated with 10⁵ CFU of *S. pyogenes* serotype M1T1. Biopsies were then incubated with DAB (peroxidase substrate), counterstained with haemalum and then examined microscopically using an Olympus BX51 microscope (Olympus Europe, Hamburg, Germany).

**COX-2 staining of histological sections**

Livers were removed from *S. pyogenes*-infected mice at 24 h of infection, fixed in 3.7% formalin solution, embedded in paraffin, and cut into 5-μm thick sections. Tissue sections were deparaffinated with Xylol and ethanol (incubation for 2 min in every dilution of ethanol; 100%, 96%, 90%, 70%, and 50%) and the quenching of endogen peroxidase was performed by washing with 0.3% H₂O₂. Sections were then incubated with DAB (diaminobenzidin substrate), counterstained with haemalum and then examined microscopically using an Olympus BX51 microscope (Olympus Europe, Hamburg, Germany).

**In vitro infection of BM-derived macrophages**

Bone marrow (BM) was flushed out of the femur and tibia of mice using ice-cold complete cell culture medium consisting of RPMI 1640 medium containing 10 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin and M-CSF (Sigma-Aldrich) in a concentration of 50 ng/ml and cultured for 7 d at 37°C, 5% CO₂. At day 7, differentiated macrophages were harvested, counted, adjusted to 10⁶ cells/ml, and used for further experiments.

**Western blot analysis**

BM macrophages were infected with *S. pyogenes* at a multiplicity of infection (MOI) of 10:1 (10 bacteria per macrophage) and incubated for 1 h at 37°C, 5%CO₂. Macrophages were then washed with sterile PBS, resuspended in complete medium containing 100 μg/ml gentamicin to kill noningested bacteria, and further incubated for 2 h at 37°C, 5% CO₂. Macrophages were then disrupted with dH₂O to release intracellular bacteria (this was considered time 0 h relative to gentamicin treatment) or 4 h later (this was considered time 4 h relative to gentamicin treatment) and the amount of viable intracellular bacteria was determined by plating on blood agar. In some experiments, macrophages were preincubated with PGE₂ (5–50 nM) 5 min prior to infection.

In some experiments, infections were performed in the presence of AH23848 (10 μM), AH6809 (10 μM) butaprost (0.1–10 μM), sulprostone (0.1–10 μM), forskolin (1–100 μM), rolipram (1–100 μM), PKI₁₄–₂₂ (5 nM and 50 nM), and (5)-p-8-(4-chlorophenylthio) asenosine-3’, 5’-cAMP (0.2 mM and 2 mM).

**RNA extraction and cDNA synthesis**

Total RNA was isolated from murine liver tissue or from cultured macrophages after homogenization in RLT buffer by using an RNeasy Midi kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. cDNA synthesis was performed using a Life Technologies RT-PCR kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions.

**RT-PCR**

The cDNA was PCR amplified using the following oligonucleotides: for COX-2, 5'-ACACTCTATCTACTGACCTC-3' and 5'-GAAGGGACACC-CTTCAGCAAF-3' (584 bp); for COX-1, 5'-CCCTGATACCCAGC- GGT-3' and 5'-ACCTGTTGGTACCGCAT-3' (494 bp); for rasp-9, 5'- CTGGAGCCAGGCAAGATGACG-3' and 5'-TGACGTTGGCCGT-GAGCAAC-3' (143 bp). The PCR mixture contained 10% PCR buffer, dNTPs (25 μM each), 5 U/μl solution of Taq DNA polymerase, primers, and cDNA samples. The following PCR conditions were used: 30 cycles of 5 min at 94°C, 15 s at 94°C, 30 s at 58°C, 30 s at 72°C, and 5 min at 72°C. PCR products were separated on 1.5% agarose gels.

**Differentiation of bone marrow-derived macrophages**

Bone marrow (BM) was flushed out of the femur and tibia of mice using ice-cold complete cell culture medium consisting of RPMI 1640 medium containing 10 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin and M-CSF (Sigma-Aldrich) in a concentration of 50 ng/ml and cultured for 7 d at 37°C, 5% CO₂. At day 7, differentiated macrophages were harvested, counted, adjusted to 10⁶ cells/ml, and used for further experiments.

**In vitro infection of BM-derived macrophages**

BM-derived macrophages were infected with *S. pyogenes* at a multiplicity of infection (MOI) of 10:1 (10 bacteria per macrophage) and incubated for 1 h at 37°C, 5%CO₂. Macrophages were then washed with sterile PBS, resuspended in complete medium containing 100 μg/ml gentamicin to kill noningested bacteria, and further incubated for 2 h at 37°C, 5% CO₂. Macrophages were then disrupted with dH₂O to release intracellular bacteria (this was considered time 0 h relative to gentamicin treatment) or 4 h later (this was considered time 4 h relative to gentamicin treatment) and the amount of viable intracellular bacteria was determined by plating on blood agar. In some experiments, macrophages were preincubated with PGE₂ (5–50 nM) 5 min prior to infection.

In some experiments, infections were performed in the presence of AH23848 (10 μM), AH6809 (10 μM), butaprost (0.1–10 μM), sulprostone (0.1–10 μM), forskolin (1–100 μM), rolipram (1–100 μM), PKI₁₄–₂₂ (5 nM and 50 nM), and (5)-p-8-(4-chlorophenylthio) asenosine-3’, 5’-cAMP (0.2 mM and 2 mM).
**Immunofluorescence analysis**

BM macrophages were seeded on glass coverslips and infected with FITC-labeled *S. pyogenes* at an MOI of 10:1 or PBS for uninfected control cells and incubated for 2 h at 37°C, 5% CO₂. After washing twice with PBS, macrophages were fixed with 3.5% formaldehyde and incubated overnight at 4°C. Cells were then washed, permeabilized with 200 μl 0.1% Triton X-100 for 5 min at room temperature, and blocked with PBS and 10% FCS. Macrophages were incubated for 45 min at room temperature with the first Ab, rabbit anti–COX-2 diluted 1:100 in PBS and 10% FCS. After washing twice with PBS, the second Ab, goat anti-rabbit IgG ALEXA-568 diluted 1:200 in PBS and 10% FCS was added and incubated for 1 h at room temperature. Cover slips were washed again with PBS, mounted on glass slides with Moviol containing DAPI (Prolong Gold, Promega, Mannheim, Germany) and analyzed by fluorescence microscopy.

For labeling *S. pyogenes* with carboxyfluorescein (Molecular Probes, Göttingen, Germany), a suspension of 5 × 10⁶ bacteria was centrifugated, resuspended in 1 ml HBBS containing 0.2 mg/ml carboxyfluorescein, and incubated 30 min 4°C in the dark. After incubation, labeled bacteria were washed several times to remove unbound dye.

**Cytokines ELISA**

The levels of IFN-γ, IL-6, and TNF-α were determined by ELISA using matched Ab pairs and recombinant cytokines as standards. Briefly, 96-well microtiter plates were coated overnight at 4°C with the corresponding purified rat anti-mouse capture monoclonal anti–IFN-γ, IL-6, or TNF-α Abs (Pharmingen, San Diego, CA) diluted at a concentration of 2 μg/ml in sodium bicarbonate buffer. Wells were blocked with 2% BSA-PBS, and samples and standards (serial concentrations of recombinant murine IFN-γ, IL-6, or TNF-α) were added and incubated overnight at 4°C. Bound cytokines were detected with biotinylated rat anti-mouse Abs against the corresponding cytokine (Pharmingen), followed by streptavidin-peroxidase conjugate and were developed with ABTS. The optical densities of samples and standards were measured at 405 nm.

**Measurement of intracellular cAMP**

For the determination of cAMP, BM-derived macrophages were lysed by incubating for 20 min with 0.1 M HCl at room temperature. Intracellular cAMP levels were determined by ELISA according to the manufacturer’s recommendations (Cayman Chemical).

**Determination of ROS**

The production of ROS was determined as previously described (20). Briefly, untreated of PGE₂-treated BM macrophages were infected with *S. pyogenes* and incubated with 1 mg/ml Nitro blue tetrazolium (NBT) dissolved in Krebs-Ringer phosphate glucose buffer (KRPG: 144 mM NaCl, 5 mM KCl, 8.5 mM Na₂HPO₄, 1.4 mM NaH₂PO₄, 1.3 mM MgSO₄, 5 mM glucose, 10 mM HEPES, pH 7.4) for 45 min at 37°C. After incubation, the cells were washed twice with KRPG buffer, fixed in 4% paraformaldehyde, and counterstained with Giemsa stain. ROS production is evidenced by the presence of blue-black formazan precipitate after microscopy examination.

**Statistical analysis**

Data were analyzed by using Excel 2000 (Microsoft Office, Microsoft, Redmond, WA) or GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Comparison between groups was made by use of two-tailed *t* test. Comparison of survival time curves was performed by use of log-rank test. The *p* values <0.05 were considered as significant.

**Results**

**COX-2 expression during acute *S. pyogenes* infections**

To investigate whether COX-2 was expressed during acute *S. pyogenes* infection, tissue biopsies collected from patients with streptococcal soft tissue infections of varying severities were stained with anti–COX-2 Abs and subjected to in situ image analysis. To determine the intensity of infection in the affected area, parallel biopsies were stained with Abs against *S. pyogenes*. The obtained results revealed a positive staining for COX-2 in all biopsies collected from streptococcal-infected tissue (Fig. 1). It is noteworthy that the strongest expression of COX-2 and highest bacterial loads was seen in patients 1 and 2, who both had severe necrotizing fasciitis, as compared with only a weak response in biopsies of patient 3, who had a milder form of soft tissue infection (i.e., cellulitis). Therefore, the levels of COX-2 expression in the infected tissue seem to positively correlate with bacterial load and severity of infection.

We next examined the expression of COX-2 in mice experimentally infected with *S. pyogenes*. For this purpose, mice were i.v. inoculated with *S. pyogenes*, sacrificed at defined time points postinfection and *Cox-2* mRNA was determined in liver tissue, the major target organ for *S. pyogenes* in this infection model (19), by RT-PCR. As shown in Fig. 2A, *Cox-2* mRNA was highly upregulated in infected liver tissue with maximal levels reached at 24 and 48 h of infection. In contrast, *Cox-2* mRNA was under the detection limit in uninfected animals, whereas *Cox-1* and *rps-9* were constitutively expressed in all samples (Fig. 2A). We then determined the presence of COX-2 protein in liver tissue isolated from infected mice by immunohistochemical staining. COX-2 staining was more intense and more abundant in liver tissue from infected mice (Fig. 2Bi) than in tissue from uninfected control animals (Fig. 2Bii). A close examination of the histological sections demonstrated that COX-2 was mainly expressed by flat cells in close contact with the hepatic sinusoids (insert in upper right corner, Fig. 2Bii). The morphology and location suggest that these cells are resident macrophages of the liver, also known as Kupffer cells.
Inhibitor, NS-398, indicating that the synthesis of PGE2 in response to infection was completely blocked by treatment of mice with the COX-2 selective inhibitor. White bars represent uninfected control animals (Bi) or uninfected animals (Bii). Tissue sections were stained with anti-COX-2-specific Abs (red) and counterstained with H&E. The insert in (Bi) shows a higher magnification of COX-2 expression in a single cell (arrow). Original magnification ×20 for Bi and Bii, and ×63 for insert in Bi. C. Levels of PGE2 in the serum of mice treated with the specific COX-2 inhibitor NS-398 or with vehicle solution. White bars represent uninfected control animals (n = 15) and black bars represent mice infected with S. pyogenes for 48 h (n = 15). Each bar represents the mean ± SD of data obtained from three independent experiments. ***p < 0.001.

S. pyogenes at 48 h of infection. As shown in Fig. 2C, PGE2 was significantly increased in the plasma of infected mice compared with uninfected control animals (p < 0.001). This increase was completely blocked by treatment of mice with the COX-2 selective inhibitor, NS-398, indicating that the synthesis of PGE2 in response to S. pyogenes infection was largely mediated by this enzyme (Fig. 2C). Taken together, the data indicate that COX-2 is strongly up-regulated in mice upon infection with S. pyogenes, which results in production of high levels of PGE2.

Induction of COX-2 and production of PGE2 by macrophages after in vitro infection with S. pyogenes

Stimulated macrophages have traditionally been considered the most potent PG producers of all leukocytes (21), with COX-2 being responsible for most of the eicosanoids secreted by macrophages (22). Therefore, we next evaluated the capacity of S. pyogenes to induce COX-2 expression in primary BM murine macrophages. BM macrophages were infected with S. pyogenes and Cox-2 mRNA levels were determined at defined time points postinfection by RT-PCR. As shown in Fig. 3A, S. pyogenes induced Cox-2 gene expression in BM macrophages at detectable levels already after 1 h of infection and levels continued to increase with maximal levels reached at 24 h postinfection. There was no basal expression of Cox-2 mRNA in uninfected cells (Fig. 3A).

COX-2 protein expression was then determined in S. pyogenes-infected BM macrophages by Western blot analysis. The results show that S. pyogenes induced expression of COX-2 in BM macrophages in a time-dependent manner (Fig. 3B). Visualization of COX-2 in S. pyogenes-infected BM macrophages by immunofluorescence staining reveals that COX-2 accumulates within vesicles in the cytoplasm of infected cells (Fig. 3C). Also human primary monocytes were found to express COX-2 after infection with S. pyogenes (Supplemental Fig. 1).

S. pyogenes strains are characterized based on their M-type, and there is a linkage between certain types and specific disease manifestations, such as M1 and M3 strains that are commonly associated with invasive infections (23). To evaluate whether BM macrophage expression of COX-2 in response to S. pyogenes infection is strain-dependent, COX-2 expression was determined in macrophages infected with M1, M3, M18, or M49 strains of S. pyogenes using a similar MOI. Induction of COX-2 seemed to be strain-independent, as equal protein expression was observed postinfection with the different strains (Supplemental Fig. 2).

Similar to the in vivo observation, upregulated COX-2 activity after exposure of BM macrophages to S. pyogenes translated into a significant augmentation in PGE2 secretion in the culture supernatant (Fig. 3D). This increase was markedly inhibited by the addition of the selective COX-2 inhibitor, NS-398, confirming the major contribution of COX-2 to the synthesis of PGE2 in this experimental setting (Fig. 3D). The levels of PGE2 production by infected BM macrophages were strongly dependent of the MOI (Fig. 3E).

Effect of COX-2 deficiency in the outcome of experimental S. pyogenes infection

To define the extent to which the levels of COX-2 expression and PGE2 production influence the outcome of S. pyogenes infection, COX-2−/− mice and their COX-2+/+ littermates were i.v. infected with S. pyogenes and their survival time was monitored overtime. In contrast to the high levels of PGE2 detected in serum of S. pyogenes-infected COX-2−/− mice, PGE2 was under detection levels in infected COX-2−/− animals (data not shown). Interestingly, COX-2−/− mice showed significantly extended survival times compared with the COX-2+/+ counterparts (Fig. 4A). Assessment of bacterial load in the liver and spleen of infected mice at 48 h of infection revealed a significantly lower bacterial count in the organs of COX-2−/−, as compared with COX-2+/+ mice (p < 0.05) (Fig. 4B).

Inflammatory cytokines have previously been shown to modulate the pathogenesis of invasive S. pyogenes infection (24). Determination of IL-6 (Fig. 4C) and IFN-γ (Fig. 4D) levels in the serum of infected COX-2−/− and COX-2+/+ mice at 48 h postinoculation revealed that the production of these cytokines was significantly attenuated in COX-2−/− mice. These results clearly indicate that the deficiency of COX-2 results in improved control of bacterial growth as well as lower levels of systemic inflammation, leading to enhanced survival of S. pyogenes-infected animals.

The above results suggest that therapeutic inhibition of COX-2 might have a beneficial effect in the outcome of severe S. pyogenes infection.
infection. To test this hypothesis, mice were treated with the specific COX-2 inhibitor, NS-398, given 2 h before and 2 h after bacterial inoculation. Mice in the control group were treated in a similar regimen but receiving vehicle solution instead of the inhibitor. The therapeutic effect of NS-398 in *S. pyogenes*-infected animals was evaluated by measuring survival times, bacterial growth, and systemic inflammatory cytokines. As shown in Fig. 4E, treatment with NS-398 significantly extended the survival times of infected mice.

![Induction and expression of COX-2 in BM macrophages infected with *S. pyogenes*. BM murine macrophages were infected with *S. pyogenes* at MOI of 10:1. After exposure of macrophages to *S. pyogenes* for 1 h, cells were washed and further incubated in the presence of gentamicin for the indicated period of time before isolating total RNA or protein lysates. At progressing times of infection, the induction of *cox-2* gene was determined by RT-PCR (A) and the expression of COX-2 protein by Western blot analysis (B). Fluorescence staining of COX-2 in *S. pyogenes*-infected macrophages is shown in (C). The intracellular distribution of COX-2 (red) in *S. pyogenes*-infected (Ci) or uninfected (Cii) macrophages was visualized by fluorescence microscopy. Macrophages nuclei are stained by DAPI (blue). Bar size, 25 μm for Ai and Aii. Insert in Ci upper right corner, original magnification ×100. D, Production of PGE2 by NS-398-treated (white bars) or Vehicle-treated (black bars) *S. pyogenes*-infected macrophages. E, Production of PGE2 by macrophages infected with *S. pyogenes* at a MOI of 1:1, 10:1, or 50:1. Each bar represents the mean ± SD or three independent experiments. *p < 0.05; ***p < 0.001 compared with uninfected control.

![Effect of COX-2 genetic deletion or COX-2 pharmacological inhibition in the course of *S. pyogenes* infection. A, Survival curves of COX-2−/− mice and their COX-2+/+ littermates after i.v. infection with 10⁵ CFU of *S. pyogenes*. B, Bacterial loads in liver and spleen of COX-2−/− (white bars) and COX-2+/+ (black bars) mice after 48 h of bacterial inoculation. Levels of IL-6 (C) and IFN-γ (D) in serum of COX-2−/− (white bars) and COX-2+/+ (black bars) mice at 48 h of infection. E, Survival curves of *S. pyogenes*-infected mice treated with either NS-398 (white symbols) or vehicle solution (black symbols) 2 h prior and 2 h after i.v. inoculation with 10⁵ CFU of *S. pyogenes*. F, Bacterial loads in the livers of *S. pyogenes*-infected mice treated with either NS-398 (white bars) or vehicle solution (black bars) at 48 h after bacterial inoculation. G, Levels of IL-6 in serum of NS-398–treated (white bars) or vehicle solution (black bars) mice at 48 h of infection. Each bar represents the mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.
mice with respect to vehicle solution-treated animals (p < 0.05). Bacterial loads were significantly lower in the liver of NS-398-treated mice (Fig. 4F) and the systemic levels of IL-6 (Fig. 4G), IFN-γ (Supplemental Fig. 3A), and TNF-α (Supplemental Fig. 3B) were also significantly decreased when compared with the group treated with the vehicle solution. No differences were observed between vehicle solution-treated and nontreated mice (data not shown).

Thromboxane A2 is an additional eicosanoid generated by the COX pathway (25). Thromboxane A2 is one of the most potent vasoconstricting and platelet-aggregating agents known (26), and may, thus, be a factor contributing to the noted COX-2–mediated effect in S. pyogenes infections. Because of the transient nature of this compound, it is difficult to accurately measure circulating levels in whole animal experimental models. Therefore, we investigated the effect of thromboxane A2 in the course of streptococcal infection by treating infected mice with the antagonist, GR 32191B, which specifically blocks the thromboxane A2 receptor, TP. However, inhibition of thromboxane A2 signaling did not modify the course of S. pyogenes infection (Supplemental Fig. 4); thus, ruling out a role for this compound in the modulation of disease severity.

**COX-2-mediated PGE2 production results in impaired macrophage clearance of S. pyogenes**

Next, we investigated the potential mechanisms underlying the detrimental effect of PGE2 on the course of S. pyogenes infection. Using an experimental murine model of infection, we have demonstrated in previous studies that the bactericidal activity of macrophages is a critical effector mechanism in host defense against S. pyogenes (27). As PGE2 has been reported to inhibit the killing of certain bacterial pathogens by macrophages (28, 29), we determined the effect of PGE2 on the capacity of BM macrophages to kill phagocytosed S. pyogenes. As seen in Fig. 5A, exposure to PGE2 significantly reduced the capacity of BM macrophages to kill intracellular S. pyogenes. The suppressive activity of PGE2 was dose-dependent with a maximal effect of PGE2 achieved at a concentration of 50 nM (Fig. 5A). We further assessed the effect of PGE2 on S. pyogenes killing by human monocytes. Similarly to

![Graph A](image1.png)

**Graph A.** BM macrophages were preincubated with increasing concentrations of PGE2 or with vehicle solution and then infected with S. pyogenes as described in Materials and Methods. The amount of viable intracellular bacteria was determined at time 0 h (white bars) and at time 4 h (black bars) of infection. p < 0.05; **p < 0.01 versus vehicle-treated control.

![Graph B](image2.png)

**Graph B.** BM macrophages were preincubated with PGE2 (50 nM) or with vehicle solution and then infected with S. pyogenes in the presence or absence of either the EP4 inhibitor, AH23848, or the EP2 inhibitor, AH6809. The amount of viable intracellular bacteria was determined at time 0 h (white bars) and at time 4 h (black bars) of infection. **p < 0.01 versus vehicle-treated control.

![Graph C](image3.png)

**Graph C.** BM macrophages were infected with S. pyogenes in the presence of increasing concentrations of the EP2 receptor agonist, butaprost (C), and the amount of viable intracellular bacteria was determined at time 0 h (white bars) and at time 4 h (black bars) of infection. ***p < 0.001 versus vehicle-treated control.

![Graph D](image4.png)

**Graph D.** BM macrophages were infected with S. pyogenes in the presence of increasing concentrations of the EP1 and EP3 receptor agonist, sulprostone (D), and the amount of viable intracellular bacteria was determined at time 0 h (white bars) and at time 4 h (black bars) of infection. **p < 0.01 versus vehicle-treated control.

![Graph E](image5.png)

**Graph E.** Survival curves of S. pyogenes-infected mice treated with either the EP2 receptor inhibitor AH6809 (white symbols) or vehicle solution (black symbols) 24 h prior i.v. inoculation with 10^7 CFU of S. pyogenes. **p < 0.001. **Graph F.** Bacterial loads in the livers of S. pyogenes-infected mice treated with either the EP2 receptor inhibitor AH6809 (white bars) or vehicle solution (black bars) at 48 h after bacterial inoculation. **p < 0.005. For each experimental set, bars represent the mean ± SD of three independent experiments.
murine macrophages, human monocytes exposed to 50 nM PGE2 were significantly impaired in their capacity to kill S. pyogenes (Supplemental Fig. 5).

Together, these data suggest that the detrimental effect of COX-2 in S. pyogenes infections is likely attributed to the PGE2-mediated suppression of macrophage killing of S. pyogenes.

Molecular mechanism underlying the PGE2-induced suppression of macrophage killing of S. pyogenes

PGE2 mediates its biological activities via ligation to four types of membrane-bound, G protein-coupled E prostanoid (EP) receptors (EP1-EP4) (30). To identify the receptors involved in the PGE2-mediated suppressive effects on macrophages, BM macrophages were pretreated with the EP2 antagonist, AH6809, or the EP4/EP3/thromboxane A2 receptor antagonist, AH23848 prior to S. pyogenes infection. Control BM macrophages were pretreated with the vehicle solution.

Pretreatment of BM macrophages with AH6809 completely inhibited the suppressive effect of PGE2 and significantly increased the killing of ingested bacteria (Fig. 5B). In contrast, no effect was seen with the EP4 antagonist AH23848 (Fig. 5B). Further support for the contribution of EP2 signaling to the suppression of BM macrophages bactericidal capacity was obtained by using the EP2 agonist, butaprost, which fully mimicked the suppressive effect of PGE2 (Fig. 5C). No such effect was evident when the EP1 and EP3 agonist, sulprostone, was used (Fig. 5D). These results clearly demonstrate that EP2 receptor is responsible for the ability of PGE2 to suppress the killing of ingested S. pyogenes in BM macrophages.

To confirm the biological significance of these in vitro results, we investigated whether treatment with the EP2 inhibitor AH6809 could improve the disease outcome in S. pyogenes-infected mice. To this end, mice were infected i.v. with S. pyogenes and subsequently treated with either 200 μg AH6809 or vehicle solution alone administered by i.p. injection. Mice treated with AH6809 survived significantly longer (p < 0.001) (Fig. 5E) and exhibited significantly lower bacterial counts in the liver at 48 h of infection than Vehicle-treated mice (p < 0.01) (Fig. 5F). No differences were observed between mice treated with vehicle solution and nontreated animals (data not shown).

After ligand binding, the EP2 and EP4 receptors signal through a stimulatory G protein (Gs) that activates the adenylyl cyclase activity and consequently cAMP formation (31, 32). The EP3 receptor, on the other hand, more often reduces cAMP via inhibitory G protein (Gi) (31, 32). To further explore the downstream mechanisms of PGE2-mediated suppressive effects, BM macrophages were incubated with increasing concentrations of PGE2 for 4 h and the level of intracellular cAMP determined. The obtained results revealed that PGE2 mediated a dose-dependent increase in the intracellular cAMP levels in macrophages (Fig. 6A). This effect of PGE2 on the levels of intracellular cAMP was mimicked by the EP2 agonist, butaprost (Fig. 6B). On the other hand, treatment of BM macrophages with the EP3 agonist, sulprostone, resulted in strong reduction of the basal intracellular concentration of cAMP (Fig. 6B). This is in agreement with reports demonstrating that signaling through the Gi-coupled EP3 inhibits production of cAMP (31, 32).

As cAMP has been shown to suppress the microbicidal capacity of macrophages against bacterial pathogens (28, 33), we explored the effect of the cAMP-elevating agent, forskolin, on the bactericidal activity of BM macrophages against S. pyogenes. We found that forskolin abrogated the ability of BM macrophages to kill ingested S. pyogenes in a concentration-dependent manner (Fig. 6C). Similar results were observed when an inhibitor of phosphodiesterase-4, rolipram, that antagonizes cAMP breakdown and therefore increase signaling through the cAMP system was used (Fig. 6D). These data implicate the EP2-adenylyl cyclase–cAMP pathway in the PGE2 mediated impaired killing of S. pyogenes.

The biological activity of cAMP is mediated by the activation of two major downstream effector molecules, the protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac) (34). In our experimental settings, the PKA/Epac activator (S)-p-8-(4-chlorophenylthio) adenosine-3',5'-cAMP suppressed the antimicrobial activity of BM macrophages to the same degree as PGE2 (Fig. 7A). To investigate whether this inhibitory effect was attributed to PKA, the best known cAMP effector, the cells were exposed to the highly specific, myristoylated peptide PKA inhibitor PKI14–22. As shown in Fig. 7A, PKI14–22 completely prevented the suppres-

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**FIGURE 6.** Suppression of *S. pyogenes* killing by PGE2 is mediated by increased intracellular levels of cAMP. *A*. BM macrophages were incubated with increasing concentrations of PGE2 for 4 h and intracellular cAMP concentrations measured as described in the Materials and Methods section. Control BM macrophages were treated with vehicle solution alone. Each bar represents the mean ± SD of three independent experiments. ***p < 0.001. B*. Intracellular cAMP concentrations in BM macrophages after 4 h treatment with either the EP2 receptor agonist, butaprost, or the EP1 and EP3 receptor agonist, sulprostone, or vehicle solution alone. **p < 0.005; ***p < 0.001. BM macrophages were infected with *S. pyogenes* in the presence of increasing concentrations of the adenylyl cyclase activator, forskolin, (C) or the phosphodiesterase-4 inhibitor, rolipram, (D) and the amount of viable intracellular bacteria was determined at time 0 h (white bars) and at time 4 h (black bars) of infection. *p < 0.05; ***p < 0.001 versus vehicle-treated control. For each experimental set, bars represent the mean ± SD of three independent experiments.
bacterial load and severity of infection; and 2) genetic ablation as well as pharmacological inhibition of COX-2 significantly enhanced the ability of mice to control S. pyogenes infection and prolonged the survival times of infected animals. These observations are consistent with other studies reporting an enhanced clearance of Pseudomonas aeruginosa from the lungs in COX-2−/− as compared with COX-2+/− mice (15), or the significant decreased number of metastatic lesions observed in Leishmania major-infected BALB/c mice after administration of the irreversible COX-2 inhibitor indomethacin (35). Similarly, inhibition of COX activity has been shown to significantly improve the survival of septic newborn piglets infected with group B Streptococcus (36).

Furthermore, our data complement a previous study of Aronoff and coworkers (37) reporting that exogenous application of misoprostol, a pharmacomimetic of PGE2, impaired the innate immune defense required for clearance of Clostridium sordellii in the rat reproductive tract. Together, the published reports and the results in this study underscore an immunosuppressive effect of PGE2 during the course of bacterial infections.

The detrimental consequences of high levels of PGE2 in the outcome of S. pyogenes infection underscored the need to understand the mechanisms underlying this effect. PGE2 is known to have deactivating properties on macrophages and to impair phagocytosis and bactericidal activity (5, 33). Indeed, we found that exposure to PGE2 reduced the capacity of murine BM macrophages and human monocytes to kill S. pyogenes. This is of interest as a reservoir of S. pyogenes in macrophages has been shown to contribute to bacterial persistence during severe soft tissue infections (18). Because an intact antimicrobial activity of macrophages have been reported to be critical for an efficient control of S. pyogenes infection (27), we propose that induction of PGE2 is a mechanism used by S. pyogenes to disable the antimicrobial activity of macrophages and thus contribute to bacterial survival in the infected host.

PGE2 can act through four functionally divergent G protein-coupled receptors (EP1, EP2, EP3, and EP4) (32, 32). Ligation of the EP1 receptor induces an increase in intracellular calcium concentration. The EP2 and EP4 receptors couple to the Gi protein resulting in increased intracellular cAMP levels by activating the adenyl cyclase, whereas the EP3 receptor reduces or increases cAMP depending of the particular splice variant expressed by the cells (40). Using pharmacological agonists/antagonists for the different EP receptors, we demonstrated that activation of Gi-coupled EP2 receptor mediates the suppressive effect of PGE2 in the capacity of BM macrophages to kill ingested S. pyogenes. Based on these results, we proposed that antagonists for EP2 receptor would be therapeutically beneficial during severe S. pyogenes infection. This was indeed the case because treatment with the EP2 receptor inhibitor, AH6809, significantly improved the outcome of infection in experimentally infected mice.

As cAMP has been reported to suppress the microbicidal capacity of BM macrophages against bacterial pathogens (28, 33), we speculated that this pathway might be involved in the PGE2-mediated inhibition of S. pyogenes killing in BM macrophages. Indeed, exposure of macrophages to PGE2 resulted in significantly increased levels of intracellular cAMP. Further evidence for a role of intracellular cAMP was provided by the suppressive effect in the microbicidal capacity of S. pyogenes-infected macrophages exerted by the cAMP-elevating agents, forskolin and rolipram.

An increase in the cellular level of cAMP is known to activate the two downstream effector molecules, PKA and Epac (34, 41). In the current study, we provide evidence that the PGE2-mediated impaired bacterial killing of infected macrophages involves the cAMP–PKA signaling pathway. Furthermore, we demonstrated...
that activation of this signaling pathway prevents the generation of ROS in infected BM macrophages. This can explain the PGE2-mediated suppressive effect in the bactericidal capacity of S. pyogenes-infected macrophages because we have previously demonstrated that killing of S. pyogenes by macrophages is largely dependent of NADPH oxidase activity and production of ROS (20). Interestingly, it has been shown in a recent study that genetic deletion of the EP3 receptor resulted in enhanced bacterial clearance and improved survival in mice infected with Streptococcus pneumoniae (42). This effect was associated with a superior capacity of EP3-deficient alveolar macrophages to generate NO and to kill S. pneumoniae (42). These observations together with the results of our study indicate that different PG receptors modulate unique macrophage bactericidal mechanisms and underscore the complexity of the prostanoids regulatory network modulating the activity of immune cells in the context of infection. Considering that a variety of epidemiological, clinical, and experimental approaches have convincingly demonstrated a significant contribution of host genetic factors predisposing to S. pyogenes infection (2, 43, 44), makes it tempting to hypothesize that potential interindividu- nal variation in the expression of PGE2 receptors might influence the level of susceptibility to S. pyogenes infection. This assumption deserves further investigation.

Nonsteroidal anti-inflammatory drugs (NSAIDs), which act as nonselective COX inhibitors, are often used to alleviate pain, inflammation, and fever in patients and some clinical studies have suggested an association between NSAIDs and worsening of streptococcal infections (45). However, retrospective studies do not support a risk for developing streptococcal severe infection as a result of NSAIDs therapy but rather support the notion that NSAIDs mask the initial nonspecific symptoms of necrotizing fasciitis; thereby, resulting in delayed diagnosis and treatment (46). In line with this postu- lation, the results of our study provide clear evidence that by reducing the production of PGs, it has been speculated that inhibition of COX may lead to a shunt to the lipoxygenase pathway as a virulence mechanism to disable the host antimicrobial defenses. Inhibitors that specifically target this pathway could ameliorate rather than aggravate the destructive pathophysiological events caused by S. pyogenes and, therefore, might lead to the development of novel adjunctive treat- ments.

Although selective COX inhibitors primarily exert their activity by reducing the production of PGs, it has been speculated that inhibition of COX may lead to a shunt to the lipoxigenase pathway resulting in the formation of lipid mediators such as leukotrienes and lipoxins. Leukotrienes are potent mediators of inflammation, and it is now clear that PGs and leukotrienes have complementary effects (47). Lipoxins represent another class of lipoxigenase-derived eicosanoids, but in contrast to leukotrienes, these possess anti-inflammatory properties (48). Hence, it is possible that a generation of lipoxins may account for some of the beneficial effects exerted by the COX-2 inhibitor, NS-398, in S. pyogenes-infected mice. This subject will be addressed in future studies.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1
Supplemental Figure 2

COX-2

β-Actin

72 kDa

42 kDa
Supplemental Figure 3

A

B

IFN-γ (pg/ml)

Sham NS-398

100 150 200

1250 1000 750

TNF-α (pg/ml)

Sham NS-398

0 250 500 750

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Supplemental Figure 4
Supplemental Figure 5

(A) Percent survival over time (days) for Vehicle and GR 32191B groups. 

(B) Log$_{10}$ CFU S. pyogenes levels for Vehicle and GR 32191B groups.
Supplemental material

Isolation and infection of blood-derived human monocytes. Monocytes were isolated from blood of healthy adult donors. Heparinized whole blood was mixed with complete cell culture medium, carefully layered over Ficoll-Paque (Amersham) and centrifuged at 900 g for 20 min at 4°C. The interface containing the mononuclear cell fraction was collected, re-suspended in complete medium and centrifuged again at 400 g for 10 min at 18°C. After washing, the pellet was re-suspended in RPMI 1640 and incubated for 45 min at 37°C. After removing non-adherent cells by gently washing, monocytes were harvested, counted, adjusted to $5 \times 10^5$ cells/ml and seeded into a 48-well tissue culture plate. Monocytes were infected with *S. pyogenes* at an MOI of 10:1 for 4 h at 37°C and 5% CO$_2$ in antibiotic-free medium and expression of COX-2 as determined as described for murine macrophages.

Legend Supplemental Figure S1. *S. pyogenes*-induction and expression of COX-2 in *in vitro* cultured macrophages is independent of the M type. Induction of COX-2 protein expression on macrophages by M1, M3, M18, and M49 *S. pyogenes* strains. Expression of COX-2 was determined by western blot analysis at 6 h of infection. Results are representative of 3 independent experiments.

Legend Supplemental Figure S2. Fluorescence staining of COX-2 in human monocytes. Human monocytes infected for 1 h with *S. pyogenes* at an MOI of 10:1 were stained with anti-COX antibodies (red) and analysed by fluorescence microscopy (i). COX-2 expression in uninfected monocytes is shown in (ii). The intracellular distribution of COX-2 in *S. pyogenes*-infected monocytes is shown in the insert in Ai. Monocytes nuclei are stained by DAPI (blue). Bar size, 25 µm for Ai and Aii. Insert in upper right corner in left panel, 100× magnification.
Legend Supplemental Figure S3. Levels of INF-γ and TNF-α in serum of *S. pyogenes*-infected mice after treatment with NS-398. Mice were treated with PBS (sham) or with NS-398 2 h prior and 2 h after intravenous inoculation with $10^5$ CFU of *S. pyogenes*. Levels of IFN-γ (*A*) and TNF-α (*B*) in serum of NS-398-treated (white bars) or sham (black bars) mice at 48 h of infection. Each bar represents the mean ± SD of three independent experiments. *, $p < 0.05$.

Legend Supplemental Figure S4. Effect of PGE$_2$ on the response of human monocytes to *S. pyogenes*. Human monocytes were pre-treated with 50 nM of PGE$_2$ or left untreated and then infected for 1 h with *S. pyogenes* at an MOI of 10:1, washed and further incubated for 4 h in gentamicin-containing medium. Monocytes were then lysed and the amount of phagocytosed bacteria determined by plating onto blood agar. The results are expressed as Log$_{10}$ viable bacterial counts. Each bar represents the ± SD of three independent experiments. ***, $p < 0.001$.

Legend Supplemental Figure S5. Pharmacological inhibition of thromboxane A2 signaling does not affect the natural course of *S. pyogenes* infection in mice. *A*, survival curves of *S. pyogenes*-infected mice treated with either the thromboxane A2 receptor antagonist GR 32191B (white symbols) or vehicle solution (black symbols). *B*, Bacterial loads in the livers of *S. pyogenes*-infected mice treated with either the thromboxane A2 receptor antagonist GR 32191B (white bar) or vehicle solution (black bar) at 48 h after bacterial inoculation.