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Although mainly expressed in neuronal cells, lipocalin-type PGD synthase (L-PGDS) is detected in the macrophages infiltrated to atherosclerotic plaques. However, the regulation and significance of L-PGDS expression in macrophages are unknown. Here, we found that treatment of macrophages with bacterial endotoxin (LPS) or Pseudomonas induced L-PGDS expression. Epigenetic suppression of L-PGDS expression in macrophages blunted a majority of PGD2 produced after LPS treatment. Chromatin immunoprecipitation assays show that L-PGDS induction was regulated positively by AP-1, but negatively by p53. L-PGDS expression was detected in whole lung and alveolar macrophages treated with LPS or Pseudomonas. L-PGDS overexpressing transgenic mice improved clearance of Pseudomonas from the lung compared with nontransgenic mice. Similarly, intratracheal instillation of PGD2 enhanced removal of Pseudomonas from the lung in mice. In contrast, L-PGDS knockout mice were impaired in their ability to remove Pseudomonas from the lung. Together, our results identify induction of L-PGDS expression by inflammatory stimuli or bacterial infection, the regulatory mechanism of L-PGDS induction, and the protective role of L-PGDS expression in host immune response. Our study suggests a potential therapeutic usage of L-PGDS or PGD2 against Pseudomonas pneumonia.

Macrophages are key players in innate immunity. When activated by endotoxin (LPS) or live bacteria, macrophages in the lung trigger a series of inflammatory responses by producing cytokines, thereby recruiting neutrophils (1). The inflammatory responses normally lead to clearance of invading bacteria. Therefore, depletion of macrophages by treating mice with liposomal clodronate impairs inflammatory responses and clearance of bacteria including Pseudomonas from the lung (2–4).

Macrophages are responsible for the toxicity caused by LPS (5), one of the major bacterial factors that trigger inflammation, which is in part attributed to the fact that although expressed in various cell types, TLR4 is highly expressed in macrophages (6–9). TLR4 is a receptor for LPS and is crucial for host innate immunity against bacterial infection (10–16). Engaged by LPS, TLR4 triggers Toll/IL-1R-mediated signaling via MyD88 adaptor protein, resulting in activation of canonical IkB kinase (IKK) and MAPKs, including ERK, JNK, and p38 kinase (17). The activated protein kinases increase transcriptional activities of NF-κB and AP-1, resulting in expression of inflammatory genes (17). On the other hand, MyD88-dependent Toll/IL-1R signaling activates noncanonical the IKK complex, which activates NF-κB and IFN regulatory factor-3 (18, 19). It has been shown that the MyD88-dependent pathway regulates many classic markers of inflammation including cyclooxygenase (COX)-2 (20).

LPS treatment induces COX-2 expression in macrophages, and NF-κB activation is sufficient for COX-2 induction (21–23). Along with the constitutively expressed isoenzyme COX-1, COX-2 converts arachidonic acid to PGH2, which serves as a precursor for other prostanooids including PGD2 (24). PGH2 is converted to PGD2 by two tissue-specific enzymes, lipocalin-type PGD synthase (L-PGDS) and hemopoietic isotype (H-PGDS). However, these two enzymes share no homology in DNA and polypeptide sequences (25). Northern blot analyses also show that the expression profiles of these enzymes are distinctive, because L-PGDS is mainly detected in CNS and related organs (26), whereas H-PGDS is in hemopoietic cells including macrophages (27).

PGD2 is involved in lung inflammation. PGD2 exacerbates asthma (28–30), suggesting that PGD2 is proinflammatory. On the other hand, injection of PGD2 or retroviral delivery of PGD synthase to the lung suppresses inflammatory processes elicited by bleomycin and monosodium urate monohydrate crystal challenges (31–33). In addition, PGD2 in a murine model of pleuritis was caused

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by carrageenan is associated with resolution of lung inflammation, suggesting that PGD₂ exerts anti-inflammatory functions. These results clearly show that the outcome of PGD₂ production depends on the inflammatory milieu.

Although not detectable in macrophages normally, L-PGDS expression can be detected in macrophages infiltrated to the atherosclerotic plaques (34), suggesting that L-PGDS is aberrantly expressed in macrophages in a pathological environment. However, the regulation of L-PGDS expression in macrophages and the significance of L-PGDS expression in a pathological condition remain unknown. In this study, we attempted to identify the mechanism that controls L-PGDS expression in macrophages. Given the role of PGD₂ in lung inflammation, we examined the effect of L-PGDS on lung inflammation caused by Pseudomonas lung infection, a common cause of nosocomial pneumonia and the most serious respiratory pathogen in cystic fibrosis patients (35). Our findings revealed a novel regulatory mechanism of L-PGDS expression in macrophages and the protective function of L-PGDS expression or PGD₂ against bacterial lung infection.

Materials and Methods

Reagents
PGD₂, PGE₂, and COX-2 inhibitor CAY10404 were purchased from Cayman Chemical. TLR4-specific Escherichia coli LPS was obtained from Alexis Biochemical, and doxorubicin was from Sigma-Aldrich. Various MAPK inhibitors including SB 220025 (p38 inhibitor), JNK inhibitor II, Alexis Biochemical, and TLR4-specific Escherichia coli LPS was obtained from LPS (1 μg/ml) or with PA103 (lanes 5 and 6), similar to A. To verify COX-2, 293 cells were transfected with a plasmid encoding a murine COX-2 (lane 1). C. BMDM (1 × 10⁶ cells) were treated with LPS (1 μg/ml) for 12 h in the presence or absence of CAY10404 (100 nM), a COX-2 specific inhibitor, and the cell culture supernatant was collected for PGD₂ measurement. Each bar represents the mean PGD₂ (nM) ± SEM (for n = 3 wells per treatment group), and the experiment was repeated three times (*, p < 0.05 compared with other group).

FIGURE 1. Induction of COX-2 and production of PGD₂ by BMDM. COX-1 and COX-2 expression were measured in BMDM and RAW 264.7 cells. A. BMDM (1 × 10⁶ cells) were treated with LPS (1 μg/ml) for 4 h (lane 2) or with PA103 to moi 1 for the indicated time periods (lanes 4, 5, and 6) before cell harvest. An equal amount of total cell lysate was analyzed by SDS-PGDS and Western blotting (WB) for COX-2 expression (top) and COX-1 expression (bottom). B. RAW 264.7 cells (1 × 10⁶) were treated with LPS (lane 3) or PA103 (lanes 5 and 6), similar to A. To verify COX-2, 293 cells were transfected with a plasmid encoding a murine COX-2 (lane 1). C. BMDM (1 × 10⁶ cells) were treated with LPS (1 μg/ml) for 12 h in the presence or absence of CAY10404 (100 nM), a COX-2 specific inhibitor, and the cell culture supernatant was collected for PGD₂ measurement. Each bar represents the mean PGD₂ (nM) ± SEM (for n = 3 wells per treatment group), and the experiment was repeated three times (*, p < 0.05 compared with other group).

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Cell culture
Bone marrow-derived macrophages (BMDM) were obtained as described elsewhere (36). In short, cellular material from femurs of mice ranging from 8 to 16 wk of age was flushed out with PBS. One million cells were treated with LPS (1 μg/ml) for 4 h in the presence or absence of CAY10404 (100 nM), a COX-2 specific inhibitor, and the cell culture supernatant was collected for PGD₂ measurement. Each bar represents the mean PGD₂ (nM) ± SEM (for n = 3 wells per treatment group), and the experiment was repeated three times (*, p < 0.05 compared with other group).

Animal model
Male and female mice weighing 20–28 g were used in this study. Transgenic mice encoding human L-PGDS cDNA under the control of the chicken β-actin promoter and the CMV immediate early enhancer on a FVB/N background were described previously (37). Male C57BL/6 mice harboring homozygous deletion of L-PGDS gene, L-PGDS knockout (KO) mice, were described previously (38). Animal experiments were performed per protocol approved by the Vanderbilt University Institutional Animal Care and Use Committee (Nashville, TN). We made every effort to minimize pain, discomfort, and the number of animals for the study.

LPS and PGD₂ administration
After sedation, mice were treated with intratracheal (i.t.) administration of PGD₂. Mouse tracheas were directly exposed by surgical resection, pierced with a 26-gauge needle, and injected with 50 μl of PGD₂ (0.1 μg/g) diluted in sterile PBS. The dose of PGD₂ was chosen based on the published dose of 15-deoxy-A₁₂,1₄-PGJ₂ (15d-PGJ₂) administered i.t. (39). The neck wound was closed with sterile sutures under aseptic conditions. For i.p. injections, a single dose of 3 μg of LPS per g of body weight was administered (40).

Bronchoalveolar lavage (BAL) fluid and total and differential cell counts
After mice were asphyxiated with CO₂, tracheas were cannulated, and lungs were lavaged in situ with sterile pyrogen-free physiological saline that was instilled in four 1-ml aliquots and gently withdrawn with a 1-ml tuberculin syringe. Lung lavage fluid was centrifuged at 400 × g for 10 min. Supernatant was kept at −70°C, the cell pellet was suspended in serum-free RPMI 1640, and total cell counts were determined on a grid hemocytometer. Differential cell counts were determined by staining cytocentrifuge slides with a modified Wright stain (Diff-Quik; Baxter) and counting 400–600 cells in complete cross-sections.

Bacterial infection and colony counting
Pseudomonas aeruginosa 103 (PA103) was cultured in a dialysate of triplicative soy broth supplemented with 10 mM nitrilotriacetic acid (Sigma-Aldrich), 1% glycerol, and 100 mM monosodium glutamate. Macrophages were incubated with Pseudomonas with 5 × 10⁶ CFU for up to 7 h. Unless specified, 1 million bacteria in 100 μl of PBS were instilled by i.t. injection with a 26-gauge needle to surgically exposed mouse tracheas. The neck wound was closed with sterile sutures under aseptic conditions. Before the lung was harvested, the right ventricle was infused with 1 ml of sterile PBS to remove blood from the lung tissue, and then the lungs were removed aseptically and homogenized in 3 ml of sterile PBS. Lung homogenate was cultured overnight on soy base blood agar plate for bacterial colony counting.

Protein isolation and Western blot analysis
Total cell lysate was prepared with radioimmunoprecipitation assay cell lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% sodium orthovannadate, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche). To obtain proteins from tissue, harvested organs were quickly frozen in liquid nitrogen, and 200 mg of tissue were suspended in radioimmunoprecipitation assay buffer. Tissue...
in the buffer was homogenized and incubated on ice for 15 min with occasional vortexing. Cell debris was removed by centrifugation at 1000 g for 10 min at 4 °C. Protein content was quantified by the Bradford assay (Bio-Rad) as specified by manufacturer. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (Bio-Rad), which was incubated with appropriate Abs. Immune complex was detected by enhanced chemiluminescence (ECL plus; Amersham). COX-1 and -2 Abs were purchased from Cayman Chemical, and other Abs used in this study were purchased from Santa Cruz Biotechnology.

**DNA constructs and L-PGDS small interfering RNA (siRNA) cell line**

To construct L-PGDS siRNA plasmids, a 19-nt-long candidate sequence was located by using a software (OligoEngine) from 528 to 546 nt (5’-GGACGAGCTGAAGGAGAAA-3’) in the open reading frame of murine L-PGDS. The candidate sequence was further analyzed by BLAST search for potential homology. Those sequences were cloned into pSUPER.retro.puro (OligoEngine), and stably transfected into RAW 264.7 cells. Transfected cells were cultured in DMEM containing 2 µg/ml puromycin (Sigma-Aldrich) for selecting stable transfecents. Western blotting of L-PGDS was performed to confirm successful siRNA colonies. A p53-expressing vector plasmid was a gift from Dr. Philip Hinds (Tufts University School of Medicine, Boston, MA). For transfection of macrophages, GenePORTER 2 (Gene Therapy Systems) was used per the suggested protocol of the manufacturer.

**Prostanoid measurement**

PGD$_2$ and other prostanooids were measured by a liquid chromatographic-electrospray ionization-mass spectrometry-mass spectrometry as previously described (41). Liquid chromatographic separation was performed isocratically on a Phenomenex Luna 3-µm C$_{18}$ 5.0- × 0.2-cm column. The mass spectrometer was operated in positive-ion electrospray ionization mode. Detection of the analytes was accomplished by selected reaction monitoring, using the following reactions: 370→317 (PGE$_2$ and PGD$_2$), 374→321 (PGD$_2$-d$_4$). The quantum was set to the following parameters: capillary V = 35 V; spray voltage = 4.3 kV;
The samples were treated with 10 μM each such as JNK inhibitor (lane 3), SB 220025 (p38 inhibitor; lane 4), and U0126 (ERK1/2 inhibitor; lane 5), as recommended by the manufacturer. Pretreated cells were subsequently treated with LPS (1 μg/ml) for 16 h to induce L-PGDS (lanes 2–5). Total cell lysate was prepared from variously treated RAW 264.7 cells for Western blotting (WB) analysis of L-PGDS and α-tubulin for equal loading. L-PGDS expression induced by LPS treatment was used as a positive control (lane 2). The experiment was repeated at least three times. B. Before virus infection, RAW 264.7 cells were transfected with an NF-κB-luciferase reporter construct, along with a tk-Renilla luciferase reporter construct. The transfected cells were infected with recombinant adenoviruses encoding either eGFP (Ad-eGFP; lanes 2 and 3) or IκBζ532,36A (Ad-IκBζ; lanes 4 and 5) with an moi of 0.5 for 1 h and subsequently treated with LPS (1 μg/ml) for 16 h to induce L-PGDS (lanes 3 and 5). Total cell lysate was analyzed by Western blotting for L-PGDS expression and α-tubulin for equal loading. The experiment was repeated at least three times. C. To verify the efficacy of Ad-IκBζ in suppressing NF-κB activity, dual luciferase assay was performed. Each bar represents the mean relative light units ± SEM (for n = 3 wells per treatment group), and the experiment was repeated three times. *, p < 0.05 compared with other group.

**FIGURE 4.** MAPKs are involved in induction of L-PGDS. A, RAW 264.7 cells (1 × 10⁶ cells) were pretreated with either vehicle, DMSO (lanes 1 and 2) or various MAPK inhibitors (10 μM each) such as JNK inhibitor (lane 3), SB 220025 (p38 inhibitor; lane 4), and U0126 (ERK1/2 inhibitor; lane 5), as recommended by the manufacturer. Pretreated cells were subsequently treated with LPS (1 μg/ml) for 16 h to induce L-PGDS (lanes 2–5). Total cell lysate was prepared from variously treated RAW 264.7 cells for Western blotting (WB) analysis of L-PGDS and α-tubulin for equal loading. L-PGDS expression induced by LPS treatment was used as a positive control (lane 2). The experiment was repeated at least three times. B. Before virus infection, RAW 264.7 cells were transfected with an NF-κB-luciferase reporter construct, along with a tk-Renilla luciferase reporter construct. The transfected cells were infected with recombinant adenoviruses encoding either eGFP (Ad-eGFP; lanes 2 and 3) or IκBζ532,36A (Ad-IκBζ; lanes 4 and 5) with an moi of 0.5 for 1 h and subsequently treated with LPS (1 μg/ml) for 16 h to induce L-PGDS (lanes 3 and 5). Total cell lysate was analyzed by Western blotting for L-PGDS expression and α-tubulin for equal loading. The experiment was repeated at least three times. C. To verify the efficacy of Ad-IκBζ in suppressing NF-κB activity, dual luciferase assay was performed. Each bar represents the mean relative light units ± SEM (for n = 3 wells per treatment group), and the experiment was repeated three times. *, p < 0.05 compared with other group.

**Chromatin immunoprecipitation (ChIP) assay**

Reagents were obtained from Upstate Biotechnology, and assay was performed as described previously (42). Briefly, we grew cells to 90% confluence with 1 × 10⁶ cells for each treatment. After treated with 1% formaldehyde for 5 min, cells were harvested, suspended in SDS-lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, protease inhibitors) and underwent sonication (4 times for 12 s each at one-fifth of the maximum power). After centrifugation at 4°C for 10 min, supernatants were diluted 1/10 with dilution buffer (16.7 mM Tris-HCl (pH 8.1), 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100) and added with DNA. The PCR products ran on either 1% agarose or 8% polyacrylamide gels. Elutes were heated at 65°C for at least 4 h to reverse formaldehyde cross-linking. The samples were treated with 10 μg of proteinase K at 45°C for 1 h. The recovered DNA was purified with a DNA cleanup kit (Qiagen), and samples of input DNA were also prepared in the same way. PCR conditions were as follows: 94°C for 240 s; 30–32 cycles at 94°C for 40 s; 54°C for 40 s; 72°C for 60 s; and final elongation at 72°C for 10 min. PCR for the input was performed with 100 ng of genomic DNA. The PCR products ran on either 1% agarose or 8% polyacrylamide gel. Primers were 5′-GGTCAAGGCACAATGTGGCTT-3′ and 5′-TCCA GAGGCAGAATGGCTCAG-3′. This primer set covers the AP-1 site area, generating a 225-bp PCR product. Abs used for immunoprecipitation were purchased from Santa Cruz Biotechnology. To exclude nonspecific precipitation of DNA bound protein, isotopic IgG (Santa Cruz Biotechnology) was used in parallel.

**Semiquantitative RT-PCR**

Total RNA was prepared with a RNeasy kit (Qiagen) per the manufacturer’s manual. Reverse transcription (RT) of 2 μg of total RNA was performed with murine leukemia virus reverse transcriptase and a random hexamer primer (PerkinElmer) to generate cDNA. Actin cDNA level from each sample was used to normalize the samples for differences in PCR efficiency. L-PGDS mRNA quantity was determined by using end-point dilution PCR, including three serial 1/5 dilutions (1/5, 1/25, and 1/125) of RT products for PCR amplification. To eliminate genomic DNA contamination, equal amounts of total RNA from each sample were PCR amplified without RT reaction. A portion of the cDNA was amplified with 0.5 U of Tq polymerase (PerkinElmer) and appropriate oligonucleotides at 94°C for 40 s, 60°C for 30 s, and 72°C for 40 s for 35 cycles with an initial 4 min denaturation at 95°C and final 10 min of extension at 72°C. The oligonucleotides used in this study were as follows: L-PGDS forward primer, 5′-GGTCTCCGGGAAAGAAACTG-3′; L-PGDS reverse primer, 5′-CA CGACAGGAGTTGGATGC-3′; L-PGDS forward primer, 5′-CA CGACAGGAGTTGGATGC-3′; β-actin forward primer, 5′-AGAGGG AAATCGTGCGTGAC-3′; and β-actin reverse primer, 5′-CAA TATGTGACCTGGCGGTG-3′.

**Recombinant adenovirus and luciferase assay**

Recombinant adenoviruses encoding either enhanced GFP or IκBζ532,36A and the NF-κB-luciferase reporter construct were described previously (23). DNA was transfected to RAW 264.7 cells by GenePORTER 2 (Gene Therapy Systems) for 24 h. Transfected cells were infected with the viruses with multiplicity of infection (moi) of 0.5 for 1 h. After removing viral inoculum, we added to the infected cells a fresh culture medium with LPS (1 μg/ml) to induce L-PGDS. Luciferase assay was performed with a dual luciferase kit and the manufacturer’s manual (Promega). NF-κB-driven luciferase activity was normalized with tk-Renilla luciferase activity.
Statistical analysis
For comparison among groups, paired or unpaired t tests and one-way ANOVA tests were used (with the assistance of InStat, Graphpad Software; p values <.05 are considered significant.

Results
COX-2 expression is responsible for PGD$_2$ production in macrophages

Because COX-1 and COX-2 synthesize PGH$_2$, the precursor of PGD$_2$ (24), we first examined the expression profiles of COX-1 and of COX-2 in macrophages. BMDM were prepared from mice and treated with LPS or P. aeruginosa $10^3$ (PA103). The treated cells were analyzed by Western blotting for COX-1 and COX-2 expression. As shown in Fig. 1A, LPS treatment of BMDM induced COX-2 expression (Fig. 1A, top, lanes 1 and 2). Similarly, PA103 treatment of BMDM induced COX-2 expression (Fig. 1A, top, lanes 3–6). However, neither LPS nor PA103 affected the constitutive expression of COX-1 (Fig. 1A, bottom). We also performed a similar experiment with a murine macrophage cell line, RAW 264.7 cells (Fig. 1B). Treatment of RAW 264.7 cells with LPS or PA103 induced COX-2 expression (Fig. 1B, top), but not affected the constitutive expression of COX-1 (Fig. 1B, bottom).

Next, to determine the impact of COX-2 expression on PGD$_2$ production, we treated BMDM with LPS in the presence or absence of a COX-2-specific inhibitor, CAY10404. At 12 h after LPS treatment, the cell culture supernatant was collected for measurement of PGD$_2$ released in the culture medium. As shown in Fig. 1C, the COX-2 inhibitor abolished PGD$_2$ production by BMDM, indicating that COX-2 activity is responsible for a majority of PGD$_2$ produced by macrophages after LPS treatment.

L-PGDS expression is induced by LPS or Pseudomonas treatment of macrophages

Because H-PGDS is expressed in macrophages (43), we examined whether PGD$_2$ produced by LPS treatment correlates with an increase of H-PGDS expression. BMDM were treated with LPS for various periods, and the total cell lysate of the treated cells was analyzed by Western blotting. As shown in Fig. 2A, H-PGDS was expressed constitutively, which was not altered by LPS treatment (Fig. 2, top). Rather, LPS treatment induced L-PGDS expression in macrophages (Fig. 2, middle).

To examine whether live bacteria induce L-PGDS expression, we performed a similar experiment with PA103. As shown in Fig. 2B, PA103 treatment also induced L-PGDS expression, without altering H-PGDS expression in macrophages. Together, these results reveal that L-PGDS expression is inducible by LPS or bacterial infection.

Induction of L-PGDS expression accounts for a majority of PGD$_2$ produced by macrophages

Next, to determine the impact of L-PGDS expression on PGD$_2$ production, we generated RAW 264.7-derived cell lines that encode a siRNA specific for L-PGDS mRNA to suppress L-PGDS expression epigenetically. As shown in Fig. 3, A and B, an L-PGDS siRNA cell line (denoted as silenced) blunted the induction of L-PGDS expression accounts for a majority of PGD$_2$ produced by macrophages.
of L-PGDS expression elicited by LPS without altering constitutive H-PGDS expression. With the L-PGDS siRNA cell line, we determined the production profile of PGD$_2$ (Fig. 3C). Compared with the control cell line, RAW264.7 cells stably transfected with an empty vector plasmid (denoted as normal), the silenced cell line produced significantly lower amounts of PGD$_2$. To confirm that the decrease of PGD$_2$ production was a specific effect of L-PGDS silencing, we tested three additional L-PGDS siRNA cell lines derived from RAW 264.7 cells and obtained similar results (data not shown). Because PGD$_2$ production correlated with COX-2 expression, as shown in Fig. 1C, we examined the possibility that low PGD$_2$ production results from impaired COX-2 expression in the silenced cell line. As shown in Fig. 3D, the silenced cell line expressed COX-2 to a similar level as the parental cell line, indicating that impaired COX-2 expression did not cause the low PGD$_2$ production in the silenced cell line. Together, these results suggest that L-PGDS is a key enzyme for the maximal production of PGD$_2$ in LPS-treated macrophages.

**JNK and p38 kinase are involved in L-PGDS expression**

Because LPS binding to TLR4 activates MAPKs and NF-kB (44, 45), we tested the potential role of MAPKs in L-PGDS expression. Before LPS treatment, RAW 264.7 cells were treated with various MAPK inhibitors (10 $\mu$M) for 1 h. At 16 h after LPS treatment, total cell lysate was analyzed by Western blotting for L-PGDS. As shown in Fig. 4A, the inhibitor of either JNK or p38 kinase, but not that of ERK1/2, suppressed L-PGDS induction, indicating that JNK and p38 kinase activities led to L-PGDS induction.

Next, to examine the potential role of NF-kB in L-PGDS expression, we tested whether inhibition of NF-kB activity affects L-PGDS expression. Before LPS treatment, RAW 264.7 cells were transfected with NF-kB reporter construct for 24 h and subsequently infected with a recombinant adenovirus encoding IxB$\alpha^{532,364}$, a dominant negative inhibitor of NF-kB (23). After 16 h of LPS treatment, total cell lysate was prepared for Western blot analysis of L-PGDS and for luciferase assay. As shown in Fig. 4B, suppressed NF-kB activity did not alter L-PGDS expression induced by LPS (Fig. 4B, lanes 3 and 5), suggesting that NF-kB is not involved in L-PGDS expression. To verify the efficacy of the recombinant adenovirus in suppressing NF-kB activity, we measured NF-kB-driven luciferase activity, showing suppressive effect of NF-kB-driven luciferase activity by the recombinant adenovirus (Fig. 4C). Together, these results suggest that L-PGDS expression is mediated by JNK and p38 kinase, but not by NF-kB.

**AP-1 is involved in L-PGDS expression**

Our finding that JNK and p38 MAPK were associated with L-PGDS expression suggests that a transcription factor activated by both JNK and p38 kinase is involved. To identify the transcription factor, we analyzed the promoter sequence of murine L-PGDS gene and located an AP-1 binding site from −302 to −292 nt of the promoter (Fig. 5A). Given that AP-1 is activated by both JNK and p38 (46), we performed ChIP assay to examine whether the putative AP-1 site is involved in L-PGDS expression. RAW 264.7 cells were treated with LPS in the absence or presence of 10 $\mu$M concentrations of either JNK kinase or p38 kinase inhibitor. Nuclear fraction was prepared, and DNA cross-linked to c-Jun, a subunit of AP-1, was immunoprecipitated by a c-Jun-specific Ab and amplified by PCR with the primers flanking the AP-1 site. As shown in Fig. 5B, LPS treatment induced c-Jun binding to the cognate site (lanes 1–4), which was blocked by either JNK inhibitor (Fig. 5B, comparing lanes 3 and 6) or p38 kinase inhibitor (Fig. 5B, comparing lanes 3 and 8). Together, our results indicate that AP-1 is involved in inducing L-PGDS expression.

**p53 induced by LPS suppresses L-PGDS expression**

Promoter analysis in Fig. 5A reveals that the AP-1 site was partially overlapped with a p53 binding site. Given the late induction of p53 in LPS-treated macrophages (47), we tested whether p53 functions as a competitor of AP-1. First, to examine whether p53 affects L-PGDS expression, we transfected RAW 264.7 cells with a p53-expressing plasmid, and the transfected cells were subsequently treated with LPS. Western blot analysis in Fig. 6A shows that ectopic expression of p53 suppressed L-PGDS expression (Fig. 6, lanes 2 and 4). Because it is possible that ectopic expression of p53 induces apoptosis of the transfected cells, resulting in a low expression of L-PGDS, we performed similar experiment by treating RAW 264.7 cells with doxorubicin for 6 h to induce endogenous p53, in which significant cell death did not occur (48). As shown in Fig. 6B, p53 expression induced by doxorubicin treatment blunted L-PGDS induction elicited by LPS. These results show that p53 functioned as an inhibitory factor in L-PGDS expression.

To investigate whether the overlapping binding sites of AP-1 and p53 are associated with the inhibitory function of p53 in L-PGDS expression, we performed ChIP assay for the bindings of p53 and c-Jun to their cognate sites. From RAW 264.7 cells treated with LPS, DNA bound to each protein was coprecipitated by the corresponding Abs and amplified by PCR with a set of primers encompassing both the AP-1 and p53 sites. As shown in Fig. 6C, LPS treatment induced c-Jun binding to the cognate site (Fig. 6C,
PGDS-treated mice was compared with vehicle-treated mice. After various treatments, total RNA was extracted from the lung of C57BL/6 mice and analyzed by semi-quantitative RT-PCR for L-PGDS mRNA expression (top), H-PGDS (middle), and β-actin as internal controls (bottom). To exclude a false positive by carryover genomic DNA, PCR was performed without RT (−RT; lane 10). The fidelity of PCR was verified by performing PCR with cDNAs of L- and H-PGDS and β-actin (plasmid; lane 9). Two representative results from each experimental group were shown. B, Alveolar macrophages from BAL fluid of mice were treated with LPS (1 μg/ml) or PA103 (moi 1) for 16 h. Total cell lysate was analyzed by Western blotting (WB) for L-PGDS and β-actin. The experiment was repeated three times.

FIGURE 7. Induction of L-PGDS in the inflamed lung and in alveolar macrophages. A, C57BL/6 mice (n = 4) were injected i.p. with LPS (3 μg/g of mouse) for indicated periods. After various treatments, total RNA was extracted from the lung of C57BL/6 mice and analyzed by semi-quantitative RT-PCR for L-PGDS mRNA expression (top), H-PGDS (middle), and β-actin as internal controls (bottom). To exclude a false positive by carryover genomic DNA, PCR was performed without RT (−RT; lane 10). The fidelity of PCR was verified by performing PCR with cDNAs of L- and H-PGDS and β-actin (plasmid; lane 9). Two representative results from each experimental group were shown. B, Alveolar macrophages from BAL fluid of mice were treated with LPS (1 μg/ml) or PA103 (moi 1) for 16 h. Total cell lysate was analyzed by Western blotting (WB) for L-PGDS and β-actin. The experiment was repeated three times.

L-PGDS expression is induced in the inflamed lung and in alveolar macrophages

Because the lung harbors abundant macrophages and PGD2 is implicated in lung inflammation (31–33), we examined whether L-PGDS is inducible in inflamed lung. A single i.p. injection of mice with LPS elicits lung inflammation (40). Thus, we injected C57BL/6 mice i.p. with LPS, and after various time points total RNA was extracted from the lungs of the treated mice for semi-quantitative RT-PCR analyses of L- and H-PGDS mRNA expression induced by LPS.

FIGURE 8. L-PGDS or PGD2 enhances clearance of PA103 from the mouse lung. A, Either transgenic mice (n = 4; L-PGDS T/G) or wild-type littermate (n = 3; WT) were injected i.t. 1 × 10⁶ CFU of PA103 per mouse. At 24 h after bacteria injection, total lung was harvested, and bacterial colonies were counted. Each bar represents the mean CFU (log 4/ml) ± SEM. ∗, p < 0.05 compared with wild type. B, Lung and liver of transgenic (T/G) and nontransgenic littermate control mice (WT) were obtained, and an equal amount of proteins was loaded for Western blot (WB) analysis of L-PGDS. Results of mice from three different experimental settings are shown. C, Experiment similar to A was performed by using C57BL/6 mice (n = 6). The mice were injected i.t. with either PGD2 (0.1 μg/g) or DMSO, along with 1 × 10⁶ CFU of PA103. At 24 h after i.t. injection of bacteria, bacterial colonies were counted from the lung. Bacterial colony number of PGD2-treated mice was compared with vehicle-treated mice. ∗, p < 0.05.

Next, to examine the effect of L-PGDS on Pseudomonas lung infection, transgenic mice overexpressing L-PGDS and nontransgenic littermate control mice received i.t. injection of PA103 (1 × 10⁶ CFU/mouse) without significant mortality. At 24 h after bacterial infection, lung was harvested and the bacteria in the lung were counted. As shown in Fig. 8A, the number of bacteria in the L-PGDS-transgenic mice was lower than in control mice, suggesting that the L-PGDS-transgenic mice are more effective in clearing Pseudomonas than are the control mice.

Because the L-PGDS is expressed in the lung of transgenic mice (Fig. 8B) and produce PGD2 (37), we tested whether PGD2 instillation to the lung also enhances bacterial clearance. C57BL/6 mice received i.t. injections of PA103, along with either PGD2 or vehicle (DMSO). At 24 h after bacterial injection, the bacteria in the lung were counted. As shown in Fig. 8C, PGD2 i.t. injection reduced the bacterial titer in the lung compared with the vehicle-treated mice. Together, these results suggest that L-PGDS expression or PGD2 contributes to removal of bacteria from the lung.

L-PGDS or PGD2 contributes to neutrophil influx to the lung

In an effort to determine the mechanism by which L-PGDS expression or PGD2 contributes to host defense against Pseudomonas, we tested whether L-PGDS expression or PGD2 instillation affects inflammatory cell influx to the lung. Because macrophages and neutrophils are predominant immune cells in the lung after PA103 challenge, neutrophils are critical in bacterial clearance, and delayed neutrophil infiltration is related with impaired clearance of Pseudomonas from the lung (4), we measured neutrophil influx to the lung. The L-PGDS-expressing transgenic mice and nontransgenic control mice received i.t. injection of PA103, and results show that L-PGDS expression or PGD2 contributes to neutrophil influx to the lung.
compared with wild type.

Because L-PGDS expression or PGD2 instillation to the lung enhances clearing Pseudomonas, we examined whether lack of L-PGDS impairs clearance of the bacteria from the lung. L-PGDS KO mice received i.t. injection of PA103 (Fig. 10A). For the experiment, we used a low dose of PA103 (5 × 10^5 CFU/mouse) because of a high mortality of L-PGDS KO mice when the mice received the similar dose used for transgenic mice. At 24 h after PA103 i.t. injection, the lung was harvested, and bacterial colonies were counted. As shown in Fig. 10B, L-PGDS KO mice were less effective in clearing PA103 from the lung.

To examine whether the impaired bacterial clearance is associated with disturbed neutrophil recruitment, we measured neutrophils in the lung after PA103 challenge. The L-PGDS KO mice and wild-type littermate control mice received i.t. injection of PA103 (1 × 10^6 CFU/mouse), and BAL fluid of the treated mice was obtained for neutrophil counting at 3 h after PA103 injection. As shown in Fig. 10C, the number of neutrophils in the lung of L-PGDS KO mice was lower than in wild-type controls. We performed similar experiment by injecting PA103 (5 × 10^5 CFU/mouse) for 24h. In this experiment, we lowered the dose of Pseudomonas because of lethality (data not shown). As shown in Fig. 10D, the number of neutrophils in KO mice was higher than in control mice at 24 h after PA103 challenge. There was no neutrophil in the lung without PA103 injection. These results suggest that ineffective clearance of bacteria from the lung of L-PGDS KO mice is associated with impaired recruitment of neutrophils to the lung.

**Discussion**

L-PGDS is constitutively expressed in the CNS and related organs (26) but is also detectable in the macrophages of atherosclerosis...
plaques (34). These results suggest induction of L-PGDS expression in macrophages in a pathological environment. However, the induction mechanism and the role of L-PGDS expression in macrophages are unknown. Given the noncanonical expression of L-PGDS in macrophages (34), we used various macrophages to study the mechanism of L-PGDS expression. Because PGD₂ is closely associated with lung inflammation, we used *Pseudomonas* lung infection as a model for studying the function of L-PGDS expression. Here, we found that LPS or *Pseudomonas* treatment induced L-PGDS expression in macrophages and in mice, which involved AP-1 and p53. In mice, L-PGDS expression or PGD₂ treatment facilitated removal of *Pseudomonas* from the mouse lung. Together, our study suggests that L-PGDS expression is induced by inflammatory stimuli and contributes to host defense against bacterial lung infection.

Our results show induction of L-PGDS expression by inflammatory stimuli. Given that atherosclerosis involves inflammation (49), our results suggest that L-PGDS expression in the macrophages of the plaques is likely due to inflammatory stimuli in the atherosclerotic plaques. Because our results show that COX-2 expression directly affects the amount of PGD₂, it is conceivable that induced L-PGDS plays a role for coping with a surge of PGH₂ due to induction of COX-2 expression in macrophages activated by inflammatory stimuli.

COX-2 expression in macrophages occurs early after LPS treatment and involves NF-κB that is rapidly activated by LPS (23). L-PGDS expression in macrophages was detectable 8 h after LPS treatment and involved AP-1 rather than NF-κB. Therefore, it is plausible that macrophages express COX-2 before L-PGDS expression by differentially using key transcription factors, ensuring that a sufficient amount of PGH₂ is available for L-PGDS to produce PGD₂. We also show that p53 suppresses L-PGDS expression by competing with AP-1 for the partially overlapping AP-1 and p53 binding sites, to which AP-1 and p53 bind sequentially after LPS treatment. Thus, our results uncovered a unique self-regulatory mechanism of L-PGDS expression.

It is not clear why neuronal cells express constitutively L-PGDS, whereas macrophages did so in a signal-dependent manner. Given that AP-1 is expressed in various cell types and activated by various stimuli, it is possible that neuronal cells express AP-1 abundantly and constitutively, resulting in the constitutive expression of L-PGDS. In RAW 264.7 cells, AP-1 expression was low but increased significantly by LPS treatment (data not shown), which may explain, at least in part, the signal dependent expression of L-PGDS in macrophages. Alternatively, unlike macrophages, a signaling or stimulation peculiar to neuronal cells causes a constant binding of AP-1 to L-PGDS promoter.

As shown in Fig. 7, L-PGDS mRNA expression was induced in mouse lung challenged with LPS. In addition, alveolar macrophages extracted from mouse lung also expressed L-PGDS when treated with either LPS or *Pseudomonas*. Considering that macrophages are a major responder to LPS (5), it is likely that alveolar macrophages are largely responsible for L-PGDS expression in the LPS-treated lung. Although it is possible that neutrophils infiltrated to the lung also contribute to L-PGDS expression, it is notable that neutrophils activated by various inflammatory stimuli including LPS do not produce a detectable level of PGD₂ (50). Currently, we are investigating whether or not other lung cells including epithelial cells express L-PGDS.

Effective bacterial clearance by L-PGDS-overexpressing transgenic mice and PGD₂-treated mice seemed to be associated with neutrophil recruitment. Supportive to this notion, L-PGDS KO mice that were less effective in clearing bacteria than control mice showed impaired neutrophil recruitment at an early time point after *Pseudomonas* challenge. Neutrophils are professional phagocytic cells that clear bacterial pathogens including *Pseudomonas* from the lung, and thus impaired recruitment of neutrophils undermines the capability of a host to remove *Pseudomonas* from the lung (4). Therefore, it is possible that L-PGDS expression and thereby increased PGD₂ production during lung inflammatory process facilitate neutrophil recruitment, contributing in part to effective removal of bacterial pathogens.

The effect of PGD₂ on lung inflammation is complex because PGD₂ either promotes or suppresses inflammation depending on the inflammatory milieu (24, 51–54). The net effect of PGD₂ could be more complicated because of the fact that PGD₂ undergoes nonenzymatic processes to generate 15d-PGJ₂, an anti-inflammatory lipid (55, 56). In RAW 264.7 cells, 15d-PGJ₂ was composed of approximately one-tenth of PGD₂ in cell culture medium (data not shown). 15d-PGJ₂ suppresses NF-κB activity by forming an adduct with NF-κB and IKK (57, 58) and by activating peroxisome proliferator-activated receptor-γ (59, 60). PGD₂ exerts its effect by binding to its receptors, PGD₂ receptor (DP; Ref. 61) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) receptor (62). 15d-PGJ₂ also binds to these receptors with a similar avidity (63). Although associated with anti-inflammatory function of PGD₂ in vitro studies, DP receptor is related with the proinflammatory function of PGD₂ in many in vivo experimental settings (64). Alternatively, CRTH2 is mostly associated with proinflammatory function of PGD₂ (64). In mice, DP receptor is expressed in various tissues including lung (64), whereas CRTH2 expression is mostly confined to hematopoietic cells such as Th2 cells, eosinophils, basophils, mast cells, and a subset of monocytes (64). Given that *Pseudomonas* lung infection predominantly elicits an influx of neutrophils in the lung (4) and that alveolar macrophages are critical in removing bacteria including *Pseudomonas* (2–4), it is possible that, in conjunction with *Pseudomonas*, PGD₂ delivered to the lung acts on the DP receptor of alveolar macrophages, increasing innate immune activity. In light of the role of lung epithelial cells against *Pseudomonas* lung infection (65), it is also plausible that PGD₂ binds to the DP receptor of both alveolar macrophages and lung epithelial cells, cooperatively clearing *Pseudomonas* from the lung. Our results did not exclude a possible involvement of anti-inflammatory 15d-PGJ₂ by directly inactivating NF-κB activity. Given the fact that a significantly high amount of 15d-PGJ₂ is required to execute these activities (57, 66), it is likely that 15d-PGJ₂ generated from PGD₂ could bind to DP receptor, contributing to PGD₂ effect in our experimental settings.

Given the induction of L-PGDS expression in macrophages by *Pseudomonas* treatment, it is likely that *Pseudomonas* infection induces L-PGDS expression in the lung as well. However, *Pseudomonas* lung infection also induces COX-2 expression in mice (67) and, as a result, produces not only PGD₂ but also other prostaglandins including PGE₂ (67, 68). Increase of PGE₂ production in the lung is associated with bacterial pneumonia (69). A seminal study shows that PGE₂ inhibits the phagocytic activity of alveolar macrophage (70). In addition, PGE₂ suppresses production of reactive oxygen species (67). These results suggest that PGE₂ exacerbates *Pseudomonas* infection. Given that inhibition of COX-2 protects mice from *Pseudomonas* infection (67, 71), it is possible that PGE₂ effects prevail over that of PGD₂. However, in light of our results showing the protective effect of PGD₂ against *Pseudomonas* lung infection, it is conceivable that PGD₂ generated during bacterial pneumonia counterbalances the otherwise detrimental effect of PGE₂, contributing to successful clearance of bacteria.

The purpose of our study is to elucidate the mechanism and the role of L-PGDS expression in macrophages. Our results revealed...
the self-regulatory mechanism of L-PGDS induction by AP-1 and p53 in inflammatory environment and the protective role of L-PGDS in inflammation and host defense against bacterial infection. Our results suggest that L-PGDS or PGD2 may be used for a treatment of infection by bacteria highly resistant to antibiotics such as P. aeruginosa.

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