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E-Prostanoid 3 Receptor Deletion Improves Pulmonary Host Defense and Protects Mice from Death in Severe Streptococcus pneumoniae Infection

David M. Aronoff,‡ Casey Lewis,† Carlos H. Serezani,‡ Kathryn A. Eaton,‡ Deepi Goel,§ John C. Phipps,§ Marc Peters-Golden,† and Peter Mancuso§

Prostaglandins (PGs) are potent lipid mediators that are produced during infections and whose synthesis and signaling networks present potential pharmacologic targets for immunomodulation. PGE₂ acts through the ligation of four distinct G protein-coupled receptors, E-prostanoid (EP) 1–4. Previous in vitro and in vivo studies demonstrated that the activation of the Gαs-coupled EP2 and EP4 receptors suppresses inflammatory responses to microbial pathogens through cAMP-dependent signaling cascades. Although it is speculated that PGE₂ signaling via the Gαs-coupled EP3 receptor might counteract EP2/EP4 immunosuppression in the context of bacterial infection (or severe inflammation), this has not previously been tested in vivo. To address this, we infected wild-type (EP3+/+) and EP3−/− mice with the important respiratory pathogen Streptococcus pneumoniae or injected mice i.p. with LPS. Unexpectedly, we observed that EP3−/− mice were protected from mortality after infection or LPS. The enhanced survival observed in the infected EP3−/− mice correlated with enhanced pulmonary clearance of bacteria; reduced accumulation of lung neutrophils; lower numbers of circulating blood leukocytes; and an impaired febrile response to infection. In vitro studies revealed improved alveolar macrophage phagocytic and bactericidal capacities in EP3−/− cells that were associated with an increased capacity to generate NO in response to immune stimulation. Our studies underscore the complex nature of PGE₂ immunomodulation in the context of host-microbial interactions in the lung. Pharmacological targeting of the PGE₂-EP3 axis represents a novel area warranting greater investigative interest in the prevention and/or treatment of infectious diseases. The Journal of Immunology, 2009, 183: 2642–2649.

A subset of patients with community-acquired pneumonia will develop severe infection requiring intensive medical therapy for complications such as respiratory failure and sepsis. For these reasons, pneumonia ranks among the 10 leading causes of mortality in the United States (1). Streptococcus pneumoniae is the most frequently isolated pathogen in severe and uncomplicated community-acquired pneumonia (2), accounting for nearly 1.6 million deaths annually (3)—more than any other bacterium (4). A better understanding of the immunological factors governing the development of pneumococcal pneumonia and its complications is needed to improve both preventive and therapeutic strategies.

PGs are small lipid molecules derived from the cyclooxygenase-dependent metabolism of the cell membrane fatty acid constituent, arachidonic acid. The PGs are recognized as potent mediators of both innate and acquired immunity. An increasingly large body of evidence suggests key roles for PGs in the modulation of pulmonary host defense (5–7). PGE₂ is synthesized in abundance both locally (in infected tissues, including the lung (8)) and systemically (e.g., within the brain) in response to microbial invasion, in which it exerts myriad effects on physiological and immunological functions (9, 10). PGE₂ can exert both pro- and anti-inflammatory effects on the immune response (11–13). The latter actions counterregulate host inflammatory responses, potentially limiting collateral damage to neighboring cells and tissues and aiding in the resolution of inflammation once pathogens are contained (14). Specifically, PGE₂ has been shown to inhibit the ability of alveolar macrophages to phagocytose and kill respiratory pathogens (5, 15), and to generate proinflammatory signaling molecules such as TNF-α (16). Although such effects might benefit the immunocompetent host, data suggest that the exaggerated production of PGE₂ in certain clinical circumstances may contribute to an enhanced susceptibility to respiratory infections. For example, excessive PGE₂ generation was reported to be an important determinant of risk for bacterial lung infection following hematopoietic stem cell transplantation in lethally irradiated mice (6).

The actions of PGE₂ follow the activation of four distinct cell membrane-associated G protein-coupled receptors, termed E-prostanoid (EP) 1, EP2, EP3, and EP4. EP1 receptor activation provokes Gαs-coupled increases in intracellular Ca²⁺; EP2 and EP4 receptors signal predominantly through Gαq, increasing cAMP; and the EP3 receptor most often reduces cAMP via Gαi coupling (17). EP3-based signaling is complicated by the existence of several receptor variants generated through the alternative splicing of mRNA transcripts (18).

In general, the suppressive effects of PGE₂ on leukocyte function follow the activation of the Gαq-coupled EP2 and EP4 receptors (19), whereas EP1 and EP3 receptors have been implicated in

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3 Abbreviations used in this paper: EP, E-prostanoid; BAL, bronchoalveolar lavage; BAF, BAL fluid; H2DCF, 2′,7′-dichlorodihydrofluorescein diacetate; i.t., intratracheal; KO, knockout; ROI, reactive oxygen intermediate; Tb, core body temperature; WT, wild type.

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Macrophages were then infected with a 0.1-ml suspension of opsonized bacteria, prepared as described previously (26), were seeded in duplicate. Animals were treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

Because EP2 and EP4 activation suppresses pulmonary host defenses through increases in cAMP (5–7), whereas EP3 receptor signaling inhibits adenylyl cyclase activity (and might therefore support antimicrobial defense mechanisms), we hypothesized that the selective deletion of the EP3 receptor would worsen the ability of infected mice to handle microbial invasion of the lungs. A worsening of host defenses against bacterial infection in EP3 null mice caused by S. pneumoniae was unexpected results were revealed by these investigations, in that EP3-/- mice were protected from pneumococcal mortality. Further investigations were performed to define the physiological and immunological host responses governed by PGE2-EP3 signaling. Our studies underscore the complex nature of PGE2 immunomodulation in the context of host-microbial interactions in the lung and the importance of correlating in vitro findings with in vivo experiments.

Materials and Methods

Animals

Female mice with a targeted disruption of the EP3 gene (20) backcrossed over 10 generations onto a C57BL/6 background (EP3 +/- mice) were a gift of S. Narumiya (Kyoto University, Kyoto, Japan) and were obtained from Ono Pharmaceuticals and bred in the University of Michigan Unit for Laboratory Animal Medicine. Age- and sex-matched WT C57BL/6 mice (EP3 +/- mice) were purchased from The Jackson Laboratory. Animals were treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

Reagents

RPMI 1640 cell culture medium and penicillin/streptomycin/amphotericin B solution were purchased from Life Technologies-InVitrogen. Tryptic soy broth was supplied by Difco. Cytochalasin D, saponin, and MTT were purchased from Sigma-Aldrich. The 2',7'-dichlorodihydrofluorescein diacetate (H2DCF) was from Invitrogen. Other reagents are listed below.

Isolation and culture of alveolar macrophages

Resident alveolar macrophages from mice were obtained via lung lavages, as previously described (23), and resuspended in RPMI 1640 to a final concentration of 1 x 10^6 cells/ml. Cells were allowed to adhere to tissue culture-treated plates for 1 h (37°C, 5% CO2), followed by two washes with warm RPMI 1640. Cells were cultured overnight in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin/amphotericin B (antibiotics) before use. The following day, cells were washed twice with warm medium to remove nonadherent cells.

Tetrazolium dye reduction assay of bacterial killing

The ability of bacteria to survive within the alveolar macrophage was quantified using a tetrazolium dye reduction assay, as described elsewhere (24, 25). In this assay, bacterial growth is determined colorimetrically based on the ability of live bacteria to convert MTT to a purple formazan salt that absorbs light at 595 nm (A595). Briefly, 2 x 10^7/ml mouse alveolar macrophages, prepared as described previously (26), were seeded in duplicate 96-well tissue culture dishes. The next day, S. pneumoniae were opsonized with 10% normal rat-derived immune serum, as previously described (26). Macrophages were then infected with a 0.1-ml suspension of opsonized S. pneumoniae (2 x 10^7 CFU/ml; multiplicity of infection, 100:1; Ref. 27) for 120 min to allow phagocytosis to occur. The bacterial killing protocol was assessed, as described elsewhere (24, 25). The intensity of the A595 was directly proportional to the number of intracellular bacteria associated with the macrophages (24). Results were expressed as percentage of survival of ingested bacteria, in which the survival of ingested bacteria = 100% - A595 control (phagocytosis) plate/A595 experimental (phagocytosis + killing) plate.

Fluorometric assay of alveolar macrophage phagocytosis

The ability of alveolar macrophages to phagocytose S. pneumoniae was assessed using a previously published protocol for determining the ingestion of fluorescent, FITC-labeled bacteria (5). Briefly, heat-killed S. pneumoniae serotype 3 were labeled with FITC, as previously described (28). A total of 1.5 x 10^8 murine alveolar macrophages was obtained from the bronchoalveolar lavage (BAL) fluid (BALF) of naive mice and seeded in replicates of 24 in 384-well tissue culture plates with opaque sides and optically clear bottoms (Costar, Corning Life Sciences). On the following day, FITC S. pneumoniae were opsonized with 10% normal rat-derived nonimmune serum. Macrophages were then infected with FITC S. pneumoniae using a multiplicity of infection of 150:1 for 180 min to allow phagocytosis to occur. Trypan blue (250 µg/ml; Molecular Probes) was added for 10 min to quench the fluorescence of extracellular bacteria, and fluorescence was determined using a Spectramax Gemini EM fluorometer (Molecular Devices). The phagocytic index was calculated, as previously described, in relative fluorescence units (5).

Measurement of reactive oxygen intermediates

Alveolar macrophages were adhered to 384-well plates at a concentration of 1.25 x 10^5 cells/well and cultured overnight in RPMI 1640 containing 10% FCS and antibiotics. On the next day, the medium was replaced with PBS containing 10 µM H2DCF and the cells were cultured for 1 h. The medium was then replaced with warm HBSS, and the cells were stimulated with heat-killed S. pneumoniae using a multiplicity of infection of 50:1. Reactive oxygen intermediate (ROI) production was assessed every 30 min for 2 h by measuring fluorescence using a Spectramax Gemini XS fluorometer (Molecular Devices) with excitation/emission setting at 493/522 nm.

NO production

Alveolar macrophages were adhered to 96-well plates at a concentration of 2 x 10^5 cells/well and cultured with DMEM supplemented with 1% sodium pyruvate (Invitrogen) containing 10% FCS and penicillin/streptomycin with or without 10 µg/ml lipoteichoic acid from Staphylococcus aureus (Sigma-Aldrich) and 10 ng/ml IFN-γ (R&D Systems) for 24 h. NO production was determined by measuring stable nitrite (NO2-) concentrations using a modified Griess reaction with a commercially available assay kit, according to the manufacturer’s instructions (Cayman Chemical).

S. pneumoniae infection

S. pneumoniae serotype 3, 6303 (American Type Culture Collection), was used for these studies. The virulence of this organism was maintained by subculturing bacteria obtained from the spleens of bacteremic mice and storing them at -80°C until use. Bacteria were then grown in Todd Hewitt broth medium (BD Biosciences) at 37°C in 5% CO2 to mid-log phase. The virulence of this organism was maintained by subculturing bacteria obtained from the spleens of bacteremic mice and storing them at -80°C until use. Bacteria were then grown in Todd Hewitt broth medium (BD Biosciences) at 37°C in 5% CO2 to mid-log phase. The bacteria were pelleted by centrifugation (16,000 x g for 5 min) and washed twice in endotoxin-free PBS (Invitrogen), and the concentration of bacteria was determined spectrophotometrically (A600). The S. pneumoniae concentration was confirmed by plating serially diluted bacteria on soy-based blood agar plates (Difco). Mice were anesthetized with an i.p. injection of 100 mg/ml ketamine (Ketaset; Fort Dodge Animal Health) and 20 mg/ml xylazine (Anased; Lloyd Laboratory) together at a dose of 10 µl/g mouse. A small incision was made to expose the trachea, and the bacterial inoculum (10^7 CFUs suspended in 30 µl) was injected intratracheally (i.t.) using a 26-gauge needle. The incision was closed using 3M Vetbond (3M Animal Care Products).

BAL and cell counts

Twenty-four and 48 h postinfection, lung leukocytes were obtained from mice by BAL following CO2 asphyxiation, as previously described (29). Blood was collected by cardiac puncture for peripheral white blood cell counts and hematocrit levels using a Hemavet cell analyzer (Drew Scientific) operated by the University of Michigan Unit for Laboratory Animal Medicine Animal Diagnostic Laboratory.
Serum lactate levels

Lactate levels were determined using the EnzyChrom lactate assay kit (Bioassay Systems) on heparinized blood samples collected from mice anesthetized with isoflurane (AErrane; Baxter) 24 h following i.t. S. pneumoniae challenge.

Histology

At 48 h postinfection, lungs were harvested, perfused i.t. with 10% buffered formalin, and immersed in formalin (McClinchey Histology Laboratory). Lungs were embedded in toto in paraffin, and 5 μm H&E-stained sections of all lung lobes were examined blindly without knowledge of their source. Histologic lesions in the lungs consisted of multifocal neutrophilic to suppurrative interstitial pneumonia that ranged from mild to focally severe; multifocal atelectasis; and, in (some cases) pulmonary edema. Pleural lesions consisted of fibrinonecrotizing and suppurrative pleuritis that ranged from mild to severe. Pneumonia and pleuritis were scored separately on a semiquantitative scale in which 0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation, and 3 = severe inflammation.

Temperature measurements

Murine core body temperatures (Tₜ) were assessed using a MicroTherma 2T handheld thermometer (Braintree Scientific) by inserting the probe 3–4 cm up from the anus at several time points (0, 30, 60, 120 min) after LPS injection and at 24 and 48 h postinfection.

Myeloperoxidase activity assay

The presence of myeloperoxidase in lung homogenates was determined 48 h after infection. Lung tissue was homogenized in sterile PBS after the tissues were perfused with sterile PBS to eliminate myeloperoxidase activity in contaminating blood. The homogenized tissue was stored at −80°C to lyse the cells. After thawing, the homogenates were centrifuged for 30 min at 10,000 × g at 4°C. Supernatants were used to determine myeloperoxidase levels (in ng/ml) using a commercially available kit, according to the manufacturer’s instructions (EnzChek myeloperoxidase activity kit; Molecular Probes).

Lung, spleen, or serum cytokine, chemokine, and PGE₂ determination

Lung homogenates, obtained from mice 48 h after pneumococcal infection, and blood harvested from mice 5 h after LPS injection were evaluated for cytokines (IL-1β, TNF-α, IL-10, IL-12 p70, IL-6, and MCP-1) by ELISA (R&D DuoSet; R&D Systems) by the University of Michigan Cancer Center Cellular Immunology Core. PGE₂ levels in lung homogenates were determined by an ELISA kit, according to the manufacturer’s instructions (Assay Designs).

LPS injection

LPS (10 mg/kg) from Escherichia coli serotype 0111:B4 (Sigma-Aldrich) was reconstituted in PBS and administered to mice via an i.p. injection. Mice were monitored over a 7-day period for survival analysis (n = 15 per group). In other animals, blood was collected for cytokine and chemokine analysis 2 h (n = 5 per group) or 5 h (n = 10 per group) after LPS injection. The peritoneal cavity of separate cohorts of mice (n per group) were used to determine myeloperoxidase levels (in ng/ml) using a commercially available kit, according to the manufacturer’s instructions (EnzChek myeloperoxidase activity kit; Molecular Probes).

Evans blue dye method for measuring vascular permeability

Vascular leak induced by LPS was estimated based on the extravasation of Evans blue dye (30). Six hours after i.p. challenge with LPS (10 mg/kg), mice received a 30 mg/ml solution of Evans blue dye i.v. (30 mg/kg via the jugular vein). Thirty minutes later, mice were euthanized and perfused with 5 ml of ice-cold PBS via the left ventricle. The lungs and kidneys were harvested, weighed, and homogenized in 1 ml of PBS. Organ homogenates were then incubated with 2 ml of formamide at 60°C for 18 h, and then centrifuged at 5000 × g for 30 min. The absorbance of the Evans blue in the supernatants was spectrophotometrically measured at 620 nm, whereas the absorbance of contaminating heme pigments was determined at 740 nm. The following formula was used to correct Evans blue absorbance for the presence of heme (Aₑ₆₂₀): Aₑ₆₂₀ (corrected) = Aₑ₆₂₀ (1.426 × Aᵥ₇₄₀ + 0.0130). Data were expressed in absorbance units per gram tissue.

Statistical analysis

Survival was evaluated for differences using a log-rank test. Where appropriate, mean values were compared using paired Student’s t test or a one-way ANOVA, followed by the Bonferroni post hoc test for mean separation. Differences were considered significant if p < 0.05. All experiments were performed on at least three separate occasions, unless otherwise specified. Data are presented as mean values ± SEM, unless otherwise noted.

Results

EP3⁻/⁻ mice are protected from death following S. pneumoniae infection

To examine the role of PGE₂ EP3 signaling in host defense against bacterial infections of the lungs, EP3−/− mice or WT C57BL/6 mice (EP3+/+) were infected i.t. with 10⁵ CFU of S. pneumoniae. As demonstrated (Fig. 1A), infected EP3−/− mice experienced a significantly lower 10-day mortality (56%) than did infected EP3+/+ animals (90%; p < 0.05).

Pulmonary clearance of S. pneumoniae is enhanced in EP3−/− mice

The difference in observed mortality between EP3+/+ and EP3−/− animals could reflect the fact that PGE₂ EP3 receptor signaling modulates the local immune clearance of pneumococcal organisms or limits bacterial dissemination to distant sites. To test these possibilities, bacterial burdens were measured in the lung and spleen of infected animals 24 and 48 h following i.t. inoculation. These time points were selected to allow measurements before the onset of animal mortality. As shown (Fig. 1B), at 48 h after infection the EP3−/− mice exhibited significantly better lung clearance of S. pneumoniae (2.39 × 10⁷ CFU/g lung tissue) than did infected WT animals (3.8 × 10⁷ CFU/g lung tissue; p < 0.05). There was a nonsignificant trend toward better clearance at the 24-h time point as well (mean CFU/g lung tissue of 1.2 × 10⁶ in EP3+/+ mice vs 3.98 × 10⁶ in EP3−/− mice; p = 0.28), whereas there were no differences in splenic bacterial loads (Fig. 1B).

Pulmonary inflammatory responses are similar in EP3−/− and WT mice

Data from the human U937 monocyte cell line suggest that PGE₂ EP3 signaling enhances leukocyte migration (31). Conversely, PGE₂ impairs neutrophil migration (32) primarily as a result of EP2 activation, with a possible contribution of EP3 (33). To assess the influence of EP3 in phagocyte influx into infected alveoli, we performed BAL on mice 48 h after inoculation with S. pneumoniae. There were nonsignificant trends toward reduced numbers of neutrophils and macrophages in the air spaces of EP3−/− mice (Fig. 2A), whereas the percentage of neutrophils and macrophages did not differ between knockout (KO) and WT mice (Fig. 2B). These similarities were confirmed through histopathological determinations of inflammatory infiltrates that also showed no significant difference in severity of pneumonia (Fig. 2C). It was noted that...
inflammatory infiltrates were more prominent in peripheral (pleural) regions in the EP3−/− mice compared with WT animals (Fig. 4, A and B). Interestingly, the amount of myeloperoxidase enzyme activity in the lung homogenates of infected animals (a surrogate marker for the presence of neutrophils) was significantly reduced in the EP3−/− mice compared with EP3+/+ controls, suggesting differences in neutrophil ingress into the pulmonary interstitial spaces rather than into alveoli (Fig. 2D).

**Improved phagocytosis and killing of S. pneumoniae by alveolar macrophages from EP3−/− mice**

Alveolar macrophages are a critical defender against S. pneumoniae in murine models of infection (34, 35). The capacity of alveolar macrophages to phagocytose and kill bacterial pathogens is subject to regulation by PGE2 (5, 15). We therefore infected alveolar macrophages from WT and EP3−/− mice ex vivo, to compare both phagocytosis and bacterial killing. The capacity of EP3-deficient alveolar macrophages to internalize serum-opsonized S. pneumoniae was ~10% greater than WT cells (Fig. 2E; p < 0.05). Furthermore, bacterial killing was enhanced in the EP3−/− macrophages, as shown in Fig. 2F. In these studies, bacterial survival within alveolar macrophages was ~57% greater in the WT cells than in EP3−/− cells (p < 0.05).

**NO production is enhanced in EP3−/− alveolar macrophages**

To address potentially causal mechanisms explaining the increased bacterial killing in EP3 null cells, alveolar macrophages were assessed for their ability to generate ROIs and NO in response to inflammatory stimuli. As shown (Fig. 3A), the production of ROIs by EP3+/+ and EP3−/− macrophages was equally robust upon exposure to S. pneumoniae. The capacity for alveolar macrophages to generate NO (determined as the stable nitrite, NO2−) was determined by stimulating the cells with a combination of IFN-γ and lipoteichoic acid (Fig. 3B). Although basal production was similar, EP3 null cells generated significantly greater quantities of NO2− than did WT cells (54.5 ± 1.76 μM vs 35.74 ± 1.11 μM, respectively; p < 0.001), with WT levels.

**Local and systemic inflammatory mediators are similar in infected EP3−/− and EP3+/+ mice**

PGE2 regulates the production of cytokines and chemokines during infection and inflammation primarily via EP2 and EP4 receptors activation (36, 37). In the absence of intact PGE2-EP3 communication, the pulmonary (Fig. 4, C and D) and splenic (data not shown) production of inflammatory mediators did not significantly differ after infection. There was a trend toward lower levels of TNF-α, IL-10, and IL-12 in the lung homogenates of EP3−/− animals. As expected, PGE2 levels were higher in the lungs (230.9 ± 37.7 ng/ml in WT and 241.5 ± 18.3 ng/ml in EP3−/− mice) than in the spleens (150.3 ± 5.9 ng/ml in WT and 134 ± 11.7 ng/ml in EP3−/− mice) of infected animals 48 h after infection (n = 5), but the differences between WT and EP3−/− mice were not significant.

**Circulating leukocyte levels are lower in EP3−/− mice than in EP3+/+ mice during infection**

The acute elevation of circulating white blood cell numbers, particularly neutrophils, is a usual response to moderate or severe bacterial infections. The blood leukocyte counts were determined in uninfected and infected animals to identify correlates of survival in the EP3−/− mice (Fig. 5). Basal counts in uninfected animals were similar. At 24 h postinfection, the total leukocyte count of WT mice did not appreciably change, although there were decreases in lymphocytes and increases in neutrophils in this group. Notably, circulating neutrophil and lymphocyte counts decreased significantly 24 h after infection in EP3−/− mice (Fig. 5B). No changes were observed in RBC or platelet numbers (data not shown).

**Serum lactate levels and acute weight loss are similar in EP3 null and WT mice**

Tissue hypoxia during streptococcal sepsis may cause an elevation in serum lactate levels (38). The amount of tissue hypoxia induced...
by pneumococcal infection, as assessed by lactate determination, was similar in EP3−/− and EP3+/+ mice (Fig. 6A). Sickness in mice often results in reduced food intake and weight loss. Although both EP3 null and WT mice lost weight over the first 48 h after infection, there was no difference in this indicator of illness severity (Fig. 6B).

**EP3−/− mice have an impaired febrile response to pneumococcal infection**

The febrile response to inflammatory noninfectious stimuli (such as LPS or proinflammatory cytokine injection) has been shown to depend on the presence of EP3 receptors in the CNS (9). However, the role of this receptor in mediating fever during actual infection with a live pathogen has not been demonstrated. Therefore, the core Tb in mice was measured before and during infection (Fig. 6C), revealing that whereas WT mice exhibit an increase in Tb 24 h after inoculation, mice lacking intact PGE2-EP3 circuitry had a nonsignificant tendency toward hypothermia at the same time point. By 48 h, Tb values were similar in surviving animals from both groups (data not shown).

**EP3−/− mice are protected from death following systemic endotoxin exposure**

To determine whether the beneficial effects of EP3 receptor deletion on mortality were limited to live infection or might be true in the face of overwhelming, noninfectious inflammation, mice were injected i.p. with sterile LPS (10 mg/kg). As demonstrated in Fig. 7A, ablation of PGE2-EP3 signaling dramatically improved animal survival in the face of severe endotoxin exposure. Consistent with this observation, EP3−/− mice exhibited similar degrees of tissue hypoxia and weight loss as WT mice, but had an impaired febrile response to infection. A, Twenty-four hours after infection, serum lactate levels were measured in WT and EP3 KO mice. B, Weights were determined for mice before and 24 and 48 h after infection. **, p < 0.01; *, p < 0.05 compared with basal weight. C, Core body temperature was measured, as detailed in the text, for WT and KO mice at time 0 and 24 h after pneumococcal infection. ***, p < 0.01 compared with time 0.
The inflammatory response to LPS exposure is blunted in EP3−/− mice

Whereas significant differences in inflammatory mediators were not observed between EP3−/− and WT mice during a local bacterial infection, it was unknown whether such differences might become evident (and significant) in an acute systemic inflammatory insult such as i.p. LPS injection. Notably, the mean serum concentration of TNF-α was significantly lower in the EP3−/− mice 5 h after LPS exposure compared with the EP3+/+ mice, with mean (±SEM) values of 326.6 ± 47.4 pg/ml vs 518.4 ± 60.8 pg/ml, respectively (p < 0.05; n = 10 mice per group; Fig. 7C). There were nonsignificant trends toward lower levels of several other mediators in the KO animals (Fig. 7C). When the same mediators were compared in the peritoneal cavity of LPS-injected mice (Fig. 7D), no significant differences were observed between mice having the EP3 receptor or not.

When serum was examined at an earlier time point (2 h after LPS injection), we observed significantly lower levels of TNF-α in the EP3−/− (497.0 ± 20.1 pg/ml) vs EP3+/+ animals (1363 ± 206.0 pg/ml; p < 0.01); lower levels of IL-1β in the EP3−/− (134.3 ± 6.6 pg/ml) vs EP3+/+ animals (182.5 ± 14.3 pg/ml; p < 0.05); and lower levels of IL-10 in the EP3−/− (275.0 ± 30.3 pg/ml) vs EP3+/+ animals (696 ± 123.0 pg/ml; p < 0.05; n = 5 mice per group; data not shown). Other mediators were not different in the serum, and no mediators significantly differed between the two mouse strains when measured in the peritoneal fluid (data not shown).

Vascular permeability during LPS-induced shock is not influenced by the EP3 receptor

The enhanced survival observed in EP3 null mice in the face of overwhelming inflammatory insult with high dose LPS suggests a possible role for the EP3R in regulating the vascular leak observed in this setting. To test this, EP3+/+ and EP3−/− mice were administered LPS (10 mg/kg i.p.), and the extravasation of Evans blue dye was measured in the lungs and kidneys 6 h later as a surrogate marker of vascular leak (Fig. 7B). No differences between genotypes were observed.

Discussion

The lipid mediator PGE₂ has been implicated in the regulation of both innate and adaptive responses to microbial invasion (7, 39, 40). The diverse immunoregulatory effects of PGE₂ result in part from the presence of four specific G protein-coupled receptors that signal in distinct fashions on a range of immune effector cells. Most studies of PGE₂ immune regulation have focused on the two receptors that primarily alter cell function through increases in cAMP concentration, namely EP2 and EP4. As a ubiquitous second messenger, cAMP has been extensively characterized for its capacity to blunt immune responses and suppress host defenses against infection (41). Less is known about the remaining two EP receptors, the Gq-coupled EP1 and the Gαi-coupled EP3, in regard to the regulation of immune defense systems.

The present studies represent the first attempt, to our knowledge, to identify a role for the EP3 receptor in the regulation of host-bacterial interactions in vivo. Because the EP3 receptor predominantly signals through the Gq protein-coupled inhibition of adenylyl cyclase (19), we questioned whether the ablation of EP3 signaling would unmask EP2- and/or EP4-dependent immunosuppressive mechanisms, resulting in a worse outcome for infected EP3−/− mice. The unexpected answer to this question, under the conditions of our murine pneumonia model, was no. Instead, EP3 receptor deletion protected mice against death from either infectious or noninfectious inflammatory insults.

The enhanced survival observed in the EP3−/− mice correlated with several potentially important differences in physiological and immune parameters. These included enhanced pulmonary clearance of bacteria with improved alveolar macrophage phagocytic and bactericidal capacities; reduced accumulation of lung neutrophils; lower numbers of circulating blood leukocytes; and an impaired febrile response to infection. It is speculative that a combination of effects such as these promoted the recovery of EP3 null animals in the face of severe bacterial infection.

A striking finding was the influence of EP3 signaling on alveolar macrophage-pneumococcal interactions. The impact of the observed increase in phagocytic capacity of the KO macrophages (~10% greater than WT cells) might be irrelevant. However, this effect is amplified by a nearly 60% better killing capacity in the null macrophages (Fig. 2, E and F). These actions most likely contributed to the enhanced pulmonary bacterial clearance observed in vivo.

The observation that EP3 signaling in macrophages significantly limits bacterial killing is novel. To identify potential intracellular targets of EP3 signaling involved in this effect, we investigated two important bactericidal mechanisms: the generation of ROIs and NO by activated macrophages. Both ROIs (8, 42) and NO...
have been implicated in controlling *S. pneumoniae* infection (43, 44).

Through EP2- and EP4-mediated increases in cAMP, PGE$_2$ prevents the activation of the ROI-generating enzyme NADPH oxidase complex in alveolar macrophages (15), an effect noted previously in a Gram-negative infection model (15). Studies were conducted to identify an influence of the EP3R on alveolar macrophage ROI generation in response to pneumococcal challenge, but no effect was revealed (Fig. 3A). In addition, exogenously added PGE$_2$ (1 μM) inhibited ROI generation equally well in both EP3$^{-/-}$ and EP3$^{+/+}$ cells when challenged with *S. pneumoniae* (data not shown).

To compare the capacities of WT and EP3 null alveolar macrophages to generate reactive nitrogen species, we measured NO in response to immune stimulation using IFN-γ and the Gram-positive bacterial cell wall component lipoteichoic acid, both potent stimulators of macrophage NO (45). We made the novel observation that cells lacking EP3 receptor generated significantly more NO than did WT cells in response to immune stimulation (Fig. 3B). We similarly observed significantly more NO production by EP3$^{-/-}$ alveolar macrophages in response to either LPS plus IFN-γ or IFN-γ alone (data not shown). Future studies are needed to define the mechanisms responsible for the negative influence of EP3 signaling on NO generation. It is notable that PGE$_2$, through cAMP-dependent signaling, has been shown to enhance the expression of the inducible NO synthase enzyme in macrophages (46), suggesting that unchecked EP2 and/or EP4 signaling (through cAMP) might be responsible for the present findings.

The fact that EP3 null mice exhibited better survival in both infectious (*S. pneumoniae*) and noninfectious (LPS) models suggests a common underlying mechanism. The reduction in lung myeloperoxidase levels in the pneumonia model (probably owing to enhanced clearance of bacteria from the lungs) and in serum myeloperoxidase levels in the pneumonia model (54). The present results suggest that EP3 regulates systemic inflammation as well (Fig. 7C and data not shown). Whether there are direct effects of EP3 receptor activation on inflammatory mediator-producing cells (such as leukocytes), or the effects are indirect, remains unanswered.

In summary, these studies provide new insights into the physiological and pathophysiological role of PGE$_2$ and the EP3 receptor in the setting of severe bacterial pneumonia and overwhelming inflammation. We observed protection of EP3$^{-/-}$ mice from lethal bacterial pneumonia, and this was associated with enhanced clearance of bacteria from the lungs; reduced circulating leukocyte numbers with depressed pulmonary neutrophil recruitment; improved bacterial phagocytosis and killing by macrophages in the alveolar space; enhanced production of NO by these macrophages; and the suppression of normal thermoregulatory responses. Similar protection from death was observed in an LPS model of sepsis, and this was associated with reduced systemic inflammatory responses. Future studies to better characterize the mechanisms underpinning these new observations are warranted. Pharmacological targeting of the PGE$_2$-EP3 axis represents a novel area warranting greater investigative interest in the prevention and/or treatment of infectious diseases.

Disclosures

The authors have no financial conflict of interest.

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