

Published in final edited form as:

*Prostaglandins Other Lipid Mediat.* 2012 May ; 98(0): 23–30. doi:10.1016/j.prostaglandins.2012.03.002.

## E Prostanoid 2 Receptor Signaling Suppresses Lung Innate Immunity against *Streptococcus pneumoniae*

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### Abstract

Pneumonia is a major global health problem. Prostaglandin (PG) E<sub>2</sub> is an immunomodulatory lipid with anti-inflammatory, immunosuppressive, and pro-resolving actions. Data suggest that the E-prostanoid (EP) 2 receptor mediates immunomodulatory effects of PGE<sub>2</sub>, but the extent to which this occurs in *Streptococcus pneumoniae* infection is unknown. Intratracheal lung infection of C57BL/6 mice possessing (EP2<sup>+/+</sup>) or lacking (EP2<sup>-/-</sup>) the EP2 receptor was performed, as were *in vitro* studies of alveolar macrophage (AM) host defense functions. Bacterial clearance and survival were significantly improved *in vivo* in EP2<sup>-/-</sup> mice and it correlated with greater neutrophilic inflammation and higher lung IL-12 levels. Upon *ex vivo* challenge with pneumococcus, EP2<sup>-/-</sup> cells expressed greater amounts of TNF- $\alpha$  and MIP-2 than did EP2<sup>+/+</sup> AMs, and had improved phagocytosis, intracellular killing, and reactive oxygen intermediate generation. These data suggest that PGE<sub>2</sub>-EP2 signaling may provide a novel pharmacological target for treating pneumococcal pneumonia in combination with antimicrobials.

### Keywords

prostaglandins; pneumonia; bacterial infection; macrophage; innate immunity; cyclooxygenase; Streptococcus

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All the authors declare that they have no competing interests.

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## Introduction

Pneumonia is the leading cause of infectious mortality in the United States<sup>1</sup> and therapeutic options are limited by emerging antimicrobial resistance<sup>2</sup>. *Streptococcus pneumoniae* is the most frequently isolated pathogen in community-acquired pneumonia<sup>3</sup> and accounts for more deaths (nearly 1.6 million per year<sup>4</sup>) than any other bacterium<sup>5</sup>. An improved understanding of immune defenses against *S. pneumoniae* is important to the discovery of improved targets for preventive and therapeutic strategies.

A critical innate immune barrier to bacterial pneumonia is the alveolar macrophage (AM)<sup>6</sup>, which patrols gas-exchanging alveoli and removes potential pathogens through phagocytosis and intracellular killing. Furthermore, AMs alert the host to the presence of invading microbes by releasing lipid and protein mediators that activate resident cells and recruit polymorphonuclear leukocytes (neutrophils) to the focus of infection<sup>7</sup>. This response is highly regulated, to limit self-inflicted damage to host cells and tissues<sup>8</sup>. Emerging evidence suggests that lipid mediators are major regulators of both the amplitude and duration of infection-triggered inflammatory responses<sup>9</sup>. For example, leukotriene (LT) B<sub>4</sub> has potent proinflammatory effects that augment innate immune functions of the AM<sup>10, 11</sup> and recruit/activate neutrophils, while prostaglandins (PGs) such as PGE<sub>2</sub> and PGI<sub>2</sub> have opposite, anti-inflammatory actions in the context of pneumonia<sup>12, 13</sup>. The recently-described resolvins and protectins are lipids that have been postulated to drive the resolution phase of inflammation<sup>14</sup>.

The cyclooxygenase (COX)-derived eicosanoid PGE<sub>2</sub> primarily down-regulates AM defense functions<sup>12, 15, 16</sup>. PGE<sub>2</sub> binds to four distinct G protein-coupled E-prostanoid (EP) receptors, termed EP1-4. The inhibitory effects of PGE<sub>2</sub> primarily result from cAMP-dependent signaling processes, triggered by EP2 and/or EP4 activation<sup>17</sup> and likely evolved to prevent inflammatory tissue damage and promote the resolution of inflammation<sup>18–20</sup>. Sadikot *et al.* demonstrated that PGE<sub>2</sub>-EP2 signaling suppressed bacterial clearance from the lungs of mice infected with *Pseudomonas aeruginosa*<sup>21</sup>. In addition, Stables *et al.* used *ex vivo* human whole blood assays to demonstrate that inhibiting either PGE<sub>2</sub> synthesis or EP2 signaling significantly improved innate immune defenses against *S. pneumoniae*, including antimicrobial-resistant strains<sup>22</sup>. That important study shed new light on the potential for targeting PGE<sub>2</sub> synthesis or signaling systems as adjunctive therapy against pneumonia. However, those studies were not designed to examine pneumococcal pneumonia *in vivo*<sup>22</sup>. Thus, mechanistic details of how PGE<sub>2</sub> regulates pulmonary host defense during pneumococcal infection remain undefined.

We hypothesized that the EP2 receptor would play a potentially maladaptive, anti-inflammatory role in severe pneumococcal pneumonia, suppressing innate host defenses to allow infection to progress. A combination of pharmacological and genetic experiments were conducted to test this hypothesis, revealing a novel and critical role for PGE<sub>2</sub>-EP2 signaling in regulating pulmonary innate immunity during pneumococcal respiratory tract infection. These data add support and understanding to the idea of targeting prostaglandin synthesis and signaling as adjunctive treatments against pneumonia.

## MATERIALS AND METHODS

### Animals

Mice harboring a targeted deletion of both alleles of the *Ptger2* encoding the EP2 receptor were originally generated by Dr. Richard Breyer (Vanderbilt University). These six-to-eight week old female EP2-deficient (EP2<sup>-/-</sup>) mice, bred on a C57BL/6 background, and age-

matched, female C57BL/6 wild type (WT) animals (EP2<sup>+/+</sup> mice) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the University of Michigan Unit for Laboratory Animal Medicine. The genotypes of mouse strains were confirmed by tail-snip DNA PCR analyses performed by Transnetyx (Cordova, TN). 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 culture medium and penicillin/streptomycin/amphotericin B solution were supplied by Gibco-Invitrogen (Carlsbad, CA). Todd-Hewitt broth was purchased from Difco (Detroit, MI). Cytochalasin D, saponin, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma (St. Louis, MO). 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF) was obtained from Invitrogen (Carlsbad, CA).

## Pneumococcal pneumonia

*S. pneumoniae* serotype 3, 6303 (American Type Culture Collection, Manassas, VA) was grown to mid-log phase in Todd-Hewitt broth, washed in PBS, and serially diluted in sterile saline in order to deliver  $1 \times 10^5$  colony forming units (CFU) intratracheally to induce pneumococcal pneumonia as described previously<sup>23</sup>. Bacterial growth in lung and spleen homogenates was determined 24 h after infection using serial dilutions as previously published<sup>23</sup>.

## Isolation and culture of alveolar macrophages

Mouse resident AMs were obtained via lung lavage as previously described<sup>23, 24</sup> and resuspended in RPMI 1640 to a final concentration of  $1-4 \times 10^6$  cells/ml. Cells were adhered to tissue culture-treated plates for 1 h (37°C, 5% CO<sub>2</sub>) followed by two washes with warm medium. Cells were cultured overnight in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin/amphotericin B prior to use. The following day cells were washed 2x using warm medium to remove nonadherent cells.

## *In vitro* macrophage assays for intracellular cAMP, nitric oxide production, and reactive oxygen intermediate generation

Macrophage nitric oxide and reactive oxygen intermediate generation were performed as recently published with modifications specified in the text<sup>23</sup>. Cellular cAMP levels were measured from AMs *ex vivo* according to our previously published protocol<sup>12</sup>.

## Alveolar macrophage phagocytosis assay

The ability of mouse AMs to phagocytose *S. pneumoniae* was assessed using a previously published protocol for determining the internalization of fluorescent, fluorescein isothiocyanate (FITC)-labeled bacteria<sup>12, 23</sup>. Heat-killed *S. pneumoniae* serotype 3 were labeled with FITC as previously described (FITC-*S. pneumoniae*)<sup>25</sup>.  $1.5 \times 10^5$  AMs were obtained from the BAL fluid of naive mice and seeded in replicates of 24 in 384-well tissue culture plates with opaque sides and optically clear bottoms (Costar, Corning Inc. Life Sciences, Lowell, MA). The next day, FITC-*S. pneumoniae* were opsonized with 10% normal rat-derived non-immune serum. Macrophages were inoculated with FITC-*S. pneumoniae* using a multiple 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 485ex/535em (Molecular Devices, Sunnyvale, CA). A phagocytic index was calculated as previously described in relative fluorescence units<sup>12</sup>.

### Macrophage assay of bacterial killing

The ability of bacteria to survive within AMs was determined via a tetrazolium dye reduction assay<sup>11,26</sup>. Bacterial growth was determined colorimetrically based on the ability of live bacteria to convert MTT to a purple formazan salt that absorbs light at 595 nm ( $A_{595}$ ). Briefly,  $2 \times 10^5$ /mL mouse AMs 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<sup>27</sup>. Macrophages were then infected with a 0.1-mL suspension of opsonized *S. pneumoniae* ( $2 \times 10^7$  CFU/mL; multiplicity of infection, 100 bacteria per AM) for 2 h to allow for phagocytosis. Bacterial killing was assessed as described<sup>11,26</sup>. The intensity of the  $A_{595}$  was directly proportional to the number of viable bacteria within macrophages<sup>26</sup>. Results were expressed as percentage survival of ingested bacteria. The survival of ingested bacteria =  $100\% \times A_{595}$  control (phagocytosis) plate/ $A_{595}$  experimental (phagocytosis + killing) plate.

### Lung homogenate, bronchoalveolar lavage, and macrophage cytokine and chemokine determination

Lung homogenates, obtained from mice 24 h after pneumococcal infection, were evaluated for IL-1 $\beta$ , TNF- $\alpha$ , IL-10, IL-12 p40, IL-6, and MCP-1 by ELISA (R&D Duoset, R&D Systems). Bronchoalveolar lavage was performed to determine cytokine and chemokine levels in bronchoalveolar lavage fluid (BALF) by ELISA as reported earlier<sup>23</sup>. Resident AMs were cultured *ex vivo* with heat-killed *S. pneumoniae* (multiplicity of infection 1000:1) for 24 h in RPMI 1640 containing 1% penicillin/streptomycin and 10% fetal calf serum (EMD Biosciences). Cell culture medium was recovered and stored at  $-70^\circ\text{C}$  until assays were performed to determine the levels of TNF- $\alpha$ , IL-10, IL-12 p70, and MIP-2 by ELISA (R&D Duoset, R&D Systems). ELISAs were performed by the University of Michigan Cancer Center Cellular Immunology Core.

### Histopathology

Lungs were prepared for histopathology studies 24 h following infection as published<sup>23</sup>. Histological and immunohistochemical staining were evaluated in a blinded fashion by a board-certified veterinary pathologist (ILB) using an Olympus BX45 light microscope with a scope-mounted Olympus DP72 12.5 megapixel digital camera and Olympus DP-BSW software. Slides were evaluated separately on a numeric 0–4 scale (0: none; 0.5: minimal; 1: mild; 2: moderate; 3: severe; 4: severe and extensive) for 4 categories, specifically neutrophilic inflammation within the lung, pleural inflammation, macrophage accumulation, and presence of bacteria. Each of these categories was separately given a 1–4 score and the scores were then summed across the parameters to give a total histological score (maximal score 16). The criteria dictating the numeric scores for each parameter were as follows. For inflammatory or cell accumulation parameters: 0: none; 0.5: single or multiple small foci most evident at higher magnification; 1: single or multiple small foci evident at low magnification; 2: larger multiple to coalescing foci; 3: multiple to coalescing foci affecting 50–80% of a lobe; 4: large coalescing foci affecting >80% of a lobe. For bacteria: 0: none visible; 1: few cocci visible within 1–2 fields; 2: small numbers of cocci visible within 2 or more fields; 3: cocci immediately visible in most inflamed fields but not in high numbers; 4: cocci easily visible in high numbers or in clusters in most inflamed fields; 5: numerous easily visible clusters in all affected fields evident at low magnification).

## Statistical analyses

Statistical analyses were conducted using Prism 5.0 software (GraphPad Software, La Jolla, CA). Survival was evaluated for differences using a Mantel-Cox log-rank test. Where appropriate, mean values were compared using a paired Student *t*-test or a one-way analysis of variance (ANOVA) followed by Bonferroni correction. Differences were considered significant if  $P < 0.05$ . Unless otherwise stated, all experiments were performed at least three separate times. Data are presented as mean values  $\pm$  standard error of the mean unless noted otherwise.

## RESULTS

### The absence of PGE<sub>2</sub>-EP2 signaling is protective in pneumococcal pneumonia

Similar to our previous study of PGE<sub>2</sub>-EP3 signaling<sup>23</sup>, female C57BL/6 mice were noted to be susceptible to pneumococcal pneumonia, exhibiting 92% mortality after intratracheal inoculation of  $1 \times 10^5$  CFU of *S. pneumoniae* (Fig. 1A). We have previously reported that PGE<sub>2</sub> is an abundant mediator in the lungs of C57BL/6 mice infected with pneumococcus<sup>28, 29</sup>. Mice unable to sense this PGE<sub>2</sub> via the EP2 receptor (EP2<sup>-/-</sup> mice) were significantly protected from infection-related death (59.3% mortality;  $P < 0.05$  compared with EP2<sup>+/+</sup> mice). The increased survival of EP2<sup>-/-</sup> mice correlated with improved clearance from the lungs 24 h following infection (Fig. 1B). A non-significant trend towards reduced dissemination of bacteria from the lungs to the spleen was also observed in the EP2 null mice (Fig. 1B).

### Enhanced neutrophilic inflammation in the alveoli of infected EP2<sup>-/-</sup> mice

PGE<sub>2</sub> impairs neutrophil migration to the infected lungs<sup>30, 31</sup> and EP2 signaling impairs cellular migration, including that of neutrophils<sup>32, 33</sup>. Consistent with these results, the present studies demonstrated that EP2 receptor null mice had greater numbers of neutrophils in BALF 24 h following inoculation when compared to EP2<sup>+/+</sup> animals (Fig. 2A). A cumulative severity score describing lung inflammation was obtained for infected mice (Fig. 2B). Consistent with the BALF results, there was a trend toward more severe acute inflammation in the absence of PGE<sub>2</sub>-EP2 signaling, with severity scores of  $3.2 \pm 1.2$  in EP2<sup>-/-</sup> mice vs.  $1.0 \pm 0.2$  in EP2<sup>+/+</sup> mice ( $P = 0.099$  by Student *t* test). This difference appeared to be due to the greater presence of neutrophils in the alveoli of EP2<sup>-/-</sup> mice (Fig. 3A–D), which was the dominant histological change. Neutrophilic inflammation predominantly involved the alveoli, with less prominent bronchiolar involvement (Fig. 3A–D). Additional changes included increased macrophages in the pulmonary interstitium and alveoli, and neutrophilic inflammation of pleural surfaces. All animals had minimally-increased macrophage cellularity, while neutrophilic inflammation was present in 5 out of 5 EP2<sup>-/-</sup> animals (minimal to moderate) but only 3 out of 5 EP2<sup>+/+</sup> (WT) animals (minimal to mild). Pleural inflammation was present in the highest scoring EP2<sup>-/-</sup> animal.

### Loss of PGE<sub>2</sub>-EP2 signaling results in enhanced IL-12 production *in vivo* during pneumonia

PGE<sub>2</sub> is a potent modulator of inflammation-induced cytokine responses via the cAMP-coupled EP2 and EP4 receptors<sup>34, 35</sup>. Studies were therefore conducted to assess the generation of inflammatory mediator generation in the lung 24 h following infections (Fig. 4). The cytokines and chemokines measured were selected based on known roles in the host response to bacterial infection and previously published evidence that their expression was regulated by PGE<sub>2</sub>. While production of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, and MCP-1 was similar in lungs of EP2<sup>-/-</sup> and EP2<sup>+/+</sup> mice, IL-12 p40 subunit levels were significantly higher in EP2<sup>-/-</sup> lungs. Similar results were observed when these mediators were quantified in BALF,

with IL-12 being significantly greater in the alveoli of EP2<sup>-/-</sup> lungs (442.6 ± 20.2 pg/ml) compared with EP2<sup>+/+</sup> mice (302.4 ± 17.1 pg/ml;  $P < 0.001$  by Student *t* test).

### **Alveolar macrophages from EP2<sup>-/-</sup> mice have enhanced antimicrobial activities against *S. pneumoniae***

Prostaglandin E<sub>2</sub>-EP2 signaling, impairs bacterial phagocytosis by AMs 12 and inhibits the NADPH oxidase-dependent generation of reactive oxygen intermediates via cAMP-dependent signaling networks<sup>16</sup>. We therefore tested whether similar effects were present in the context of pneumococcal infection. The intracellular cAMP concentrations in AMs obtained from uninfected EP2<sup>+/+</sup> mice was significantly higher than that observed in EP2<sup>-/-</sup> macrophages (3.7 ± 0.31 vs. 2.5 ± 0.21 pmol/ml,  $P = 0.02$ ; n = 4). As evidenced in Fig. 5A, EP2 receptor-deficient AMs phagocytosed serum-opsonized *S. pneumoniae* more effectively than did cells from EP2<sup>+/+</sup> mice. The phagocytic activity of EP2<sup>-/-</sup> macrophages relative to EP2<sup>+/+</sup> cells was 173 ± 11.2% ( $P = 0.02$  by Student *t* test). In addition, bacterial killing by EP2 null macrophages was 4.25-fold more effective compared with EP2<sup>+/+</sup> cells ( $P = 0.007$  by Student *t* test; Fig. 5B).

It was previously shown that AMs from mice lacking the EP3 receptor also had a significantly enhanced capacity to kill intracellular pneumococci<sup>23</sup> and this correlated with a greater ability to generate nitric oxide during infection<sup>23</sup>. Notably, the EP3 null mice did not have a different capacity to make reactive oxygen intermediates during infection, compared with EP3<sup>+/+</sup> macrophages<sup>23</sup>. In the present work we made a novel, and opposite, observation: EP2 null cells had the same ability to generate nitric oxide as EP2<sup>+/+</sup> AMs but had a significantly greater capacity to produce reactive oxygen intermediates upon infection with *S. pneumoniae* (Fig.s 5C and D).

### **Alveolar macrophages from EP2<sup>-/-</sup> mice exhibit increased proinflammatory mediator generation in response to *S. pneumoniae***

It is established that multiple AM functions are regulated by PGE<sub>2</sub>-EP2 signaling 12, 16, 29, 36, 37 but the extent to which EP2 signaling regulates the production of inflammatory mediators by AMs in response to pneumococcal infection has not been previously studied. Thus, AMs from WT and EP2<sup>-/-</sup> mice were cultured *in vitro* and inoculated with heat-killed pneumococci for 24 h. Inflammatory mediators were measured in the culture supernatants by ELISA (Fig. 6). Because no difference was observed in whole lung homogenates (or BALFs) in the chemokine MCP-1 (above), we assessed AM production of MIP-2, a chemoattractant for neutrophils that is involved in host defense against *S. pneumoniae*<sup>38</sup>. Notably, AMs lacking EP2 showed enhanced TNF-α and MIP-2 production following stimulation with pneumococci (Fig. 6). These cells made only small amounts of IL-10 and IL-12, making comparisons difficult, but there was a trend towards greater IL-12 production by EP2<sup>-/-</sup> AMs.

## **DISCUSSION**

*S. pneumoniae* is the most common bacterial cause of community-acquired pneumonia in the world and remains a significant source of morbidity and mortality<sup>39</sup>. This study newly defines the important contribution of PGE<sub>2</sub>-EP2 signaling to the regulation of innate pulmonary host defenses during pneumococcal pneumonia. Whereas PGE<sub>2</sub> may serve a homeostatic function by promoting the resolution of inflammation when infection is moderate in degree<sup>18, 40</sup>, its immunosuppressive actions might be maladaptive when an infection is overwhelming. Our data indicate that PGE<sub>2</sub>-EP2 signaling modulates the first-line innate immune processes on the respiratory surface (*e.g.*, AM and/or epithelial cell functions) and contributes to their failure during severe infection.

Several previous studies have utilized pharmacologic inhibition or genetic deletion of COX or its isoforms and demonstrated improved outcome from infection *in vivo* or increased innate immune cell functions *in vitro*<sup>12, 21, 22, 41–47</sup>. Although no human studies have assessed the ability of COX inhibitors to improve bacterial clearance or the outcome of localized infections of the lung or other organs, such studies in the setting of sepsis have not met with great success<sup>48–50</sup>. Since specific prostanoids and prostanoid receptors can exert opposing biological effects, targeting the upstream enzyme COX may represent an excessively broad or nonspecific approach to immunostimulation. By contrast, greater specificity may be obtained by targeting specific downstream prostanoid receptors, such as EP2.

The anti-inflammatory (and potentially immunosuppressive) actions of PGE<sub>2</sub> are driven by EP2- and EP4-dependent increases in cAMP generation<sup>29, 51, 52</sup>. Recent evidence suggests that certain pathogens have evolved mechanisms to use this host response to their own advantage<sup>21, 23, 53–56</sup>. This phenomenon appears to be important in the pathogenesis of invasive streptococcal infections<sup>22, 29, 43, 57–61</sup> and pharmacological targeting of PGE<sub>2</sub> synthesis and/or signaling has gained significant interest in the treatment of severe streptococcal infections.

In 1982, Short *et al.* demonstrated increased survival in animals when PGE<sub>2</sub> synthesis was inhibited during Group B *Streptococcus* sepsis<sup>45</sup>. It was later reported that mice infected in the lungs with a lethal inoculum of *S. pneumoniae* were protected from infection and cleared the bacteria better when PGE<sub>2</sub>-EP3 signaling was genetically disrupted, possibly as a result of enhanced nitric oxide-dependent killing of the bacteria<sup>23</sup>. In addition, Goldmann *et al.* noted that the inducible COX isoform COX-2 was quantifiably the most up-regulated gene in mouse macrophages infected with *S. pyogenes*<sup>62</sup>, later reporting that COX-2 is also up-regulated in human and mouse tissues infected by this pathogen<sup>43</sup>. They went on to demonstrate that PGE<sub>2</sub> signaling via EP2 receptors suppressed host defenses against *S. pyogenes*<sup>43</sup>. Abdeltawab, *et al.* applied an unbiased systems genetics approach that identified two PGE<sub>2</sub> synthase enzymes as key participants mediating susceptibility to *S. pyogenes* infection<sup>57</sup>.

Our studies provide new mechanistic information about PGE<sub>2</sub> as an immunomodulator. PGE<sub>2</sub> regulates AM functions via EP2-dependent signaling mechanisms<sup>12, 21</sup>, but this has not been studied in the context of pneumococcal infection. We now demonstrate that genetic ablation of normal PGE<sub>2</sub>-EP<sub>2</sub> signaling increases survival of C57BL/6 mice against a lethal model of pneumonia. This was associated with greater bacterial clearance from the lungs and exaggerated neutrophilic inflammation. It is notable that the EP2<sup>-/-</sup> mice were better able to survive infection and control bacterial growth, despite accumulating more histologically-evident inflammation. These data suggest that the greater neutrophilic immune response was beneficial, possibly because the infection was more “contained” than in the wild type mice. The trend toward reduced dissemination to the spleen in the EP2<sup>-/-</sup> mice as compared to the WT mice is also supportive of this assertion (Fig. 1B). Recent data support an important role for neutrophils in pneumococcal host defense<sup>63</sup>. The mechanisms responsible for the increased neutrophil presence remain to be detailed. PGE<sub>2</sub>-EP<sub>2</sub> signaling has been shown to restrict neutrophil chemoattraction<sup>32</sup>. We also speculated that PGE<sub>2</sub>-EP<sub>2</sub> signaling might influence chemokine production during infection. Indeed, AMs infected with pneumococcus produced large quantities of the neutrophil chemoattractant MIP-2, and this was significantly greater for EP2<sup>-/-</sup> cells (Fig. 6).

The host inflammatory response to infection is a double-edged sword that must be robust enough to clear the infection (and therefore improve outcome) but not so intense that it harms the host (and causes morbidity or mortality)<sup>64</sup>. Our mouse model of pneumococcal

pneumonia was highly lethal but the local cellular inflammatory response was patchy and relatively mild. Similar to human infection, we observed bacterial escape and dissemination from the lung during pneumonia, which possibly contributed to the mortality. Thus, enhancing innate immune responses improved local bacterial clearance to improve outcome without inducing overwhelming lung inflammation. Whether inhibiting PGE<sub>2</sub>-EP2 signaling would negatively impact host morbidity and/or mortality by promoting sepsis in a model of more severe pulmonary infection or one with a greater degree of dissemination deserves close attention in future studies.

It was also observed that AMs from EP2<sup>-/-</sup> mice expressed more TNF- $\alpha$  than did wild type cells upon challenge with *S. pneumoniae*, and there was a trend toward greater IL-12 production as well. This cytokine pattern, which suggests enhanced M1 polarization of AMs during infection, was further supported by the observation of increased IL-12 p40 levels in the lung parenchyma of infected EP2<sup>-/-</sup> mice. It is notable that the IL-12 p40 subunit has been shown to be important in host defense against *S. pneumoniae*<sup>65</sup>. AMs are the principle source of this IL-12 is unclear. Our findings may also be relevant to the robust neutrophilic inflammation, since IL-12 has been shown to augment host defense against *S. pneumoniae* in a neutrophil-dependent manner<sup>63</sup>. Further studies are needed to determine whether the increased neutrophils and/or IL-12 are causally responsible for the better outcome of EP2-deficient mice.

AMs from EP2<sup>-/-</sup> mice had significantly enhanced phagocytosis and intracellular killing of pneumococci and this correlated with lower levels of the second messenger molecule cAMP within the cells. Whether the difference in cAMP was causally related to the differences in host defense functions remains unknown but is consistent with what is known about PGE<sub>2</sub>-EP2 signaling and the functions of cAMP within macrophages<sup>16, 17</sup>. Infected AMs also generated greater amounts of microbicidal reactive oxygen intermediates when PGE<sub>2</sub>-EP2 signaling was ablated. These data mechanistically differed from our work with EP3-deficient mice, where studies demonstrated a key role for the EP3 receptor in regulating AM nitric oxide generation and killing of *S. pneumoniae*<sup>23</sup>. We speculate that pharmacological blockade of both EP2 and EP3 receptor subtypes might additively or synergistically improve outcome during bacterial pneumonia, and this is a subject for future research.

This study did not address cellular sources of PGE<sub>2</sub> production during pneumococcal infection, which is a limitation. It is believed that macrophages<sup>66</sup> and airway epithelial cells are important sources of PGE<sub>2</sub> during infection *in vivo*<sup>59, 67</sup>. We did not measure macrophage production of PGE<sub>2</sub> as part of our infection studies *in vitro* and therefore cannot rule out differences in PGE<sub>2</sub> production between WT and EP2<sup>-/-</sup> cells.

Another limitation of these studies was the use of a single background mouse strain (C57BL/6), because genetic variation among different inbred mouse strains has been shown to affect both the susceptibility to pneumococcal infection<sup>68</sup> and the sensitivity to PGE<sub>2</sub> immunoregulation<sup>69</sup>. Future studies are needed to determine the extent to which the present findings are specific to this experimental model. Another limitation was the focus on the single eicosanoid compound PGE<sub>2</sub>, since many COX-derived lipid mediators are likely to be relevant in the setting of severe bacterial pneumonia. While the present work was specifically designed to address the role of PGE<sub>2</sub>-EP2 signaling in this setting, continued investigations are needed to identify how other prostanoids influence disease pathogenesis.

In conclusion, these studies reinforce and refine the emerging paradigm that PGE<sub>2</sub> can impair innate immune function during severe infection and suggest that targeting specific receptors could help prevent or treat pneumococcal pneumonia.



## Acknowledgments

### Funding source:

This work was supported by the National Institutes of Health [HL078727 to DMA, HL077417 to PM, HL058897 to MP-G, and T32ES007062 to EO] and the Flight Attendants Medical Research Institute [CIA-103071 to PM].

The authors wish to thank Dr. Carlos H. Serezani for his critical reading of this manuscript. We thank Joel Whitfield from the University of Michigan Cancer Center Cellular Immunology Core for technical assistance.

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### Highlights

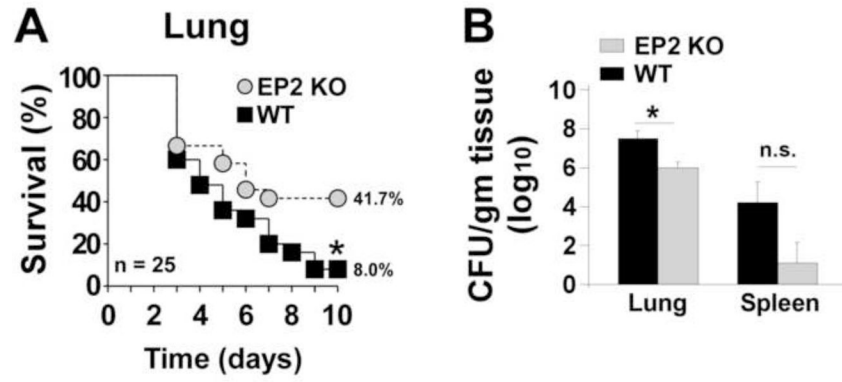
EP2 receptor-deficient mice are protected from death from pneumococcal pneumonia.

EP2 null mice have enhanced inflammatory responses to *S. pneumoniae* in the lungs.

Alveolar macrophage phagocytosis of *S. pneumoniae* is suppressed by PGE<sub>2</sub>-EP2 signaling.

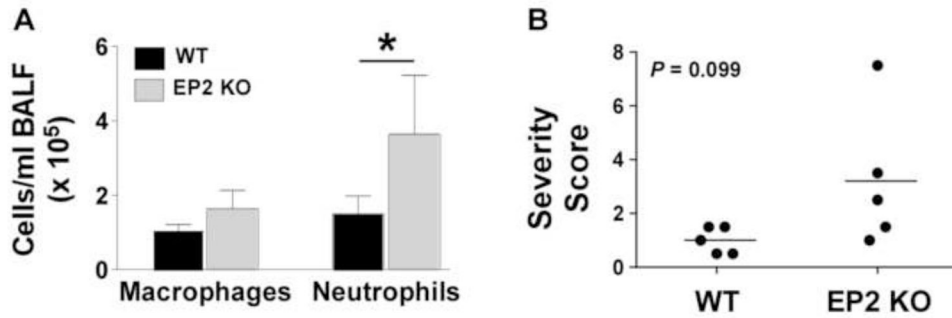
PGE<sub>2</sub> restricts pneumococcal killing by alveolar macrophages via the EP2 receptor.

The EP2 receptor limits inflammatory mediator production by infected macrophages.



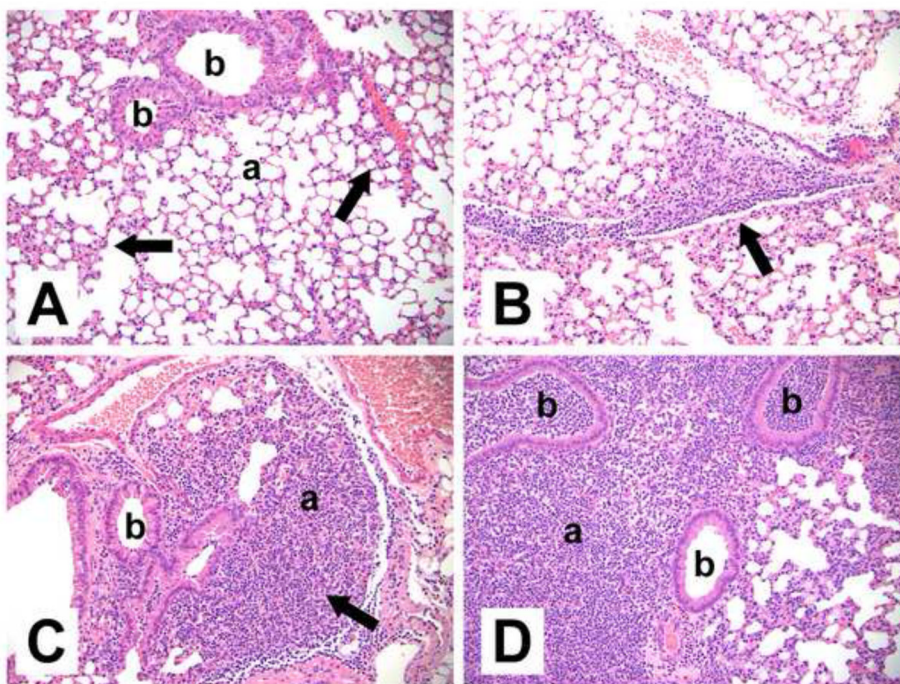
**Fig. 1. Improved survival and enhanced bacterial containment after pneumococcal infection in mice lacking the EP2 receptor**

Female wild type (WT) C57BL/6 or EP2 receptor knockout (EP2 KO) mice were infected intratracheally (A) with *S. pneumoniae* as detailed in the *Materials and Methods* section. Survival was monitored for 10 days. The number of mice per group is shown. \* $P < 0.05$  by Mantel-Cox log-rank test. (B) Bacterial loads (CFU *S. pneumoniae* per gram of homogenized tissue) in the lung and spleen of WT and EP2 KO mice 24 h after infection (n = 5 mice per experimental group). \* $P < 0.05$  by Student *t*-test; n.s., not significant. Data from one representative experiment of two independent experiments yielding similar results are shown.



**Fig. 2. Enhanced acute inflammation in EP2 receptor deficient mice after *S. pneumoniae* lung infection**

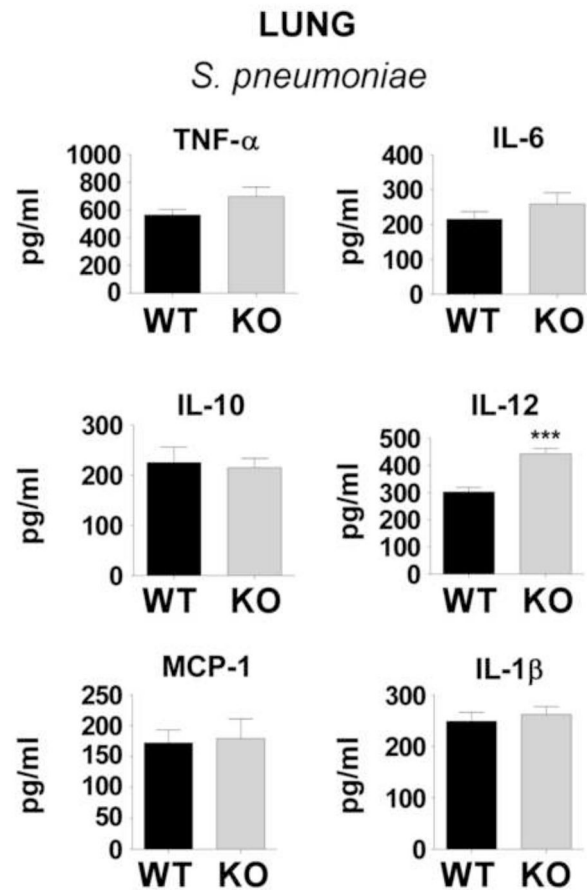
(A) Bronchoalveolar lavage fluid (BALF) cell counts were determined in wild type (WT) and EP2 knockout (KO) mice as detailed in the *Materials and Methods* section 24 h after intratracheal inoculation with *S. pneumoniae*. \* $P < 0.05$  by Student *t*-test ( $n = 5$  mice per group). (B) Pulmonary inflammation was quantitated using a summary severity score calculated through blinded histopathological analysis as described in the *Materials and Methods* section. EP2 KO mice demonstrated a trend towards more severe acute inflammation in the lungs 24 hr after infection. \* $P = 0.099$  by Student *t*-test. Data from one representative experiment of two independent experiments yielding similar results are shown



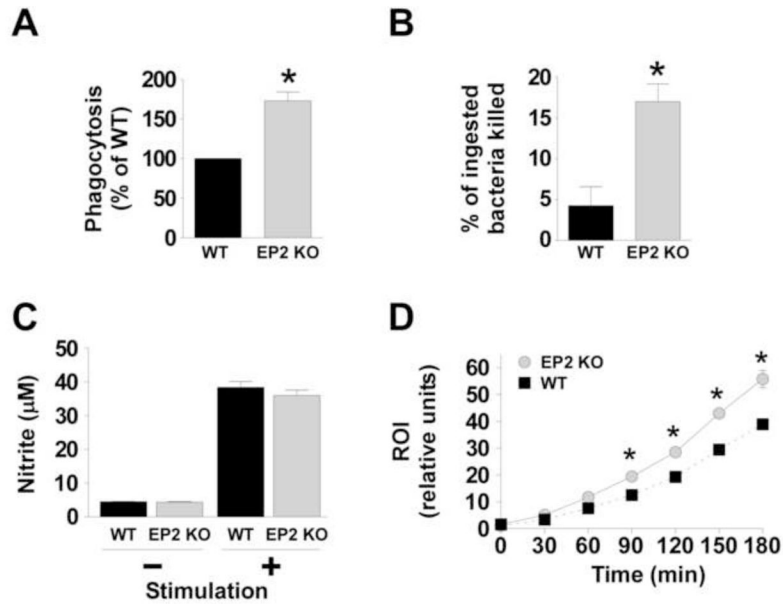
**Fig. 3. Representative histopathological changes in the lungs of *S. pneumoniae*-infected wild type (WT) and EP2 knockout (KO) mice 24 h after inoculation**

Abbreviations: b: bronchiole, a: alveoli. (A) Infected WT mouse without significant inflammation (summary score 0.5). The majority of alveolar airspaces are clear. There are small areas of increased interstitial macrophagic cellularity (arrows). (B) Infected WT mouse with mild, focal neutrophilic inflammation (summary score 1.0), adjacent to a pulmonary blood vessel and along interlobular septae (arrow). (C) Infected EP2<sup>-/-</sup> mouse with mild-moderate inflammation (summary score 1.5) focally obscuring the alveoli (arrow). (D) Infected EP2<sup>-/-</sup> mouse with focally intense inflammation (summary score 7.5) consisting of neutrophils extensively filling alveoli and bronchioles, pleural involvement (not shown), and increased interstitial cellularity. Hematoxylin and eosin. Original magnifications x200.



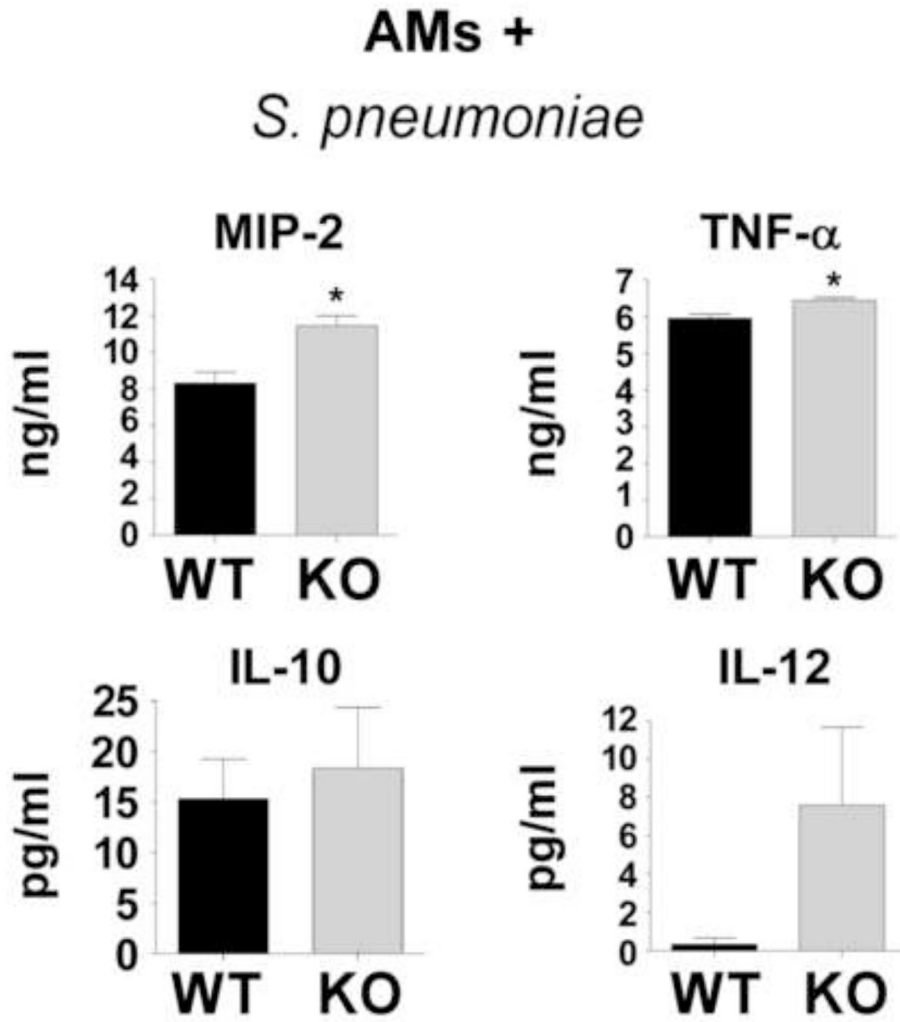


**Fig. 4. Inflammatory mediators in whole lung homogenates of wild type (WT) C57BL/6 mice or EP2 knockout (KO) mice 24 h after inoculation**  
Cytokines and chemokines were measured by ELISA as detailed in the *Materials and Methods* section 24 h after intratracheal inoculation with *S. pneumoniae*. \*\*\* $P < 0.001$  by Student *t*-test ( $n = 5$  mice per group). IL, interleukin, MCP, monocyte chemotactic protein-1.



**Fig. 5. Enhanced *in vitro* alveolar macrophage defense functions in EP2 knockout (KO) mice compared with wild type (WT) alveolar macrophages**

Alveolar macrophages were obtained from uninfected WT and EP2 KO mice as described in the *Materials and Methods* section. (A) Cells were inoculated with heat-inactivated, FITC-labeled *S. pneumoniae* (multiple of infection 150 bacteria per macrophage) and phagocytosis was measured after 3 hrs. \* $P < 0.05$  by Student *t*-test. Data represent the mean  $\pm$  SEM of a minimum of 3 independent experiments performed in octuplet. (B) The survival of internalized *S. pneumoniae* was determined as noted in the *Materials and Methods* section. \* $P < 0.05$  by Student *t*-test. Data represent the mean  $\pm$  SEM of a minimum of 3 independent experiments performed in triplicate. (C) The capacity for alveolar macrophages to generate nitric oxide (measured as nitrite) was assessed for WT and EP2 KO cells following stimulation with or without 10  $\mu$ g/ml of lipoteichoic acid and 10 ng/ml IFN- $\gamma$  for 24h. (D) Alveolar macrophages from WT mice (black squares and dotted line) or EP2 KO mice (grey circles and solid line) were cultured with 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF) for 1h then stimulated with heat-killed *S. pneumoniae* using a multiplicity of infection of 50:1. Reactive oxygen intermediate (ROI) production was assessed fluorometrically and expressed as relative fluorescence units. The data represent the mean of 3 experiments completed in quadruplicate for each time point. \* $P < 0.05$  by Student *t*-test.



**Fig. 6. Alveolar macrophage cytokine and chemokine production following stimulation with *S. pneumoniae***

Alveolar macrophages were obtained from uninfected WT and EP2 knockout (KO) mice and inoculated *in vitro* with *S. pneumoniae* as described in the *Materials and Methods* section. After 24 hrs of inoculation, bacteria- and cell-free supernatants were assayed for inflammatory mediators by ELISA. \* $P < 0.05$  by Student *t*-test. Data represent the mean  $\pm$  SEM of  $n = 3$  independent experiments.