Efferocytosis impairs pulmonary macrophage and lung antibacterial function via PGE_2/EP2 signaling

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The ingestion of apoptotic cells (ACs; termed "efferocytosis") by phagocytes has been shown to trigger the release of molecules such as transforming growth factor β, interleukin-10 (IL-10), nitric oxide, and prostaglandin E_2 (PGE_2). Although the antiinflammatory actions of these mediators may contribute to the restoration of homeostasis after tissue injury, their potential impact on antibacterial defense is unknown. The lung is highly susceptible to diverse forms of injury, and secondary bacterial infections after injury are of enormous clinical importance. We show that ACs suppress in vitro phagocytosis and bacterial killing by alveolar macrophages and that this is mediated by a cyclooxygenase–PGE_2–E prostanoid receptor 2 (EP2)–adenylyl cyclase–cyclic AMP pathway. Moreover, intrapulmonary administration of ACs demonstrated that PGE_2 generated during efferocytosis and acting via EP2 accounts for subsequent impairment of lung recruitment of polymorphonuclear leukocytes and clearance of *Streptococcus pneumoniae*, as well as enhanced generation of IL-10 in vivo. These results suggest that in addition to their beneficial homeostatic influence, antiinflammatory programs activated by efferocytosis in the lung have the undesirable potential to dampen innate antimicrobial responses. They also identify an opportunity to reduce the incidence and severity of pneumonia in the setting of lung injury by pharmacologically targeting synthesis of PGE_2 or ligation of EP2.

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The lung is highly susceptible to injury from systemic (e.g., sepsis) or inhalational (e.g., acid aspiration) exposures, and acute lung injury, manifesting clinically as adult respiratory distress syndrome (ARDS), exacts a huge human toll (1). ARDS is characterized by apoptosis of pulmonary epithelial and endothelial cells as well as of infiltrating neutrophils recruited during the inflammatory response to injury (2). The surface binding/ingestion of ACs during macrophage efferocytosis has been shown to trigger release of antiinflammatory molecules, including TGF-β, IL-10, nitric oxide, and PGE_2 (3, 4), while inhibiting their secretion of proinflammatory mediators such as TNF-α, IL-1, KC, IL-8, and leukotriene C_4 (3, 5). An antiinflammatory program may be critical to restoration of homeostasis and prevention of fibrosis after acute lung injury, as exemplified by the demonstration that intratracheal instillation of ACs enhanced the resolution of acute pulmonary inflammation induced by lipopolysaccharide (6). However, it might also impair the lung's capacity to defend itself against infection. Patients being treated in intensive care units for ARDS frequently develop bacterial pneumonia (7), but the role of efferocytosis in its pathogenesis is difficult to distinguish from that of other existing risk factors, including the altered lung architecture by itself, mechanical ventilation, nutritional deficiencies, and potentially immunosuppressive medications. This study was designed to specifically characterize the impact of efferocytosis on antibacterial defense of the lung and on its resident phagocyte, the alveolar macrophage (AM).

RESULTS AND DISCUSSION

Using a variety of recognition receptors for opsonins or pathogen-associated molecular patterns,

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AMs ingest and kill pathogens, thereby serving a critical function in immune defense of the delicate gas-exchanging region of the lung. One such receptor that plays an important role in antimicrobial responses (8) and that has been extensively investigated (9) is the Fcγ receptor (FcR) for IgG antibodies. To determine if efferocytosis suppresses in vitro FcR-mediated phagocytosis, rat AMs were preincubated with different ratios of ACs and then challenged with IgG-opsonized erythrocytes (RBCs) or *Escherichia coli* for 90 min. As a source of ACs, we used Jurkat T cells treated with camptothecin using a protocol resulting in 25.6% of cells in early apoptosis with only 3.1% contamination by late apoptotic or necrotic cells (Fig. 1 A). Because Fadok et al. (3) previously demonstrated that PGE_2 production by human macrophages occurred after 90 min of incubation with ACs, we initially used this pretreatment interval. Microscopic visualization (unpublished data) indicated that AMs bound and ingested ACs, as previously reported (10). Preincubation of AMs for 90 min with various ratios of ACs dose-dependently inhibited subsequent FcR-mediated phagocytosis of both RBCs and *E. coli* (Fig. 1 B), with ≥50% inhibition being observed at AC/AM ratios of 3:1. The inhibition by ACs (3:1) of FcR-mediated ingestion of both targets was also time dependent over a 15–90-min pretreatment interval, and a 16-h pretreatment resulted in near complete suppression (Fig. 1 C). Similar inhibitory effects were obtained when rat thymocytes, rat PMNs, or RLE-6TN rat lung epithelial cells were used as the source of ACs (unpublished data). Preincubation with either viable or necrotic cells had no effect on subsequent FcR-mediated phagocytosis (Fig. 1 D). After their ingestion, macrophages must kill bacteria. Preincubation with ACs (3:1) for 90 min significantly enhanced the intracellular survival of phagocytosed bacteria, reflecting an impairment of AM microbicidal activity against IgG-opsonized *Klebsiella pneumoniae* (Fig. 1 E). Together, these results demonstrate that preexposure to ACs markedly impairs the ability of AMs to carry out two crucial functions involved in immune defense against bacterial pneumonia: microbial phagocytosis and killing.

We next assessed whether the inhibition of FcR-mediated phagocytosis by efferocytosis in AMs was dependent on soluble mediators. Pretreatment of naive AMs with cell-free supernatant harvested from parallel AM cultures incubated for 90 min with ACs (3:1) inhibited subsequent FcR-mediated phagocytosis to the same degree as did direct addition of

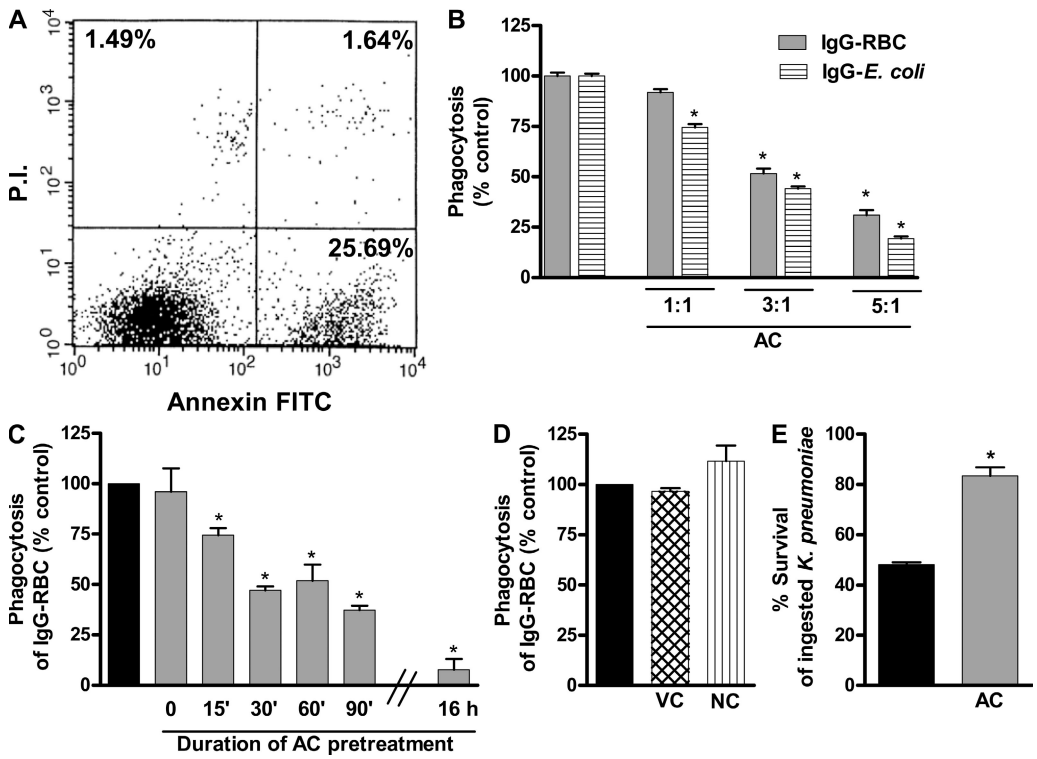

Figure 1. Efferocytosis inhibits FcR–mediated phagocytosis and bacterial killing by AMs. (A) Jurkat T cells were incubated with 8 μg/ml camptothecin for 5 h and apoptotic cells were detected by AnnexinV-FITC/PI and analyzed by flow cytometry. Early ACs represent 25.69% of cells. (B) Phagocytosis of IgG RBCs or IgG *E. coli* was determined after a 90-min pretreatment with ACs at the indicated AC/AM ratios. (C) Phagocytosis of IgG RBCs was determined after pretreatment for the indicated times with ACs added at a ratio of 3:1. (D) Phagocytosis of IgG RBCs was determined after a 90-min pretreatment with viable (VC) or necrotic (NC) Jurkat cells added at a ratio of 3:1. (E) AMs were preincubated with or without ACs (3:1) for 90 min and then infected with *K. pneumoniae* (50:1). Microbicidal activity was determined and expressed as the percentage survival of ingested bacteria. Results represent the mean ± SEM from three independent experiments, each performed in quintuplicate (B–D) or the mean ± SEM of quintuplicate values from a single experiment representative of three independent experiments (A and E). *, $P < 0.05$ versus control.

ACs themselves (Fig. 2 A), implicating a soluble factor. Both efferocytosing macrophages (3, 4) and ACs themselves (11) can generate and release TGF-β, and it has been reported that TGF-β can induce PGE_2 production (12, 13). However, the inability of a neutralizing antibody against TGF-β to reverse phagocytosis suppression induced by AC pretreatment for either 90 min (Fig. 2 A) or 16 h (not depicted) suggests that efferocytosis-induced inhibition was independent of TGF-β.

Inasmuch as the interaction with ACs leads human macrophages to also secrete PGE_2 (3), and we have reported that endogenously produced PGE_2 inhibits FcR-mediated phagocytosis by AMs (14), we evaluated the role of endogenous prostanoids in the suppression of FcR-mediated phagocytosis. Pretreatment with the cyclooxygenase (COX) inhibitors indomethacin and aspirin completely abrogated the inhibition of FcR-mediated phagocytosis by ACs, and such inhibition was reproduced by addition of exogenous PGE_2 (Fig. 2 A). Indeed, AMs secreted PGE_2 in response to ACs and this was inhibited by aspirin (Fig. 2 B). These results suggest that endogenous PGE_2, produced after binding/ingestion of ACs, is a candidate COX-derived mediator of the immunosuppression of FcR-mediated phagocytosis in AMs. Freire-de-Lima et al. (15) reported that peritoneal or RAW 264.7 macrophages incubated with apoptotic Jurkat cells for 18 h generated increased amounts of TGF-β, and PGE_2 production was inhibited when TGF-β responses were blocked using a dominant negative receptor. In our experimental system, however, we observed no increase over control in TGF-β levels measured in culture supernatants derived from incubations of ACs/AMs after either 90 min or 16 h (unpublished data). Ren et al. (16) recently demonstrated in dendritic cells that nitric oxide, but not IL-10, TGF-β, or PGE_2, mediates the immunosuppressive effects induced by ACs. These findings suggest that the soluble mediators involved in the suppression of various immune responses after efferocytosis may depend largely on the type and activation state of the phagocytes.

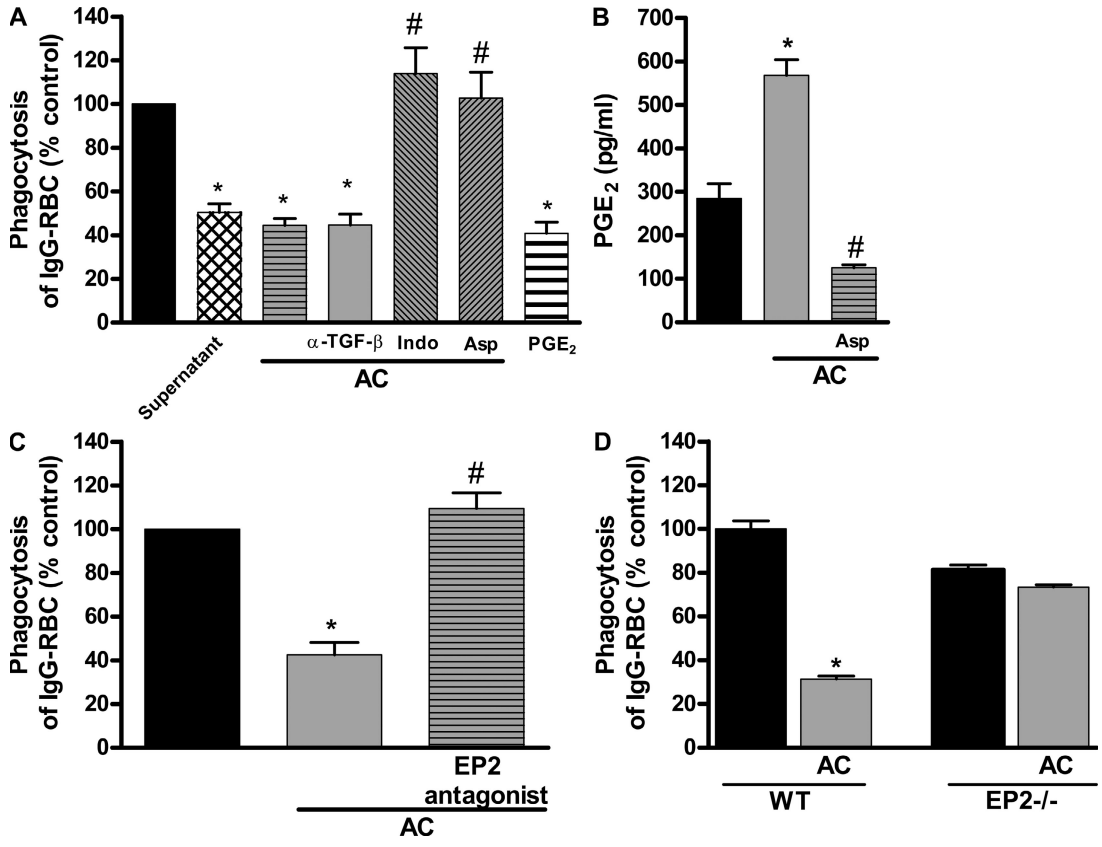

Figure 2. PGE_2 mediates the suppressive effects of efferocytosis on AM antimicrobial functions via EP2. (A) AMs were pretreated with culture supernatant derived from parallel incubations of ACs/AMs (3:1), with 5 μM PGE_2, or with 3:1 ACs in the absence or presence of 6 μg/ml of anti–TGF-β blocking antibody or 5 μM of the COX inhibitors indomethacin (Indo) and 200 μM of aspirin (Asp). They were subsequently challenged with IgG RBCs and phagocytosis was determined. (B) AMs were incubated with medium alone or with ACs in the presence or absence of aspirin. PGE_2 in supernatant was quantitated by immunoassay after 30 min. (C) AM phagocytosis of IgG RBCs was determined after a 90-min pretreatment with medium alone or with ACs (3:1) in the absence or presence of 100 μM of the EP2 antagonist AH-6809. (D) AMs from $EP2^{-/-}$ or WT control mice were preincubated with or without apoptotic thymocytes (5:1) for 90 min before challenge with IgG RBCs and phagocytosis was determined. Results represent the mean ± SEM from three independent experiments, each performed in quintuplicate (A–C) or the mean ± SEM of quintuplicate values from one experiment representative of three independent experiments (D). *, $P < 0.05$ versus control; #, $P < 0.05$ versus AC.

We have reported that the suppressive effects of PGE_2 on AM antimicrobial functions are primarily mediated by its binding to the EP2 receptor, a Gαs-coupled receptor that activates adenylyl cyclase activity with resulting cAMP formation (14, 17, 18). To assess the specific contribution of PGE_2, among other prostanoids, to the efferocytosis-induced inhibition of FcR-mediated phagocytosis and to specifically dissect the participation of EP2 in this process, AMs were pretreated with ACs in the presence or absence of the EP2 antagonist AH-6809 and then challenged with IgG RBCs. Inhibition of FcR phagocytosis by ACs was completely abrogated by the EP2 antagonist (Fig. 2 C). Moreover, AC pretreatment of AMs harvested from mice genetically deficient in the EP2 receptor was unable to significantly inhibit FcR-mediated phagocytosis as it was in cells from WT mice (Fig. 2 D). The suppressive effect of ACs on AM phagocytosis was also abrogated when these cells were pretreated with the adenylyl cyclase inhibitor SQ 22536 (Fig. 3 A). In addition, inhibition of phagocytosis by ACs directly correlated with increased intracellular levels of cAMP, which was also abrogated by aspirin and SQ 22536 (Fig. 3 B). Bactericidal capacity in the context of efferocytosis was likewise markedly augmented by coincubation with a COX inhibitor, an EP2 antagonist, or an adenylyl cyclase inhibitor (Fig. 3 C). These results demonstrate that PGE_2, acting via an EP2–adenylyl cyclase–cAMP pathway, mediates the suppressive effects of efferocytosis on FcR-mediated antimicrobial functions by AMs.

To confirm the biological significance of these in vitro results, we modeled a secondary lung infection after initial pulmonary exposure to ACs such as what would be seen in acute lung injury. For these experiments, ACs were generated by dexamethasone treatment of murine thymocytes for 6 h, which yielded 40.3% early apoptotic and 4.93% late apoptotic plus necrotic cells (Fig. 4 A). Initially, different numbers of ACs were coadministered intratracheally in C57BL/6 WT mice along with a standard inoculum of the important respiratory pathogen *Streptococcus pneumoniae*, and 48 h after challenge the bacterial burdens in lung homogenates were evaluated. As shown in Fig. 4 B, there was no difference in pulmonary bacterial clearance of mice that were infected and simultaneously exposed to ACs using this protocol compared with those infected alone. Because our in vitro results indicated that the efferocytosis-induced inhibition of FcR-mediated phagocytosis was time dependent and reached a level of 89% when phagocytic target challenge was performed after an interval of 16 h (Fig. 1 C), we devised a second model in which *S. pneumoniae* was administered intratracheally 16 h after various numbers of apoptotic thymocytes were instilled intranasally. As ~55% of the total numbers of cells obtained after treatment of thymocytes with dexamethasone remain viable, viable cells were also administrated intranasally as an experimental control. Results showed that pretreatment with ACs, but not viable cells, using this protocol dose-dependently impaired pulmonary bacterial clearance (Fig. 4 C) and also led to the dissemination of *S. pneumoniae* into the bloodstream (Fig. 4 D) 48 h after infection. Finally, to test the role

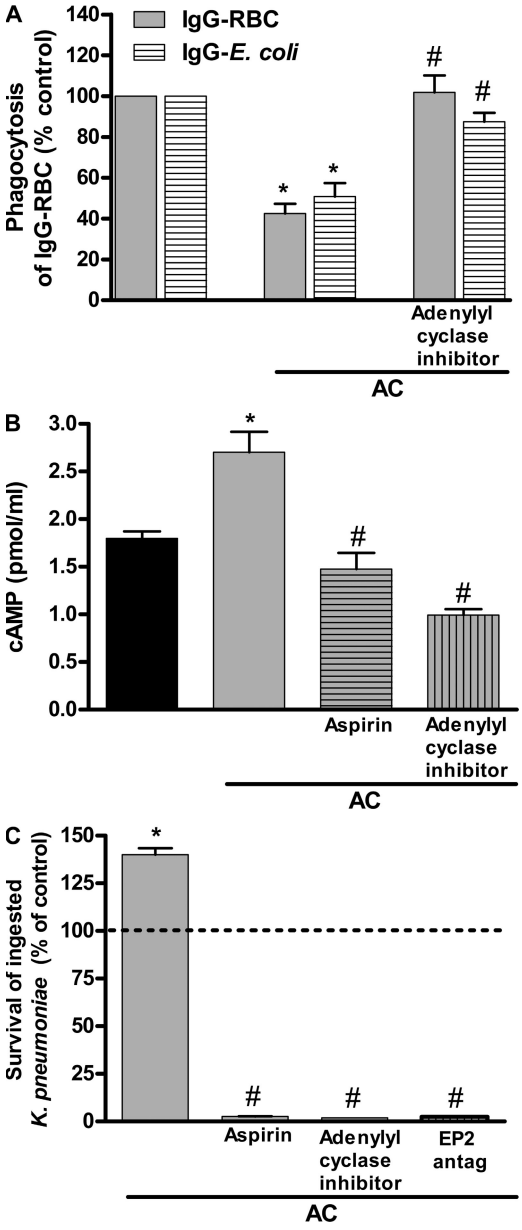

Figure 3. Adenylyl cyclase generation of cAMP mediates the suppressive effects of efferocytosis on AM antimicrobial functions. (A) Phagocytosis of IgG RBCs or IgG *E. coli* was determined after 90-min pretreatment with ACs (3:1) in the absence or presence of 10 μM of the adenylyl cyclase inhibitor SQ 22536. (B) AMs were pretreated or not with 200 μM of aspirin or 10 μM of the adenylyl cyclase inhibitor SQ 22536 before addition of ACs or vehicle for 30 min. Intracellular cAMP concentrations were determined by enzyme immunoassay. (C) AMs were preincubated with 100 μM of the EP2 antagonist AH-6809, 200 μM of aspirin, 10 μM of the adenylyl cyclase inhibitor SQ 22536, or vehicle before the addition of *K. pneumoniae* (50:1). Microbicidal activity was assessed and intracellular survival of bacteria is expressed as a percentage of the control, to which no AC were added. Results represent the mean ± SEM from three independent experiments, each performed in quintuplicate. *, $P < 0.05$ versus control; #, $P < 0.05$ versus AC.

of the EP2 receptor in this impairment of in vivo pulmonary defense against *S. pneumoniae* by AC pretreatment, we compared the lung and the bloodstream bacterial burdens in WT versus $EP2^{-/-}$ mice. In contrast with WT mice, the pulmonary bacterial burden in AC-pretreated $EP2^{-/-}$ mice was no greater than in non–AC-pretreated controls but was 2.5 logs lower than in AC-pretreated WT mice (Fig. 4 E). In addition, $EP2^{-/-}$ mice exhibited no bacteremia (Fig. 4 F). Because these mice lack preexisting antibodies against *S. pneumoniae*, bacterial recognition and clearance by phagocytes in the in vivo model is likely independent of FcR, indicating that the PGE_2/EP2/cAMP axis also suppresses innate

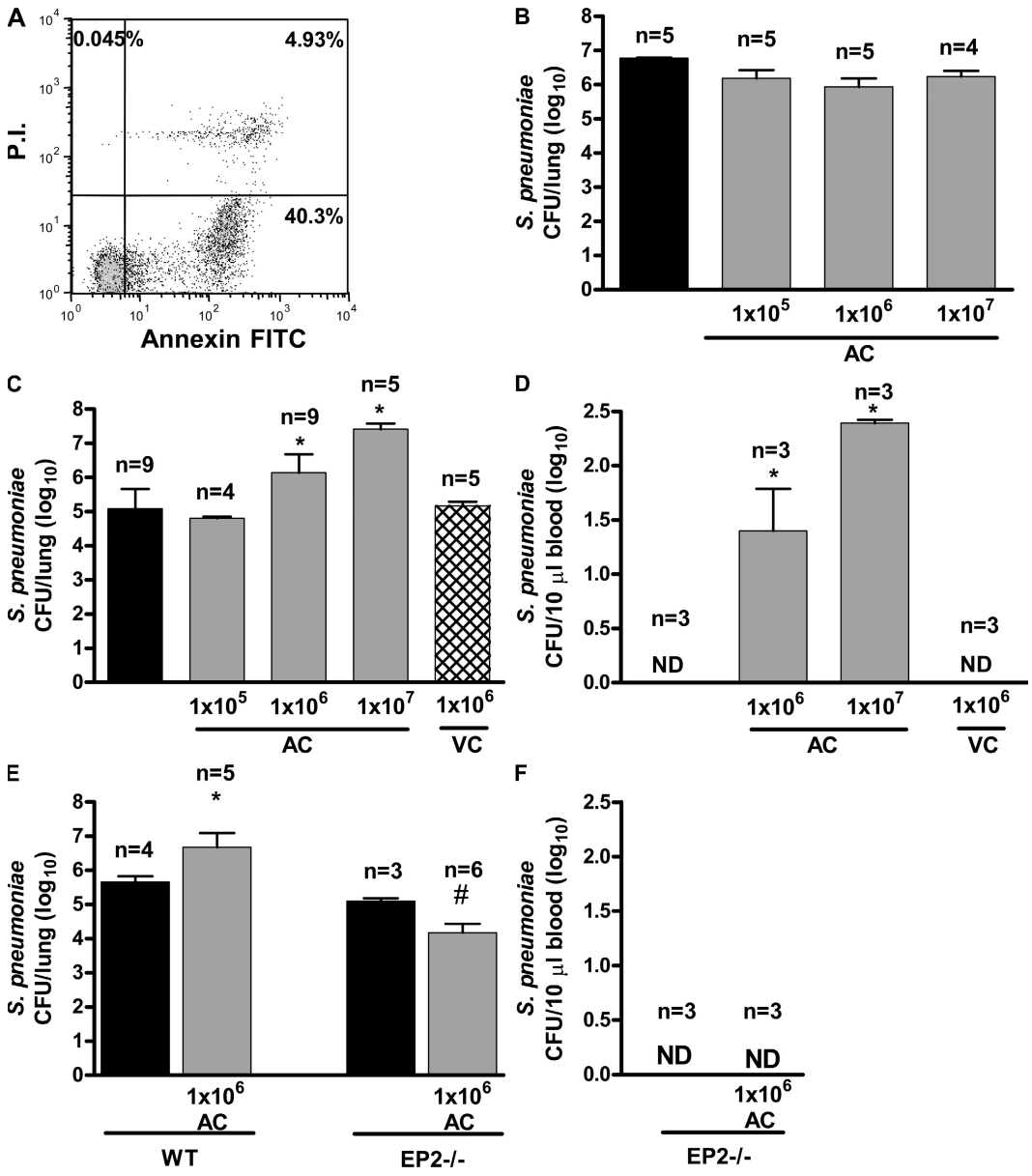

Figure 4. Intrapulmonary administration of ACs impairs host defense in a mouse model of pneumococcal pneumonia. (A) Thymocytes were incubated with 1 µM dexamethasone for 6 h and ACs were detected by AnnexinV-FITC/PI and analyzed by flow cytometry. Early ACs comprise 40.3% of total cells. (B) 10^6 CFU of *S. pneumoniae* and varying numbers of apoptotic thymocytes were coadministered intratracheally in WT mice. Lung homogenates were assessed for bacterial CFUs 48 h later. (C) Indicated numbers of apoptotic or viable thymocytes were instilled intranasally in WT mice and, 16 h later, 10^6 CFU *S. pneumoniae* were administered intratracheally. Lung homogenates were assessed for bacterial CFUs 48 h after *S. pneumoniae* challenge. (D) Bacterial CFUs were determined in blood obtained 48 h after *S. pneumoniae* challenge from the same WT mice studied in C. (E) WT and $EP2^{-/-}$ mice were subjected to intranasal administration of apoptotic thymocytes 16 h before intratracheal challenge with *S. pneumoniae* as described in C. Lung homogenate CFUs 48 h after bacterial challenge are presented. (F) Bacterial CFUs were determined in blood obtained 48 h after *S. pneumoniae* challenge from the same $EP2^{-/-}$ mice studied in E. Results represent the mean ± SEM of one experiment representative of two. The number of animals analyzed in each group is indicated above each bar. ND, none detected. *, $P < 0.05$ versus control; #, $P < 0.05$ versus AC.

immune responses when bacterial recognition proceeds via other relevant recognition receptors such as toll-like receptors (19), collectins (20), or scavenger receptors (21).

We next sought to address the impact of PGE_2/EP2 signaling on lung levels of antiinflammatory mediators. TGF-β has previously been implicated as an important antiinflammatory mediator in the lung in vivo (6). We verified that lung homogenate TGF-β and IL-10 levels were indeed dose-dependently increased 16 h after the administration of thymocytes in uninfected WT mice. In contrast, levels of NO_2^- (the stable oxidized derivative of nitric oxide) were unchanged (unpublished data). As expected, PGE_2 levels in lung homogenates of infected animals were increased after administration of ACs (Fig. 5 A). *S. pneumoniae* infection in WT mice increased lung homogenate levels of TGF-β and NO_2^- but not IL-10 (unpublished data). In this experimental context, administration of ACs had no effect on lung levels of either NO_2^- or TGF-β (Fig. 5 B) but significantly increased levels of IL-10 (Fig. 5 C). This increase in IL-10 generation after AC administration was not seen in $EP2^{-/-}$ animals (Fig. 5 C). These studies show that PGE_2/EP2 signaling drives the enhanced IL-10 production associated with efferocytosis in vivo.

In view of the contribution of PMN recruitment to antibacterial defense of the lung, we next assessed whether efferocytosis influenced PMN influx to the lungs of infected mice. Intrapulmonary instillation of apoptotic thymocytes had no impact on total and differential cell counts in bronchoalveolar lavage fluid (BALF) obtained 16 h later from noninfected WT mice (unpublished data). However, pretreatment with ACs 16 h before infection with *S. pneumoniae* dose-dependently reduced the numbers of total cells (not depicted) and PMNs (Fig. 5 D), but not mononuclear cells (not depicted), in BALF harvested 48 h after infection. $EP2^{-/-}$ mice infected with *S. pneumoniae* exhibited significantly lower numbers of total BALF cells and PMNs than WT animals, an effect likely related to reduced alveolar capillary permeability (unpublished data). However, in contrast to their WT counterparts, AC pretreatment failed to attenuate PMN recruitment in $EP2^{-/-}$ mice (Fig. 5 D). These results suggest that PGE_2/EP2 signaling also mediates the impairment in PMN recruitment to the infected lung, which likely contributes to defective bacterial clearance.

In a mouse model of Chagas disease, administration of ACs was found to enhance *Trypanosoma cruzi* parasitemia, an effect blocked by COX inhibitors; however, this study did not use the natural route of inoculation via the skin (12). Ours is the first in vivo study to use a natural route of microbial inoculation, the first to address antibacterial defenses, and

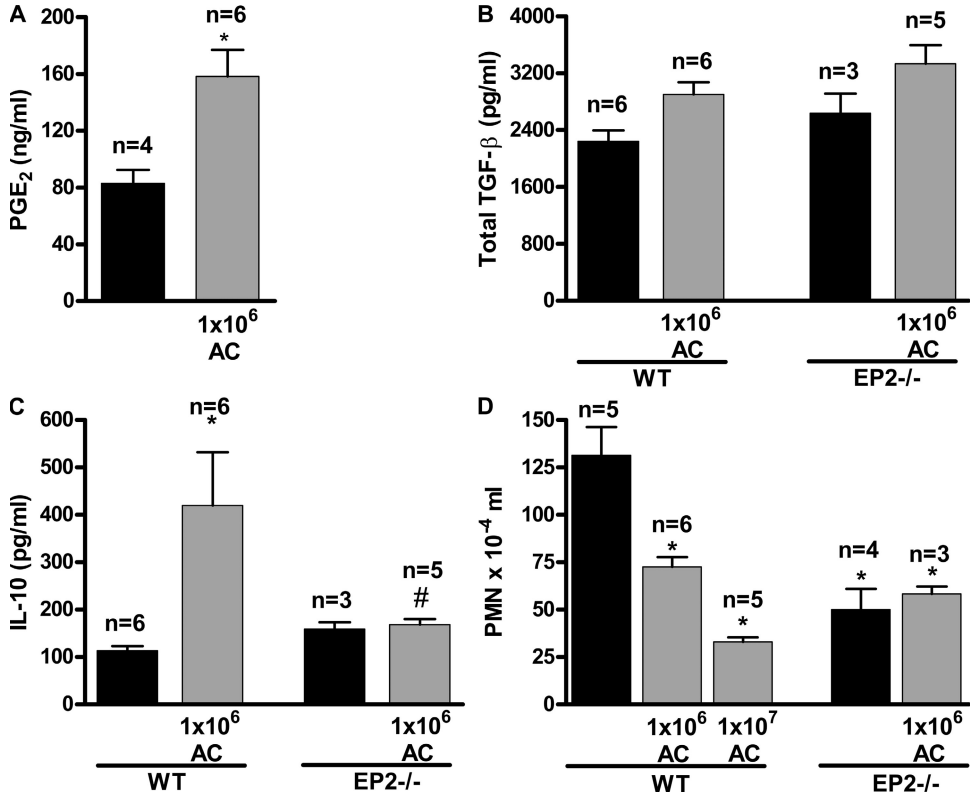

Figure 5. PGE_2/EP2 signaling impairs PMN recruitment and promotes in vivo generation of IL-10 in a mouse model of pneumococcal pneumoniae. 10^6 apoptotic thymocytes were instilled intranasally in WT and $EP2^{-/-}$ mice and, 16 h later, 10^6 CFU *S. pneumoniae* were administered intratracheally. (A–C) PGE_2 (A), total TGF-β (B), and IL-10 levels (C) were quantified in the supernatant of lung homogenates from animals studied in Fig. 4 (C and E). (D) PMNs in BALF from WT and $EP2^{-/-}$ mice were counted. Results represent the mean ± SEM of one experiment representative of two (A–C) or of one experiment (D). The number of animals analyzed in each group is indicated above each bar. *, $P < 0.05$ versus control; #, $P < 0.05$ versus AC.

the first to examine a pulmonary infection. As World Health Organization statistics document that respiratory infections account for more life years lost around the world than any other category of disease (22), the implications of these findings for global health may be substantial. Although ARDS is the most conspicuous example of such a scenario, chronic lung diseases such as cystic fibrosis (23) and emphysema (24) also involve ongoing apoptosis and confer a high risk of infection (25, 26). The capacities of AMs to bind and ingest ACs (10, 27), as well as to generate prostanoids including PGE_2 (28), are known to be reduced relative to those of peritoneal macrophages. For this reason, it was not obvious that either efferocytosis itself or PGE_2 generated under such conditions would mediate suppression of antimicrobial defense in AMs in vitro or in the lung in vivo. Nevertheless, the studies reported here demonstrate that efferocytosing AMs generate sufficient PGE_2 as well as sufficient EP2-mediated signaling responses to this lipid mediator to largely account for impairment of subsequent antibacterial responses in vitro and in vivo. As a variety of COX inhibitors are currently available, and as EP2 antagonists are under active development, our findings suggest that therapeutic targeting of this pathway has the potential to augment innate immunity in the lung under conditions characterized by apoptosis.

MATERIALS AND METHODS

Reagents. RPMI 1640 and penicillin/streptomycin/amphotericin B solution were purchased from Invitrogen. Camptothecin and dexamethasone were obtained from EMD. Tryptic soy broth was supplied by BD. Aspirin, cytochalasin D, indomethacin, *o*-phenylenediamine dihydrochloride, and SDS were obtained from Sigma-Aldrich. AH-6809, PGE_2, and NO_2^- colorimetric assay kits were obtained from Cayman Chemical, and PGE_2 and cAMP EIA kits were obtained from Assay Designs. SQ 22536 was obtained from BIOMOL International L.P. Anti–TGF-β1 antibody was obtained from R&D Systems. TGF-β and IL-10 ELISAs were obtained from BD. Compounds requiring reconstitution were dissolved in DMSO.

Animals. $EP2^{-/-}$ mice on a C57BL/6 background (29) (Ono Pharmaceutical), WT C57BL/6 mice (The Jackson Laboratory), and Wistar rats (Charles River Laboratories) 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.

Cell isolation and culture. Resident AMs from mice and rats were obtained via lung lavage and cultured as previously described (14).

Apoptotic cells. Jurkat T cells (American Type Culture Collection) and glycogen-elicited PMNs (30) were incubated with 8 μg/ml camptothecin for 5 and 3 h, respectively. Rat or mouse thymocytes were incubated with dexamethasone for 6 h (31), and RLE-6TN rat lung epithelial cells (American Type Culture Collection; gift of V. Thannickal, University of Michigan, Ann Arbor, MI) were serum starved overnight to induce apoptosis. Apoptotic cells were detected by AnnexinV-FITC/PI staining (BD) and analyzed using a FACSCalibur (BD). Jurkat cells were rendered ~85% $AnnexinV^+/PI^+$ by freeze thawing (necrotic cells).

Phagocytosis and bacterial killing assays. Phagocytosis of IgG RBCs or IgG *E. coli* was assessed as previously described (14). The ability of *K. pneumoniae* to survive intracellularly after phagocytosis was assessed as previously described (32, 33).

Measurement of cAMP, PGE_2, IL-10, TGF-β, and NO_2^- levels. Intracellular cAMP levels in AM lysates (34, 35), and PGE_2 (35), IL-10, and TGF-β levels in culture supernatants or lung homogenates were quantified by ELISA. NO_2^- (the stable oxidized derivative of nitric oxide) was determined using the Greiss reaction (36).

In vivo experiments. WT and $EP2^{-/-}$ mice were subjected to intratracheal or intranasal administration of ACs or viable cells in PBS. Either simultaneously or 16 h thereafter, 10^6 CFU *S. pneumonia* were administered intratracheally. Lung homogenates and blood were assessed for bacterial CFUs 48 h after *S. pneumoniae* challenge (*S. pneumoniae* provided by P. Mancuso, University of Michigan, Ann Arbor, MI). In another set of experiments, BAL was performed with 3 ml HBSS 16 h after instillation of ACs or 48 h after infection with *S. pneumoniae*. Cell counts and differentials were determined by light microscopy.

Statistical analysis. Data are presented as the mean ± SEM. Comparisons among groups were assessed with ANOVA followed by Bonferroni analysis. Differences were considered significant if P-values were <0.05.

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