Diesel exhaust particles override natural injury-limiting pathways in the lung

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ENVIRONMENTAL EXPOSURE to microbial products through the inhalation of bacterial fragments, such as LPS, has long been associated with exacerbations of airway disease, and levels of LPS within the environment can correlate with severity of asthma (30, 38). Asthma and COPD are commonly exacerbated by bacterial and viral infections (3, 24) that can often coexist and synergize with pollutant exposure (8, 46). Inhalation of small particulate matter is also a major cause of airway inflammation, and rising air pollution levels are associated with elevated mortality and increased hospital admissions in individuals with respiratory diseases such as asthma and COPD (2, 10, 45).

Diesel exhaust particles (DEP) are the major particulate matter of air pollution and comprise a nanoparticulate carbonaceous core, multiple organic components such as polyaromatic hydrocarbons (PAH), transition metals, and adsorbed materials such as pollen and dust. Their small size (5-20 nm; 100-nm aggregates) means that they are often denoted nanoparticles and favors their deposition in the lung (7, 11). Acute and chronic inhalation of DEP in healthy individuals, and those with preexisting respiratory disease, results in respiratory toxicity with consequent development of lung edema, infiltration of polymorphonuclear leukocytes, and the production of proinflammatory cytokines and reactive oxygen species (ROS) (5, 36, 44, 47).

The active inflammation that occurs in asthma and COPD leads to the recruitment of monocytes to the alveolar wall (28). Furthermore, urban particulate matter has been shown to cause the release of monocytes from the bone marrow (17, 23). We have previously shown that effective inflammatory responses to bacterial-derived stimuli are mediated by cooperative signaling between monocytes and tissue cells (31, 32, 39, 50). The direct effects of DEP, with the production of proinflammatory cytokines and ROS in single cell types, are well established (4, 9, 25, 27, 29, 37, 43, 49), but less is known about their actions in models of the cell networks that underpin inflammatory responses. It is also likely that air pollutants such as DEP will coexist in the environment with microbial products, and that these pollution particulates can act as carriers for microbes and allergens, delivering them to sites in the airways. The coexistence of DEP and microbial products such as LPS and flagellin may modulate the inflammatory response to these pathogenic stimuli.

In this study, we show that DEP exposure induced a modest proinflammatory response in models of airway inflammation when monocytes were cocultured with airway epithelial cells. DEP also caused a potentiation of the responses of epithelial cell/monocyte cocultures to microbial stimuli. We have shown previously that IL-1 is crucial to the induction of inflammation in cocultures stimulated with microbial stimuli (31, 32), but importantly, here we reveal that DEP altered the inflammatory milieu such that targeting of the IL-1 pathway was markedly less able to reduce inflammatory responses when DEP were present.

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma-Aldrich or Invitrogen (Paisley, UK), except where specified. OptiPrep density gradient was from Axis Shield (Oslo, Norway). The Monocyte Isolation Kit II was from Miltenyi Biotec (Bergisch Gladbach, Germany). Purified LPS (LPS; Escherichia coli serotype R515) was from Alexis (Nottingham, UK). Endotoxin-free flagellin from Pseudomonas aeruginosa was a generous gift from Dr. Yolanda Sanchez (GlaxoSmithKline, King of Prussia, PA), and flagellin purified from Salmonella typhimurium was purchased from Invivogen (Toulouse, France). Both flagellin types exhibited similar in vitro properties. IL-1ra was from National Biological Standards Board (NIBSC, Potters Bar, UK), and hydrocortisone was purchased from PromoCell (Heidelberg, Germany). The immortalized epithelial cell line BEAS-2B cells were purchased from American Type Culture Collection (Manassas, VA). Matched ELISA antibody pairs were from R&D Systems (Abingdon, UK). PE-conjugated anti-human TLR4 or CD14 and appropriate isotype controls were purchased from eBioscience (San Diego, CA). The Hyband-c-gated anti-human TLR4 or CD14 and appropriate isotype controls were purchased from R&D Systems (Abingdon, UK). PE-conjugated anti-human TLR4 or CD14 and appropriate isotype controls were purchased from eBioscience (San Diego, CA).

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phospho IkBα, human IL-1β mouse monoclonal antibody, and anti-rabbit HRP-conjugated secondary antibody were from Cell Signaling Technologies. Anti-actin was from Sigma-Aldrich. A soluble TNFα receptor fusion protein was purchased from R&D Systems.

DEP. In these studies we have used a standardized DEP, SRM 2975 from National Institute of Standards and Technology (Dept. of Commerce, United States). The DEP has been prepared from an industrial fork-lift truck and has the advantage that the particulate matter has been analyzed and evaluated for PAH concentrations, mass fraction, and thus controlling for variables such as nitric oxide, ozone, and sulfur dioxide content. Diesel particles are typically of the order of 60 nm in diameter; however, they agglomerate in the environment and in culture. Particles were prepared by ultrasonication, but cells were exposed to a mixture of agglomerates of various sizes and single nanoparticles.

Maintenance of BEAS-2B cell line. BEAS-2B epithelial cells were maintained in 75-cm² flasks (Nunc, Denmark) in RPMI 1640 with 10% BioWhittaker or Promocell low endotoxin (<0.01 EU/ml) FBS and 1% penicillin G (100 U/ml) and streptomycin (100 µg/ml). The composition of cell culture media was the same throughout, unless stated otherwise. BEAS-2B cells routinely tested negative for mycoplasma.

Monocyte preparation. Peripheral venous blood was taken with written informed consent from healthy volunteers in accordance with a protocol approved by the South Sheffield Research Ethics Committee. Whole blood was anticoagulated with 3.8% trisodium citrate. Plasma and platelets were removed by centrifugation, and following dextran sedimentation, peripheral blood mononuclear cells (PBMCs) were separated from granulocytes by centrifugation over density gradients using either plasma/Percoll or OptiPrep as previously described (19). Monocytes were further purified by negative magnetic selection using the Monocyte Isolation Kit II from Miltenyi Biotec (Bergisch Gladbach, Germany) to a purity of 91 ± 0.5% (means ± SE) CD14⁺ cells.

Coculture models. BEAS-2B cells were stimulated in 24-well plates when ~80% confluent. At the time of incubation, the cells were washed with PBS, and the cell culture media was replaced. DEP SRM 2975 were suspended in PBS at 5 mg/ml. Before use, stock suspensions of DEP were sonicated twice for two periods of 10 min. Cocultures of BEAS-2B cells with 5,000 purified monocytes per well, or the equivalent monocyte cultures, were stimulated with varying concentrations of DEP in the presence or absence of LPS or flagellin (at the indicated concentrations). In subsequent experiments, 50 µg/ml DEP was used, and, in some experiments, 10 µg/ml IL-1ra, 1 µg/ml hydrocortisone, or 0.5 µg/ml TNFα receptor fusion protein (sTNFR1) were added to the cell cultures at the same time as the DEP. Final volumes in each well of a 24-well plate always totaled 500 µl during experiments. Stimulated cells were incubated for 24 h at 37°C and humidified 5% CO₂.

ELISA. Cell-free supernatants were prepared and stored at −80°C until CXCL8 or IL-1β cytokine generation was determined by ELISA using matched pairs of antibodies at optimized concentrations as previously described (31). Absorbance was measured at 450 nm using an MRX plate reader (Thermo Labsystems, Vantaa, Finland) and Biolinx software version 2.20 (Frankfurt am Main, Germany). Samples were typically diluted so that the optical density fell within the optimal portion of a log-linear standard curve. Limits of detection (LD) for the ELISAs were 31.25 pg/ml. Sample values whose were below the LD were assigned the LD value for analysis.

Western blotting. BEAS-2B cells or monocytes alone were stimulated in 12-well plates with 50 µg/ml DEP, 1 pg/ml LPS, or both DEP and LPS for 15, 30, and 60 min. Protein was obtained by lysing samples as previously described (40) and analyzed by SDS gel electrophoresis blotted onto Hybond-c-extra nitrocellulose membrane. Samples were probed with 1:1,000 anti-p38 monoclonal IgG (rabbit), 1:5,000 anti-actin (rabbit), or 1:1,000 anti-IL-1β (mouse) monoclonal antibody. Binding was detected using a 1:2,000 anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody, respectively, and enhanced chemiluminescence detected with Amer sham ECL detection reagents. Quantitative signals were derived by densitometric analysis using NIH image 1.62 analysis software, and data displayed as ratio compared with actin loading control.

Flow cytometry. Cells were stimulated with 50 µg/ml DEP, 1 pg/ml LPS, or both DEP and LPS for 1, 4, or 24 h. BEAS-2B cells were detached with non-enzymatic cell dissociation solution, pelleted by centrifugation (1,000 g), and resuspended in buffer (PBS, 0.25% bovine serum albumin, 10 mM HEPES). Monocytes were harvested by gentle pipetting. Cells were stained with 1:10 PE-conjugated anti-human TLR4 or CD14 antibody or their relevant 1:10 PE-conjugated mouse IgG2a and IgG1 kappa isotype controls, for 30 min in the dark on ice. Antibody binding was detected in the FL-2 channel of a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) and quantified as specific mean fluorescence after subtraction of the respective isotype control. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR) and quantified as specific mean fluorescence.

ATP determination. 1 µM ATP was suspended in dH₂O, with or without 50 µg/ml DEP, for 3 h. The mixture was centrifuged at 1,000 g for 3 min to mimic the method used to obtain cell-free supernatants and then serially diluted. Samples were detected using an ATP determination kit (Invitrogen, Paisley, UK) as per the manufacturer’s instructions. The ATP Determination Kit is a bioluminescence assay for quantitative determination of ATP with recombinant firefly luciferase and its substrate β-luciferin. Luminescence was determined using a Packard Biosciences Fusion universal microplate analyzer (PerkinElmer, Beaconsfield, UK).

Statistics. Data were analyzed using GraphPad Prism v5 (San Diego, CA). Three or more groups of data were compared using two-way analysis of variance with the appropriate posttests with a statistically significant value of P < 0.05.

RESULTS

We have previously shown that mechanisms of airway inflammation can be dissected using cocultures of epithelial cells and monocytes, and have revealed the existence of IL-1-dependent inflammatory networks potentially amenable to therapeutic targeting (31, 32, 39, 50). Active inflammation in COPD and asthma delivers monocytes to the airway wall, and, accordingly, we initially investigated whether DEP pollution activated epithelial cell/monocyte cocultures and compared inflammation, via neutrophil recruitment, and hence is highly relevant to the pathology of asthma and COPD. Moreover, this chemokine is classically induced by TLR activation, and production is primarily dependent on activation of NF-κB and p38 MAPKs, making it an ideal marker for activation of pathways associated with engagement of the innate immune system (6, 33). Stimulation of epithelial cells or monocyte monocultures for 24 h with DEP at concentrations of up to 250 µg/ml did not result in induction of inflammation as measured by CXCL8 production (Fig. 1A). We therefore established cocultures of epithelial cells and monocytes in 24-well plates and stimulated these for 24 h. In these cocultures, we observed that DEP induced
significant CXCL8 production (Fig. 1A). However, this response was modest compared with a classic microbial inflammatory activator, LPS (Fig. 1B).

**DEP potentiates the proinflammatory response of cocultures to the TLR agonists LPS and flagellin.** Individuals with high DEP exposure are also likely to be inhaling various amounts of environmental contaminants such as LPS, and patients with COPD may have chronic or recurrent bacterial infections delivering microbial stimuli to the airway. Flagellin is the building block of flagella, used by many bacteria for motility, and a potent stimulus of epithelial cell activation (12, 16). We therefore determined whether responses to flagellin were amplified in coculture models, and whether LPS or flagellin effects were amplified in the presence of DEP. For the experiments shown in Fig. 2, we selected a low concentration of LPS (0.01 pg/ml) that alone had minimal ability to activate cocultures. Stimulation of cocultures for 24 h with LPS and DEP in combination caused a synergistic increase in CXCL8 release (Fig. 2A). This synergistic increase in CXCL8 was also evident when lower concentrations of DEP were utilized (Supplementary Fig. S1. Supplementary data for this article is available online at the AJP-Lung web site.). Responses to flagellin were also greater in cocultures of epithelial cells and monocytes than the relevant monoculture controls, and again these responses were enhanced in the presence of DEP (Fig. 2B). The ability of DEP to amplify responses to endotoxin was confined to low concentrations of endotoxin causing only modest activation of epithelial cell/monocyte cocultures. Where cocultures generated ≥15 ng/ml CXCL8 in response to tested concentrations of LPS, these responses were not potentiated in the presence of DEP (data not shown).

TLR engagement results in rapid activation of signaling to MAPks and NF-κB. Accordingly, we investigated whether cotreatment of monocytes or epithelial cells with TLR agonists and DEP resulted in enhanced activation of these pathways. Although exposure of cocultures to DEP resulted in enhancement of cytokine generation in response to LPS, DEP did not induce measurable enhancement of TLR-associated intracellular signaling in monocytes or epithelial cells, specifically p38 MAPk phosphorylation (Supplementary Fig. S2) or NF-κB as measured by phosphorylation of IκBα (data not shown), over a 1-h time course. We next examined whether DEP might exert effects on TLR signaling over a longer time course (24 h), to investigate the possibility that DEP exposure might increase the expression of TLRs and accessory signaling molecules. We found that monocyte TLR4 and CD14 expression was not

<ref>Fig. 1. Cooperation between epithelial cells and monocytes results in the synergistic production of CXCL8 in response to diesel exhaust particles (DEP) or LPS. The epithelial cell line BEAS-2B (epithelial cell) was grown to 80% confluence in 24-well plates, and cocultures were created through the addition of 5,000 monocytes/well. Monocultures and cocultures were stimulated with DEP (A) or LPS (B) at the indicated concentrations, in the presence or absence of 5,000 monocytes/well. After 24 h, levels of CXCL8 in the supernatant were determined by ELISA. Data shown are means ± SEM of n = 3 replicates. Each replicate was performed at a separate passage with freshly prepared monocytes from different donors. Significant differences between monolayer and epithelial cell/monocyte cocultures are indicated by *P < 0.05, **P < 0.01, and ***P < 0.001 as measured by 2-way ANOVA and Bonferroni’s posttest.</ref>

<ref>Fig. 2. DEP potentiates the proinflammatory response of LPS and flagellin in epithelial cell and monocyte cocultures. Epithelial cell/monocyte cocultures were established as described in Fig. 1. Cells were stimulated with 50 μg/ml DEP in the presence or absence of 0.01 pg/ml LPS (A) or 0.5 μg/ml S. typhimurium flagellin (B). After 24 h, levels of CXCL8 in the supernatant were determined by ELISA. Data shown are means ± SEM of n = 5–6 replicates. Each replicate was performed at a separate passage with freshly prepared monocytes from different donors. Significant differences in CXCL8 are indicated by ***P < 0.001 compared with DEP or LPS alone, as measured by 2-way ANOVA and Bonferroni’s posttest.</ref>
enhanced, but in fact was significantly reduced, by exposure to DEP over 24 h (Supplementary Fig. S3). Expression of TLR4 and CD14 was not detected on BEAS-2B cells by flow cytometry, and was not altered by DEP exposure (data not shown).

DEP exposure alters the role of IL-1 in the initiation of inflammation. We have previously shown that effective activation of cocultures by LPS required IL-1 production from the monocytes (32). Figure 3A confirms this finding and also shows that activation of cocultures can be inhibited by the steroid hydrocortisone. Figure 3B shows that this dependence on IL-1 for activation of cocultures was also evident in responses to flagellin, with the addition of either the IL-1 receptor antagonist (IL-1ra) or hydrocortisone inhibiting CXCL8 production. These data provide further evidence for the importance of this IL-1-regulated inflammatory network. Our subsequent experiments determined whether the synergistic interactions of DEP and LPS or flagellin altered the dependence of this system on IL-1. We therefore constituted cocultures of epithelial cells and monocytes, stimulated these for 24 h with LPS or flagellin, in the presence or absence of DEP or IL-1ra, and measured CXCL8 production at 24 h. Strikingly, IL-1ra lost its ability to effectively inhibit cytokine generation induced by either LPS or flagellin when DEP were also present (Fig. 4).

We investigated the mechanisms by which DEP exposure might alter the efficacy of IL-1ra. We determined that this was not the result of absorption of IL-1ra by DEP, since IL-1ra incubated with DEP still retained biological activity when tested in other CXCL8 assays (Supplementary Fig. S4). Figure 5 shows that DEP exposure resulted in a marked decrease in detectable IL-1β release in cocultures stimulated with LPS. Once again, we confirmed that this was not due to IL-1β absorption by DEP (data not shown), and we therefore investigated why IL-1β levels were reduced in activated cocultures when DEP were present.

IL-1 signaling is dependent on a complex sequence of events. IL-1β is produced in a pro-form by inflammatory stimuli. Pro-IL-1β is predominantly cleaved by caspase 1, the proteolytic component of the inflammasome. Release of mature IL-1β occurs via a mechanism involving microvesicle shedding from the cell membrane (26). IL-1β release is heavily dependent on the activation of P2X7 by its cognate ligand, ATP, which results in efflux of potassium, activation of the inflammasome, and release of active IL-1 protein (34).

We had established that IL-1β levels were reduced in cocultures stimulated with LPS in the presence of DEP, and that this was not consequent on absorption of IL-1β by DEP. We therefore determined whether IL-1β production was maintained in LPS-stimulated monocytes when DEP were present. Examination of IL-1β release into the supernatants of stimulated cocultures was performed at 24 h to capture total IL-1β produced; however, IL-1β is a classic “early” cytokine, produced rapidly when TLRs are activated. We therefore examined IL-1β intracellular production and processing in primary human monocytes stimulated for 6 h with LPS in the presence or absence of DEP. Figure 6 shows that LPS induced intracellular production of the 35-kDa pro-IL-1β, and also the production of mature 17-kDa processed IL-1β. The presence of DEP over this time course did not significantly alter the production or cleavage of IL-1β.

We next determined whether IL-1β produced in monocytes in the presence of DEP was able to be released. We returned to our coculture model and stimulated epithelial cell/monocyte cocultures for 24 h with LPS, in the presence or absence of DEP. In the final 30 min, we added BzATP, a stable and potent activator of P2X7. This induced IL-1β release from cocultures whether or not they had been stimulated with DEP, although in keeping with our earlier data, the cocultures that had been stimulated with DEP showed a much lower baseline level of IL-1β (Fig. 7A).

IL-1β release is thought to be largely dependent on P2X7 signaling (13, 34, 52). To investigate if DEP might interfere with IL-1β release by inhibiting signaling through this pathway, we incubated ATP in water or with DEP for 3 h in a cell-free system and measured the amount of recoverable ATP. We observed that treatment of ATP with DEP resulted in a marked loss in recoverable ATP (Fig. 7B). Cooperative responses of epithelial cells and monocytes to LPS and combined DEP and LPS can be inhibited by a neutralizing soluble TNFα receptor fusion protein. TNFα has been implicated as an important mediator of proinflammatory cytokine release from bronchial epithelial cells (22). In a previous study from our group examining cocultures of airway smooth muscle cells and monocytes, we observed that inhibition of TNFα with sTNFR1 did not impair LPS-mediated...
activation of CXCL8 production. We considered that the altered inflammatory response seen when DEP was added to our epithelial cell/monocyte cocultures might be reflected by altered roles for TNFα. Both DEP, and to a lesser extent LPS, caused an increase in TNFα in our coculture system (Supplementary Fig. S5), and we observed that sTNFR1 was able to partially inhibit CXCL8 production in BEAS-2B/monocyte cocultures stimulated either with LPS alone or with both DEP and LPS (Fig. 8).

DISCUSSION

We have previously shown that effective induction of inflammation in the lung requires cooperative responses between monocytes and tissue cells (31, 32, 39, 50). In this study, we show that DEP exert a modest inflammatory action on their own, which also requires cooperative signaling between monocytes and epithelial cells. We show that, in our models of monocyte/epithelial cell cocultures, DEP can also enhance the responses to very low levels of TLR agonists. Finally, we show that the presence of DEP causes a reduction in IL-1β production in LPS-activated cocultures, potentially consequent upon reduced IL-1β release from monocytes. These data reveal considerable complexity in the proinflammatory mechanisms by which DEP may modulate lung function.

The direct toxicity of DEP with the production of proinflammatory cytokines and ROS in single cell types is well established (25, 27, 29, 37, 43, 49). Our data demonstrate that DEP alone are, however, a relatively modest stimulus of inflammation. Activation of CXCL8 generation from epithelial cells by DEP required the presence of monocytes, but in contrast to the responses induced by LPS or flagellin, DEP induced little CXCL8, and showed no evidence of a concentration-dependent induction of inflammatory responses. Similar cooperative interactions between human epithelial cells and alveolar macrophages with enhanced production of GM-CSF and IL-6 but not CXCL8, IL-1β, and TNFα, have been described in response to urban particulate matter (EHC-93) (15).

Although this cytokine response is modest compared with that of TLR agonists, a greater significance can be attributed to our finding that varied concentrations of DEP are capable of accentuating the cellular responses of very small doses of LPS that in their own right have only modest or no actions in our coculture system. This is consistent with in vivo studies that have demonstrated a synergistic interaction between DEP and endotoxin with the enhanced production of lung free radicals (1), proinflammatory cytokines, and the subsequent neutrophil infiltration into the lungs (21, 48). Thus low levels of pollution exposure may enhance the proinflammatory potential of very small concentrations of microbial products, with implications for the pathology of airway inflammation in asthma and COPD. Our data suggest that determining the safe levels of
environmental pollution exposure may be complicated, and that in inflamed tissues, very low levels of pollutants may impact on disease more significantly than expected through these cooperative networks.

The lung environment is constantly exposed to inhaled microbial products that are effectively handled and eliminated by the host. In addition to LPS, many other microbial agonists may reach the lung through inhalation or local infection. Indeed, flagellin is in its own right a potent stimulus of epithelial cell activation (12, 16), and here we show for the first time that flagellin responses (from 2 different bacteria, demonstrating that this is a generic response) were also amplified when epithelial cells and monocytes are allowed to interact. Our work also reveals that DEP are capable of potentiating proinflammatory cytokine release in response to flagellin, showing that exposure to pollution in the form of DEP can exert a potentially important amplification of responses to a range of microbial stimuli.

The mechanism of this potentiation may well involve ROS generation, since DEP are known to induce ROS, and ROS is known to prime and enhance signaling of pathways such as that regulating NF-κB (14, 42). We were unable to prevent the ability of DEP to enhance responses to LPS with the ROS scavenger, N-acetyl cysteine (data not shown), or the NADPH oxidase inhibitor, diphenylene iodonium (DPI). However, DPI proved toxic to BEAS-2B cells, and we were unable to test it at optimal concentrations (data not shown).

Fig. 6. A and B: LPS-induced intracellular IL-1 production is maintained in monocytes stimulated in the presence of DEP. Five-hundred thousand monocytes were stimulated in 12-well plates with the indicated agonists for 6 h. Cell lysates were analyzed for pro- and active IL-1β or actin expression by Western blot. Quantitative signals were derived by densitometric analysis using NIH image 1.62, and data displayed as ratio compared with actin loading control. Data shown are means ± SE of n = 4 replicates, each experiment being performed with freshly prepared monocytes from different donors. A representative blot is shown in C. Significant differences compared with unstimulated monocytes are indicated by **P < 0.01 and ***P < 0.001 as measured by 1-way ANOVA and Tukey’s posttest.

Fig. 7. Epithelial cell/monocyte cocultures stimulated with combined DEP and LPS can still release IL-1 in response to exogenous ATP addition, but DEP reduces recoverable ATP levels. A: epithelial cell/monocyte cocultures were established as described in Fig. 1. Cells were then stimulated with 50 μg/ml DEP, 1 ng/ml LPS, or dual DEP and LPS for 23.5 h. Exogenous ATP (300 μM BzATP) was added for the final 30 min of culture. After 24 h, levels of IL-1 in the supernatant were determined by ELISA. LD denotes the limit of detection. Data shown are means ± SE of n = 5 experiments. Each experiment was performed at a separate passage of BEAS-2B cells with freshly prepared monocytes from different donors. Significant differences with or without BzATP are indicated by *P < 0.05 and ***P < 0.001, as measured by 2-way ANOVA and Bonferroni’s posttest. B: 1 μM ATP was suspended in dH2O with or without 50 μg/ml DEP, for 3 h. The mixture was centrifuged at 1,000 g for 3 min to mimic the method used to obtain cell-free supernatants and then serially diluted. Samples were detected using an ATP determination kit as per the manufacturer’s instructions. Luminescence was determined using a Packard Biosciences Fusion universal microplate analyzer. Data shown are means ± SE of n = 3 replicates. Significant differences are indicated by *P < 0.05 and ***P < 0.001 as measured by 2-way ANOVA and Bonferroni’s posttest.
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Both LPS- and flagellin-induced activation of epithelial cell/monocyte cocultures are inhibited by neutralization of IL-1 signaling. This work provides increased evidence of a generic mechanism of cooperative signaling in response to TLR agonists, and has implications for therapeutic targeting in patients with COPD who often have recurrent bacterial infections and can be colonized by flagellated bacteria. In our model, activation of monocytes by LPS or flagellin causes IL-1 production, which in turn activates the tissue cells to release large quantities of inflammatory mediators (these data and Refs. 31, 32, 50). Strikingly, TLR coculture responses become markedly less dependent on IL-1 signaling in the presence of DEP. This represents a major change in the underlying inflammatory response when DEP is present.

In our coculture system, monocyte-derived IL-1 is largely responsible for subsequent activation of the tissue cells and effective production of proinflammatory mediators such as CXCL8. However, we failed to detect IL-1β in epithelial cell/monocyte cocultures stimulated with LPS and DEP in combination. Addition of BzATP at the end of the culture period still caused IL-1β release even in DEP/LPS-treated cells, indicating that IL-1β release could be achieved and that the release pathways were functional. We found that incubation of DEP with IL-1ra or IL-1β did not inhibit their recovery or biological actions. However, incubation of DEP with ATP rapidly reduced the measurable levels of ATP. The processing and release of IL-1β has been shown to be dependent on ATP signaling via P2X7 in monocytes and macrophages (34), with recent data indicating that monocytes have constitutively active caspase 1 and constitutive release of ATP (34), facilitating IL-1β production. This remains a contentious and complicated area, since P2X7-independent release of IL-1β has been described by ourselves (51) and others (18).

Our data would therefore be consistent with a model in which ATP secretion in cocultures, perhaps from the epithelial cells, amplifies monocyte IL-1β production; however, in the presence of DEP sequestration or catalyzed breakdown of ATP reduces total IL-1β production and release.

On the contrary, TLR coculture responses in the presence of DEP were partially inhibited by the soluble TNFα receptor antagonist (sTNFR1). This demonstrates a major change in the nature of the inflammatory response when DEP is present, whereby IL-1β is no longer a key initiator of inflammatory responses, and the proinflammatory properties of DEP are partially maintained by the production of TNFα. We have also demonstrated that LPS-induced activation of epithelial cell/monocyte cocultures is in part dependent on the production of TNFα. In the models shown here, we have selected LPS concentrations that are at the lower end of the effective dose range, to reveal and explore the potential for DEP to interact with these agonists. Where previously we used greater LPS concentrations in coculture models of airway smooth muscle cells, we observed no role for TNFα in induction of inflammation (32). Furthermore, when LPS is used to activate cocultures of monocytes and endothelial cells, TNFα production is actually suppressed (20, 50). Our data indicate that TNFα may therefore exert roles either selectively in the activation of epithelial cells by monocytes or more likely play roles when modest inflammatory responses are induced by low concentrations of agonists; but, when higher concentrations of agonists are used, IL-1β signaling may dominate.

We appreciate that cell lines are only a model of human primary epithelial cells, and we believe the study of primary cells is important. Here we have utilized the BEAS-2B cell line, as opposed to other cell lines, because they closely resemble bronchial epithelial cells by electron microscopy with the presence of keratin, formation of tight junctions, and mucin production (35, 41). We are currently working to establish reliable cultures of primary bronchial epithelial cells to study our models in more detail. However, we have discovered that hydrocortisone, at the concentration present in standard commercial BEC culture media, profoundly dampens the responses of monocytes to LPS, as measured in terms of CXCL8 production. We have also observed that the presence of FCS facilitates endotoxin responses of monocytes without inducing their baseline activation, but FCS markedly activates primary BEC, and this activation of BEC is not completely suppressed by hydrocortisone. In addition, bovine pituitary extract, an important growth factor in primary epithelial cell media, activates primary human monocytes. We are continuing to try to determine experimental conditions to allow optimal study of primary epithelial cell/monocyte cocultures to reliably extend our observations to primary cells.

In conclusion, our data proposes a model of DEP-modulated inflammation, in which IL-1β is no longer a key initiator of inflammatory responses, and in which the proinflammatory roles of DEP take the place of IL-1β in the initiation of inflammation. Effective targeting of DEP-modulated inflammation may be achievable in part by targeting the TNFα or ROS axes of inflammation.

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