Interleukin-1β Regulates CXCL8 Release and Influences Disease Outcome in Response to *Streptococcus pneumoniae*, Defining Intercellular Cooperation between Pulmonary Epithelial Cells and Macrophages

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The success of *Streptococcus pneumoniae* (the pneumococcus) as a pulmonary pathogen is related to its restriction of innate immune responses by respiratory epithelial cells. The mechanisms used to overcome this restriction are incompletely elucidated. Pulmonary chemokine expression involves complex cellular and molecular networks, involving the pulmonary epithelium, but the specific cellular interactions and the cytokines that control them are incompletely defined. We show that serotype 2 or 4 pneumococci induce only modest levels of CXCL8 expression from epithelial cell lines, even in the absence of a polysaccharide capsule. In contrast, coculture of A549 cells with the macrophage-like THP-1 cell line, differentiated with vitamin D, or monocyte-derived macrophages enhanced CXCL8 release. Supernatants from the THP-1 cell line prime A549 cells to release CXCL8 at levels similar to cocultures. Interleukin-1Ra (IL-1Ra) inhibits CXCL8 release from cocultures and reduces the activity of macrophage-conditioned media, but inhibition of tumor necrosis factor alpha (TNF-α) had only a minimal effect on CXCL8 release. Release of IL-1β but not TNF-α was upregulated in cocultures. IL-1 type 1 receptor knockout C57BL/6 and BALB/c mice confirmed the importance of IL-1 signaling in CXC chemokine expression and neutrophil recruitment *in vivo*. In fulminant disease, increased IL-1 signaling resulted in increased neutrophils in the airway and more invasive disease. These results demonstrate that IL-1 is an important component of the cellular network involving macrophages and epithelial cells, which facilitates CXC chemokine expression and aids neutrophil recruitment during pneumococcal pneumonia. They also highlight a potential clinical role for anti-IL-1 treatment to limit excessive neutrophilic inflammation in the lung.

*S*treptococcus pneumoniae (the pneumococcus) is a major cause of respiratory tract infection and invasive bacterial disease (6). *S. pneumoniae* is also a common commensal of the upper respiratory tract, but innate host defenses prevent disease in most colonized individuals (6, 40). The success of the innate response against pneumococcus is emphasized by the relatively low incidence of community-acquired pneumonia or invasive disease in comparison to the frequency of upper respiratory tract colonization (16, 39).

The respiratory tract epithelium plays a critical role in the recognition of bacterial pathogens and in the induction of the inflammatory response (20). Epithelial cells express a range of Toll-like receptors (TLR) and other pattern recognition receptors (13). The clinical importance of these pathways of innate recognition of *S. pneumoniae* has been highlighted by the identification of genetic mutations or polymorphisms in these signaling pathways, which confer altered susceptibility to invasive pneumococcal disease (29, 31). Engagement of a range of pattern recognition receptors, including TLRs, nucleotide-binding oligomerization domain (NOD) proteins, the NOD-like receptor (NLR) family, and pyrin domain-containing 3 (NALP3), by pneumococci activates NF-κB and induces CXCL8 (interleukin-8 [IL-8]) which leads to recruitment of polymorphonuclear leukocytes (neutrophils) (8, 25, 41, 49, 65). This is a key feature of pneumococcal infection when resident pulmonary defenses are overwhelmed (15).

Epithelial cell responses are enhanced by cooperative signaling with other cell types. Bronchoalveolar lavage fluid from lungs infected with pneumococci stimulates epithelial cell NF-κB activation (44), and alveolar macrophages enhance CXCL8 production by epithelial cells in lung explants (66). It is postulated that, in order to avoid excessive inflammatory responses to commensal organisms, airway epithelial cells express constitutively low levels of TLRs, but studies of TLR2 expression indicate that it may be upregulated when inflammation is present (35, 64). Previous studies have demonstrated that monocytes enhance epithelial cytokine responses to various TLR agonists (9, 37). Increases in proinflammatory cytokine and chemokine release in cocultures of pulmonary epithelial cells and monocytes have been noted in response to a range of lipopolysaccharides (9, 42, 52), staphylococcal exotoxins (30), *Mycobacterium tuberculosis* (63), and respiratory syncytial virus (RSV) (58). Thus, cell-cell communication

between monocytes and epithelial cells is an important early step in the immune response to respiratory tract infections.

S. pneumoniae possesses a number of virulence factors which may confound front-line immune responses (28), including a polysaccharide capsule which limits bacterial phagocytosis by macrophages (2) and inhibits bacterial attachment to respiratory epithelial cells (1, 22, 51). In this study, we have characterized the cellular and molecular factors involved in the regulation of CXCL8 expression by respiratory tract epithelial cells in response to *S. pneumoniae* infection. We demonstrate that IL-1β secretion by a macrophage-like cell line is required for maximal secretion of CXCL8 by epithelial cells in response to *S. pneumoniae in vitro*. We also establish important roles for IL-1β in regulating the expression of CXC chemokines and rate IL-1 signaling to the pulmonary infiltration of polymorphonuclear cells *in vivo* in murine models of *S. pneumoniae* infection.

MATERIALS AND METHODS

Materials. Gentamicin was purchased from Roussel Laboratories (Uxbridge, United Kingdom), vitamin D_3 (1,25-dihydroxy-cholecalciferol) from Sigma-Aldrich (Poole, United Kingdom), and recombinant human IL-1β, recombinant human soluble tumor necrosis factor (TNF) receptor type 1, and recombinant human IL-1 receptor antagonist (IL-1Ra) from PeproTech EC Ltd. (London, United Kingdom).

Bacterial strains and growth conditions. *Streptococcus pneumoniae* strains used in this study were serotype 2 strain D39 and its isogenic unencapsulated derivative FP22 (43) and serotype 4 strain TIGR4 and its unencapsulated derivative FP23 (2). D39 and TIGR4 were obtained from the American Type Culture Collection (ATCC) bacteriology collection, FP22 from Tim Mitchell (University of Glasgow), and FP23 from Gianni Pozzi and Franco Ianelli (University of Sienna). Nontypeable conjunctival clinical strains 08-1773 (MLST type 448) and 02-3522 (pre-MLST) were from Tim Mitchell and the Scottish *Haemophilus*, *Legionella*, *Meningococcus*, and *Pneumococcus* Reference Laboratory (SHLMPRL). Bacteria were grown to exponential phase in brain heart infusion broth (Oxoid Unipath, Basingstoke, United Kingdom) with 20% heat-inactivated fetal calf serum (FCS) (Autogen Bioclear, Wiltshire, United Kingdom), and aliquots were stored at −80°C. The concentration (CFU/ml) was determined by Miles Misra count for each strain as previously described (14). Freshly thawed aliquots were spun at $9,000 \times g$ for 2 min, and the bacterial pellet was washed twice in phosphate-buffered saline (PBS) prior to use.

Human cell culture. All cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC), and all culture media were from BioWhittaker (Lonza, Belgium). A549 cells, a human lung carcinoma cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose, 2 mmol/liter L-glutamine, and 10% FCS. Detroit 562 cells, a human pharyngeal carcinoma cell line, were maintained in Eagle's minimal essential medium (EMEM) with 2 mmol/liter L-glutamine and 10% FCS. Epithelial cells were seeded at 2×10^5 cells/ml in 24-well plates (Costar) and grown to confluence for 24 h prior to use.

THP-1 cells, a monocytic leukemic cell line, were maintained in RPMI 1640 containing 2 mmol/liter L-glutamine and 10% FCS. Differentiation to a macrophage phenotype was achieved by resuspending cells at 2×10^4 cells/ml in fresh medium with the addition of vitamin D_3 (VD_3) at 10^{-7} M and incubation for 72 h in 5% CO_2 at 37°C (26, 50). This differentiation protocol produces a nonadherent immature macrophage phenotype more similar to an inflammatory macrophage than a highly differentiated tissue macrophage in terms of its response to microbial factors (12). Peripheral mononuclear cells were isolated from human blood as described previously (14), from healthy donors after informed consent and approval by the South Sheffield Research Ethics Committee. The day after isolation, nonadherent cells were removed by washing in RPMI, and adherent monocyte-derived macrophages (MDM) were collected by gentle scrap-

ing. Hemocytometer counts of all cell cultures were performed on the day of experimentation to determine approximate cell numbers per well. In cocultures, nonadherent VD_3-differentiated THP-1 or 1-day-old MDM were added to adherent A549 epithelial cells at a ratio of 1:10 (using data for cell numbers derived from the hemocytometer counts of representative wells of A549 cells), and cells were cultured in RPMI with 10% FCS (with low endotoxin levels).

Infection of cell cultures. Growth medium was removed from epithelial cell monolayers, and cells were washed twice with PBS. Fresh growth medium (1 ml per well) was applied, and cells were infected with bacterial strains at a multiplicity of infection (MOI) of 10 for epithelial cells and 100 for VD_3-differentiated THP-1 cells or MDM (to ensure the same number of bacteria were added to each well). Pneumococci were opsonized in RPMI 1640 supplemented with 10% antipneumococcal immune serum and incubated on a rotating device for 30 min at 37°C prior to infections for all experiments involving THP-1 cells and in experiments investigating the effect of opsonization on epithelial cell responses (2). Infected cell cultures were centrifuged at $150 \times g$ for 5 min prior to incubation at 37°C in 5% CO_2. Culture medium (1 ml) was removed from wells at each time point, spun at $10,000 \times g$ for 10 min, and stored at −80°C prior to cytokine assay. Epithelial cells showed significant loss of viability following overnight incubation with *S. pneumoniae*, and therefore gentamicin (100 µg/ml) was added to all wells at the 2-h time point to prevent bacterial overgrowth and loss of epithelial cell viability. As a positive control for CXCL8 production, some wells were treated with 10 ng/ml IL-1β for the indicated time periods.

Bacterial adherence assay. Infected epithelial cell monolayers (MOI of 10) were centrifuged at $150 \times g$ for 5 min prior to incubation for 2 h at 37°C in 5% CO_2. In certain experiments, specific variables were altered to determine their effect on adherence. These included increasing the MOI to 50, centrifuging at $1,200 \times g$, using opsonized pneumococci, and incubating for different durations. At 2 h (or at 1 h or 4 h in experiments with altered incubation periods), the medium (containing nonadherent bacteria) was removed from each well, and nonadherent bacterial counts were determined by Miles Misra (36). Epithelial cells (including any adherent bacteria) were then removed from each well with trypsin and versine, and adherent bacterial counts were determined by Miles Misra. The proportion of adherent bacteria could then be calculated (62).

Investigation of the nature of proinflammatory mediators generated in cocultures. To generate macrophage-conditioned media, VD_3-differentiated THP-1 cells (2×10^4/well) were exposed to opsonized D39 *S. pneumoniae* (MOI of 100:1) (macrophage-conditioned medium D [MCM-D] or mock-infected MCM [MCM-MI]) for 24 h (100 µg/ml gentamicin was added at 2 h to all wells). MCM-D and MCM-MI were removed from all wells at 24 h and spun at $10,000 \times g$ for 10 min, and the supernatants (1 ml) were then applied to epithelial cell monolayers. In blocking experiments, IL-1Ra (200 ng/ml) and/or soluble TNF receptor type 1 (sTNFR1; 50 ng/ml) were added to conditioned media after transfer onto epithelial cell monolayers at time zero. Plates were then incubated in 5% CO_2 at 37°C, and media were removed from wells at 6 h, spun at $10,000 \times g$ for 10 min, and then stored at −80°C prior to cytokine assay. Samples of conditioned media were saved at time zero (following centrifugation) in order to determine baseline levels of CXCL8 prior to transfer onto epithelial monolayers. These baseline values were subtracted from the total CXCL8 concentration at 6 h to calculate the amount produced by stimulated epithelial cells alone.

Blockade of candidate proinflammatory molecules. Coculture wells were prepared as described above with A549 epithelial cells and THP-1 at a ratio of 10:1, and blocking agents (IL-1Ra, 200 ng/ml) (58, 63) and/or sTNFR1 (50 ng/ml) (38) were added at time zero. Wells were then infected with opsonized pneumococci (MOI of 10), spun at $150 \times g$ for 5 min, and then incubated at 37°C in 5% CO_2. Medium was removed at 6 h, spun at $10,000 \times g$ for 10 min, and stored at −80°C. Gentamicin (100 µg/ml) was added to all wells at 2 h.

Role of soluble mediators. To physically separate THP-1 and A549 cells, THP-1 was added to cell culture inserts, pore size of 1 μm (BD Falcon), with A549 cells in the well below the insert, at ratio of 10:1 of A549:THP-1 cells. Opsonized D39 pneumococci were added at equal doses to both the insert and lower well compartments to achieve an overall MOI of 10:1 pneumococci:A549 cells. Plates were then spun at $150 \times g$ for 5 min and incubated at 37°C in 5% CO_2, and then media were removed at 6 h, spun at $10,000 \times g$ for 10 min, and stored at -80°C. Gentamicin (100 μg/ml) was added to all wells at 2 h.

Murine pneumonia models. IL-1 type 1 receptor knockout mice (18), allele *Il1r1*^{Tm1Imx}, were kindly provided by Immunex Corporation to M.J.H.N. and were backcrossed for at least 10 generations against both BALB/cOlaHsd and C57BL/6NHsd separately. Heterozygotes were bred to yield homozygotes, and working colonies of homozygotes were maintained. BALB/cOlaHsd or C57BL/6NHsd (Harlan) were used as wild-type controls. Adult mice of both sexes were used. All animal experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 and local ethical approval.

Pulmonary infection of mice was with 5×10^5 or 1×10^7 CFU serotype 4 *S. pneumoniae* prepared and delivered by direct tracheal instillation as described previously (15). At 24 h postinfection, mice were killed by overdose of sodium pentobarbitone, and bronchial alveolar fluid, lung, and blood were collected for determination of cell differential, cytokines, and viable bacteria in lung and blood as described previously (15).

Cytokine assays. CXCL8, IL-1β, and TNF-α levels in the culture supernatants were measured using human CXCL8, IL-1β, and TNF-α DuoSet enzyme-linked immunosorbent assay (ELISA) kits, respectively (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's guidelines. Murine CXCL1, CXCL2, and TNF-α were measured in bronchoalveolar lavage (BAL) fluid using murine CXCL1 and CXCL2 DuoSet ELISA kits (R&D Systems, Abingdon, United Kingdom) and murine TNF-α ELISA Ready-SET-Go! reagent set (eBioscience, Hatfield, United Kingdom), respectively, according to the manufacturers' guidelines.

Optical densities were determined using an Opsys MR microplate reader (Dynex Technologies). The lower limit of detection was approximately 30 pg/ml for CXCL8, 20 pg/ml for human IL-1β, 30 pg/ml for human TNF-α, 16 pg/ml for murine CXCL1, 32 pg/ml for murine CXCL, and 16 pg/ml for murine TNF-α.

Statistics. All results are recorded as means $\pm$ standard errors of the means (SEM) unless otherwise stated. Statistical testing was performed using Prism 5.02 software (GraphPad Software Inc.). Unpaired *t* test, Mann-Whitney test, or one-way analysis of variance (ANOVA) (with Bonferroni's posttest) were used to analyze differences between groups, as appropriate. Significance was defined as *P* values of <0.05.

RESULTS

Epithelial cell responses to *S. pneumoniae* are limited by the polysaccharide capsule. Epithelial cells contribute to innate host defense, but *S. pneumoniae* contains several adaptations, which can modulate its interaction with epithelial cells (13, 19, 32, 59, 62), in particular the expression of the polysaccharide capsule (1, 21, 22, 51, 57). The mechanisms used to integrate the epithelial cell response into the innate host response, despite microbiological restriction, are uncertain. We confirmed that there was minimal adherence of encapsulated serotype 2 or 4 *S. pneumoniae* to epithelial cell lines (see the supplemental material at http://www.shef.ac.uk/medicine /infectionandimmunity/staffprofiles/dockrell.html, Fig. S1A to S1D). Binding was not significantly enhanced by opsonization of bacteria, by increasing the MOI to 50:1, by increasing the incubation period, or by centrifuging the bacteria onto the cells at $1,200 \times g$ rather than $150 \times g$ (data not shown). Mutants lacking capsule, either on a serotype 2 or 4 background (2, 43), demonstrated significantly greater binding than the parental capsulated strains (see the supplemental material at http://www.shef.ac.uk/medicine

/infectionandimmunity/staffprofiles/dockrell.html, Fig. S1A to S1D).

CXCL8 is critical for the effective recruitment and activation of polymorphonuclear cells at sites of inflammation (3, 54). Microbial factors stimulate epithelial cells via a range of receptors to produce CXCL8 (5, 35, 49). We investigated the ability of nonopsonized pneumococci to stimulate CXCL8 release from epithelial cell monolayers. There was no significant difference between the CXCL8 response of mock-infected epithelial cells and those exposed to encapsulated pneumococci, which was low in comparison with cells stimulated with IL-1β (11) (Fig. 1A and B). We demonstrated significantly greater CXCL8 production in response to unencapsulated pneumococci; however, when we compared the encapsulated serotype 2 and 4 strains to unencapsulated clinical strains, we did not note any significant difference in CXCL8 release (see the supplemental material at http://www.shef.ac.uk/medicine /infectionandimmunity/staffprofiles/dockrell.html, Fig. S2), although it was not clear whether these results reflected the marked genetic differences between these unencapsulated strains and other pneumooccal serotypes. Opsonization modestly enhanced the epithelial cell CXCL8 response to encapsulated pneumococci (see the supplemental material at http://www.shef.ac.uk/medicine /infectionandimmunity/staffprofiles/dockrell.html, Fig. S3). We therefore opsonized pneumococci in all subsequent experiments. We also observed significantly greater CXCL8 responses to the unencapsulated serotype 4 strain, FP23, than to other strains, possibly in keeping with enhanced proinflammatory cytokine responses to piliated pneumococci (4).

Coculture of epithelial cells with a macrophage-like cell line enhances CXCL8 secretion following exposure to *S. pneumoniae*. Cytokine and chemokine responses can be enhanced in epithelial cell cultures by monocyte- or macrophage-derived factors (9, 30, 37, 42, 52, 53, 58, 63, 66). CXCL8 expression by the THP-1 macrophage cell line or epithelial cells in response to pneumococci was consistently less than the epithelial cell response to IL-1β (Fig. 1C and D). In contrast, we demonstrated a marked enhancement of CXCL8 responses for macrophage-epithelial cell cocultures. The generation of CXCL8 by cocultures treated with pneumococci at the 6- and 24-h time points was significantly greater than the sum of the CXCL8 responses of A549 epithelial cells and THP-1 cells alone. The responses were apparent by 6 h after infection and were sustained up to 24 h after infection. We therefore used the 6-h time point in subsequent experiments. Similar results were obtained with the Detroit epithelial cell line, which also showed enhanced CXCL8 expression in coculture conditions (data not shown). Interestingly, although the macrophage-like cell line showed a significantly greater CXCL8 response to encapsulated bacteria than to unencapsulated bacteria, the presence of the polysaccharide capsule had little effect on the magnitude of the coculture response to pneumococci. We also noted that opsonization corrected the inhibitory effect of the capsule on the response of epithelial cell monocultures to pneumococci.

IL-1 production by the THP-1 macrophage-like cell line stimulates CXCL8 expression from epithelial cells following exposure to *S. pneumoniae*. We next addressed whether cell contact between THP-1 cells and epithelial cells was a necessary or contributory factor. When epithelial cells were separated from monocytes using semipermeable transwells, we observed no significant reduction in CXCL8 secretion (Fig. 2), suggesting that direct cell contact was not required and that a soluble factor was responsible
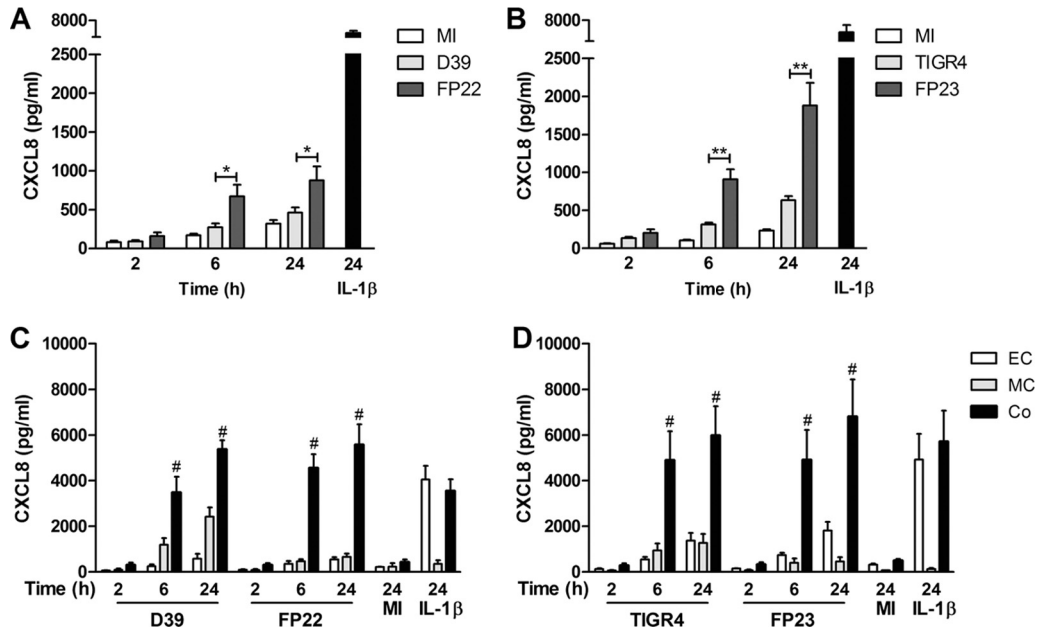
FIG 1 Presence of macrophages enhances epithelial cell CXCL8 production. (A and B) A549 epithelial cells were either mock infected (white bars) or challenged with encapsulated (pale-gray bars) or unencapsulated (dark-gray bars) nonopsonized serotype 2 (A) and serotype 4 (B) *S. pneumoniae* (MOI of 10) or IL-1β (black bars). CXCL8 levels in the cell culture supernatants were measured by ELISA at the indicated time points ($n = 4$ to 6 independent experiments). (C and D) A549 epithelial cells alone (EC), THP-1 cells alone (MC), and A549-THP-1 cocultures (Co) were challenged with opsonized *S. pneumoniae* for up to 24 h. CXCL8 levels in the cell culture supernatants were measured by ELISA at 2-, 6-, and 24-h time points. (C) Encapsulated (D39) and unencapsulated (FP22) serotype 2 pneumococci ($n = 7$); (D) encapsulated (TIGR4) and unencapsulated (FP23) serotype 4 pneumococci ($n = 5$). Mock-infected (MI) and IL-1β data at the 24-h time point is shown for comparison. One-way ANOVA (all *P* values are <0.05) with Bonferroni's posttest: *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.05$ for CXCL8 level in coculture versus the sum of CXCL8 levels from A549 and THP-1 cells alone).

for priming the epithelial cells. Bronchial alveolar fluid from the lungs of mice infected with *S. pneumoniae* has been shown to stimulate NF-κB activation and resultant chemokine expression by epithelial cells, although the cellular source of the factors responsible are incompletely characterized (44). Previous work from our group has indicated in a number of models that IL-1 may play important roles in the induction of inflammatory responses (9, 38). When we transferred supernatants from the mock-infected THP-1 cells onto epithelial monolayers, we observed little release of CXCL8, while supernatants from the *S. pneumoniae*-exposed THP-1 cells induced levels of CXCL8 secretion comparable to infected cocultures (Fig. 3). We also investigated the mediators responsible for activation of A549 cells by bacterial-exposed THP-1 cells and focused on the two dominant early proinflammatory cytokines, TNF-α and IL-1 (24, 27, 45, 52). IL-1Ra, but not soluble TNF-α receptor 1, treatment blocked the ability of macrophage-conditioned media to stimulate CXCL8 production by the epithelial cell line.
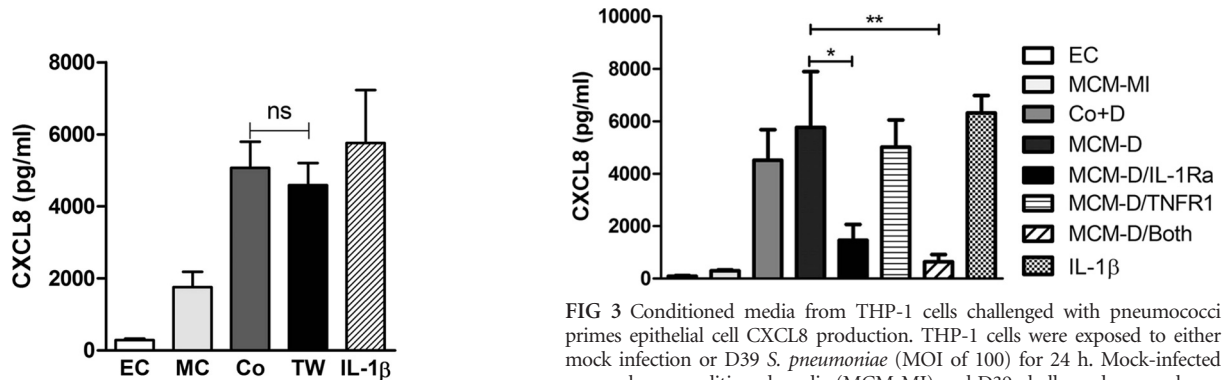

FIG 2 Physical separation of macrophages from epithelial cells does not impair priming of CXCL8 production. A549 epithelial cells alone (EC), THP-1 cells alone (MC), A549-THP-1 cocultures (Co), and A549 and THP-1 cells separated by transwells (TW) were challenged with D39 pneumococci (MOI of 10). CXCL8 levels in the cell culture supernatants were measured by ELISA at 6 h. IL-1β (positive control) data are shown for comparison (IL-1β). One-way ANOVA with Bonferroni's posttest ($n = 6$). ns, not significant.

FIG 3 Conditioned media from THP-1 cells challenged with pneumococci primes epithelial cell CXCL8 production. THP-1 cells were exposed to either mock infection or D39 *S. pneumoniae* (MOI of 100) for 24 h. Mock-infected macrophage-conditioned media (MCM-MI) and D39-challenged macrophage-conditioned media (MCM-D) were collected at 24 h. Monolayers of A549 epithelial cells were then incubated with medium alone (EC), MCM-MI, THP-1 cells and D39 (MOI 10) (Co+D), MCM-D, MCM-D plus IL-1Ra (MCM-D/IL-1Ra), MCM-D plus sTNF type 1 receptor (MCM-D/TNFR1), MCM-D plus both inhibitors (MCM-D/Both), or IL-1β. CXCL8 levels in the cell culture supernatants were measured by ELISA at 6 h ($n = 3$); one-way ANOVA ($P < 0.001$) with Bonferroni's posttest: *, $P < 0.05$; **, $P < 0.01$.
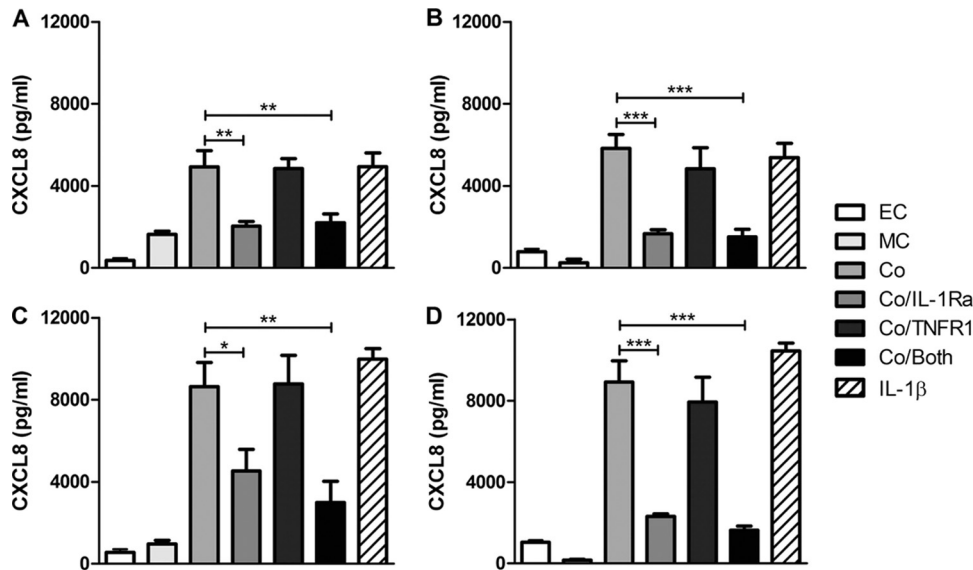
FIG 4 IL-1Ra blocks enhancement of CXCL8 production in cocultures. Monolayers of A549 epithelial cells alone (EC), THP-1 cells alone (MC), A549-THP-1 cocultures (Co), cocultures with IL-1Ra (Co/IL-1Ra), cocultures with sTNF type 1 receptor (Co/TNFR1), and cocultures with both inhibitors (Co/both) were challenged with encapsulated D39 (A) and unencapsulated FP22 serotype 2 (B) pneumococci and encapsulated TIGR4 (C) and unencapsulated FP23 serotype 4 (D) pneumococci. IL-1β-stimulated cocultures are also shown for comparison. CXCL8 levels in the cell culture supernatants were measured by ELISA at 6 h ($n = 3$ to 4); one-way ANOVA (all P values are <0.002) with Bonferroni's posttest: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

IL-1Ra inhibits CXCL8 release from cocultures challenged with *S. pneumoniae*. We next addressed whether the contribution of IL-1 and TNF-α altered with bacterial strain. IL-1Ra reduced CXCL8 secretion by cocultures in the presence of both encapsulated and unencapsulated strains and also the piliated type 4 strains (4). TNF-α blockade had no effect on CXCL8 secretion in the presence or absence of IL-1Ra at the 6-h time point (Fig. 4) and made only a minor contribution to CXCL8 release in cocultures at 24 h, which reached statistical significance only for the unencapsulated type 4 pneumococci (see the supplemental material at http://www.shef.ac.uk/medicine/infectionandimmunity/staffprofiles/dockrell.html, Fig. S4). Overall these findings suggested that IL-1 was the major factor enhancing CXCL8 release. We also confirmed that if we used primary monocyte-derived macrophages (MDM) 1 day after differentiation from monocytes, they were also able to enhance CXCL8 production from epithelial cells and that IL-1 also contributed to this release (Fig. 5).

IL-1β release is enhanced in cocultures. We next confirmed IL-1β and TNF-α release from the THP-1 cell line following challenge with D39 pneumococci, excluding the possibility that the lack of inhibition by TNF-α blockade was merely a consequence of lack of production (Fig. 6A and B). However, only IL-1β was enhanced in cocultures. The same pattern of cytokine release was observed in response to type 4 pneumococci (data not shown). We also showed that neither IL-1β nor TNF-α were induced following application of macrophage-conditioned media to epithelial cell monolayers, thus confirming the THP-1 cells as the major source of IL-1β and TNF-α (Fig. 6C and D).

Modulation of IL-1 signaling alters production of CXC chemokines and polymorphonuclear cell recruitment during *S. pneumoniae* infection *in vivo*. To confirm the relevance of these findings, we next investigated pneumonia models in mice with differing sensitivity to IL-1 signaling (23). C57BL/6 mice recruit

polymorphonuclear cells only when resident host defenses against pneumococci become overwhelmed (17). BALB/c mice develop an early neutrophilic response against pneumococci, which is greater than that seen in C57BL/6 mice. $Il1r1^{-/-}$ C57BL/6 mice
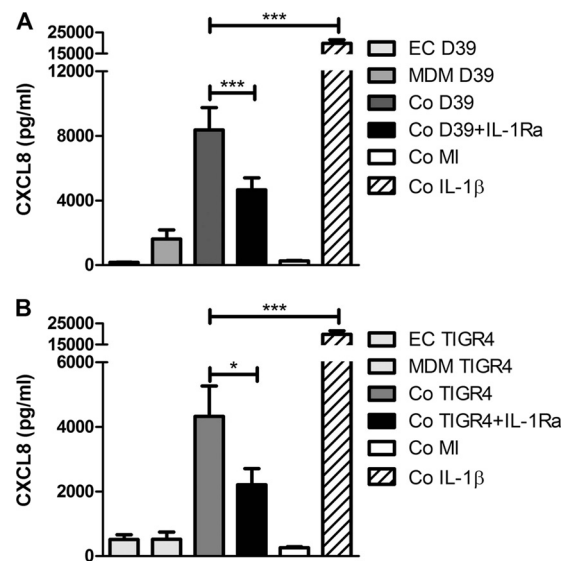

FIG 5 Enhanced epithelial cell CXCL8 production is also seen in cocultures containing primary monocyte-derived macrophages. A549 epithelial cells alone (EC), monocyte-derived macrophages alone (MDM), and A549-MDM cocultures (Co) were challenged with opsonized *S. pneumoniae* D39 (A) or TIGR4 (B) (MOI of 10), with or without IL-1 receptor antagonist (IL-1Ra) for 6 h, and CXCL8 levels in the cell culture supernatants were measured by ELISA and normalized for cell number (data show release/1×10^5 A549 cells). Mock-infected (MI) and IL-1β data are shown for comparison. One-way ANOVA (all P values are <0.05) with Bonferroni's posttest: *, $P < 0.05$; ***, $P < 0.01$. P values of <0.01 for CXCL8 levels in coculture versus the sum of CXCL8 levels from A549 cells and MDM alone ($n = 3$ to 5) from 3 independent experiments.
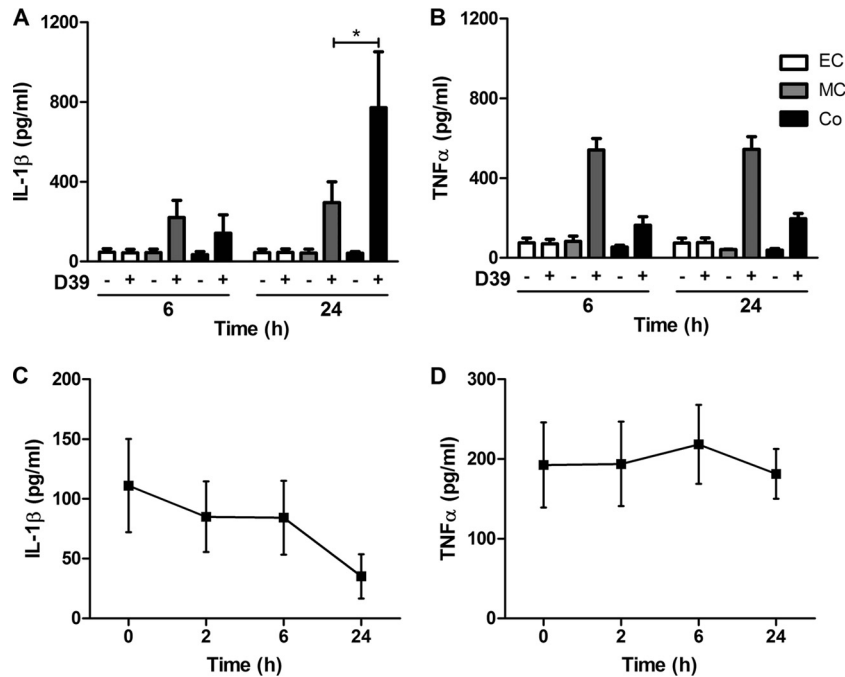
FIG 6 IL-1β but not TNF-α levels are enhanced in cocultures following challenge with pneumococci. A549 epithelial cells (EC) were exposed to media alone (−) or D39 pneumococci (+), as were THP-1 cells (MC) and A549-THP-1 cocultures (Co) and IL-1β (A) and TNF-α (B) levels measured by ELISA at 6 and 24 h ($n = 3$); one-way ANOVA (all P values are <0.02) with Bonferroni's post test: *, $P < 0.05$. A549 epithelial cells were exposed to conditioned media from D39-challenged THP-1 cells, and IL-1β (C) and TNF-α (D) levels were measured by ELISA at 0, 2, 6 and 24 h.

have normal leukocyte numbers but a reduction in early bacterial clearance in a model of pneumococcal pneumonia (18, 46), while $Il1r1^{-/-}$ BALB/c mice have never been studied in a model of pneumococcal pneumonia to our knowledge. We confirmed that polymorphonuclear cell recruitment was greater for BALB/c mice than for C57BL/6 mice following challenge with a similar dose of pneumococci (Fig. 7A and D). In each genetic background, $Il1r1^{-/-}$ mice recruited fewer polymorphonuclear cells.

We then addressed the expression of CXCL1 (KC) and CXCL2 (MIP-2α), which are the murine paralogs of CXCL8 in each mouse strain. CXCL1 was significantly reduced in each $Il1r1^{-/-}$ mouse strain (Fig. 7B and E). There was a nonsignificant decrease in CXCL2 for $Il1r1^{-/-}$ C57BL/6 and no alteration in $Il1r1^{-/-}$ BALB/c mice (Fig. 7C and F). Although numbers of mice were small, the $Il1r1^{-/-}$ C57BL/6 mice had a trend toward increased bacterial numbers in lung and blood, in keeping with prior observations (46), but there was no difference in bacterial numbers in $Il1r1^{-/-}$ BALB/c mice (see the supplemental material at http://www.shef.ac.uk/medicine/infectionandimmunity/staffprofiles/dockrell.html, Fig. S5).

Modulation of IL-1 signaling can reduce the severity of invasive disease in fulminant pneumococcal infection. We next increased the severity of infection in the BALB/c mice, which increased the number of polymorphonuclear cells in the BAL fluid. In this model, polymorphonuclear cell recruitment was again reduced in the $Il1r1^{-/-}$ mice (Fig. 8A), although levels of recruitment were much greater than with the lower-dose infection model (compare Fig. 8A with Fig. 7D). Interestingly CXCL1/2 expression was not lower in the lungs of $Il1r1^{-/-}$ mice with severe disease (Fig. 8B and C). This suggested other compensatory changes in the cytokine network might have occurred to enable neutrophil recruitment, and we observed that levels of TNF-α, a known stim-

ulus for CXCL1 chemokine expression during pneumococcal infection (55), was increased in $Il1r1^{-/-}$ BALB/c mice (Fig. 8D). We found no alteration in colony counts in the lung but a significant decrease in colony counts in the blood of $Il1r1^{-/-}$ BALB/c mice (Fig. 8E and F).

DISCUSSION

In this study, we demonstrate a major role for macrophage-alveolar epithelial cell signaling during the initial phase of host defense against *S. pneumoniae*. We describe a critical role for IL-1 in stimulating CXCL8 release in response to *S. pneumoniae* and show that a macrophage cell line and primary macrophages are important sources of this cytokine. *In vivo* models have confirmed that IL-1 signaling stimulates CXCL1/2 chemokine expression in mice and facilitates recruitment of polymorphonuclear leukocytes to the lung during infection.

Respiratory epithelial cells are an important component of pulmonary host defense, possessing pattern recognition receptors, such as TLRs, which enable sensing of microorganisms and link recognition of bacteria to NF-κB activation and transcription of cytokines and chemokines (13, 20, 44). Hyporesponsiveness in this system, as recently demonstrated for an association between IRAK4 polymorphisms and susceptibility to Gram-positive infections in patients with critical illness, limit the effectiveness of the innate immune responses (56). We show that direct exposure of A549 cells to pneumococci induces only low-level CXCL8 secretion. Capsule limits the adherence of pneumococci to epithelial cells (21, 51), and this was confirmed again in our study. Nevertheless, cytokine expression correlates poorly with adherence; previous studies have found that the epithelial cell inflammatory response is not associated with the level of bacterial adherence (7),
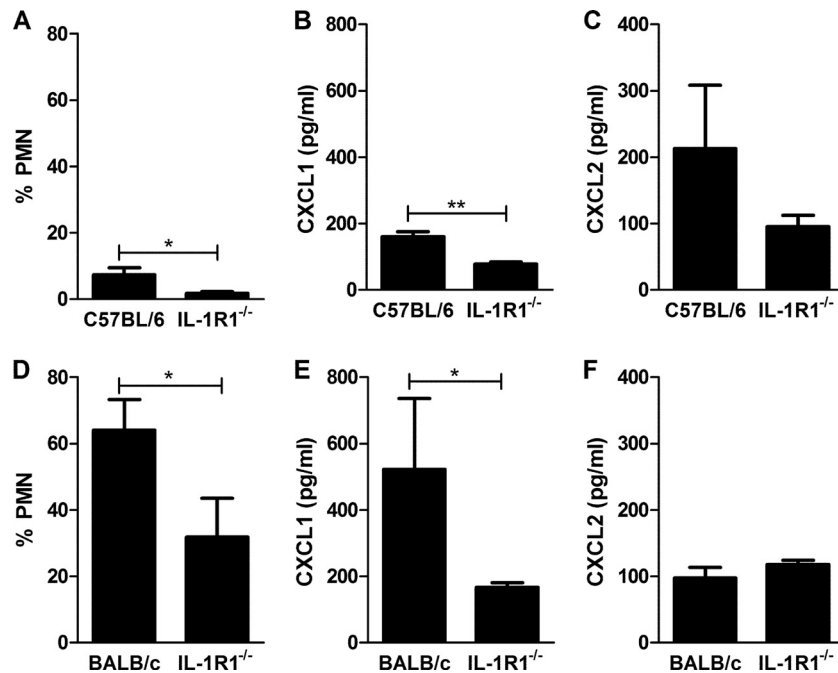
FIG 7 Modulation of IL-1 signaling alters production of CXC chemokines and polymorphonuclear cell (PMN) recruitment during *S. pneumoniae* infection *in vivo*. (A) Percentage of PMNs in bronchial alveolar lavage (BAL) fluid from C57BL/6 control mice and mice deficient in IL-1 type 1 receptor on a C57BL/6 background (IL-1R1$^{-/-}$) 24 h after intratracheal instillation of 5×10^5 CFU type 4 pneumococci. Levels of CXCL1 (B) and CXCL2 (C) in BAL fluid from the same experiment as described for panel A. (D) Percentage of PMNs in BAL fluid from BALB/c control mice and mice deficient in IL-1 type receptor on a BALB/c background (IL-1R$^{-/-}$) challenged with pneumococci as described for panel A. Levels of CXCL1 (E) and CXCL2 (F) in BAL fluid from the same experiment as that described for panel D. *, $P < 0.05$; **, $P < 0.01$, unpaired *t* test.

nor is it associated with the binding capacity of particular pneumococcal strains (48). Some bacteria can adhere and invade epithelial cell surfaces by downregulation of capsule, while some encapsulated strains can coat themselves in IgA fragments to overcome the electrostatic effects of a negatively charged capsule (21, 62).

Epithelial cell responses during inflammation are, however, dependent on a cellular network in which additional cell types prime epithelial cells to upregulate chemokine release (37, 42). These networks contain multiple potential cellular sources of a broad range of cytokines, including monocyte synthesis and secretion of IL-1β and TNF-α, which can prime alveolar epithelial cells to release CXCL8 (37, 42, 53). In the context of the early stages of pneumococcal infection, both IL-1β and TNF-α are important in stimulating NF-κB activation in pulmonary epithelium (44), but the sources of these cytokines are not fully defined. There is evidence that macrophages enhance CXCL8 expression in lung explants, but which cell types produce the cytokine is uncertain (66). We now show that a macrophage phenotype, resembling monocytes or inflammatory macrophages (12), releases IL-1 to stimulate CXCL8 release from a type II alveolar epithelial cell line. In contrast, macrophage-expressed TNF-α was not a significant factor in priming epithelial cells to produce CXCL8 in cocultures containing the macrophage cell line. The response induced by primary macrophages also involved IL-1, but the degree of inhibition by IL-1Ra was less marked, suggesting that other factors could also play a role.

These findings are consistent with data from previous studies which observed that IL-1Ra inhibits epithelial cell CXCL8 secretion in response to conditioned medium from *M. tuberculosis*-infected human monocytes (63) and in response to conditioned media from RSV-infected monocytes (58). Teichoic acid in the pneumococcal cell wall is a potent stimulus for IL-1 expression from monocytes (45). Teichoicated species in the cell wall could be the predominant microbial factor driving the enhanced CXCL8 response in cocultures, with an additional role played by strain variable factors, such as pili, a known stimulus for TNF-α production, at later stages of infection or during the fulminant mouse model when IL-1 signaling was impaired.

In interpreting the results of our study, it is necessary to bear in mind the complex nature of cell-cell signaling and cytokine networks. It is possible that the synergistic response in coculture conditions involves a component of bidirectional signaling between macrophages and epithelial cells (60). Although macrophage-conditioned media enhanced CXCL8 secretion from epithelial cells, signals could flow in the reverse direction. IL-1β expression was enhanced in cocultures. Activating signals from stimulated epithelial cells may potentiate the response of monocytes or macrophages (and possibly other immune cells *in vivo*), with inflammatory mediators acting in a paracrine fashion to stimulate production of other effector molecules. Once the initial recruitment of polymorphonuclear leukocytes has occurred, these cells can release IL-1β to fuel further chemokine release from macrophages and nonmyeloid cells, as demonstrated recently in a murine model of arthritis (10).

Cocultures of A549 and THP-1 cell lines provide a useful and well-recognized *in vitro* model of the alveolar space. We can confirm that the VD$_3$-differentiated THP-1 cells have a differentiation state which resembles a monocyte or macrophage in the early stage of tissue differentiation (12). We verified findings with a
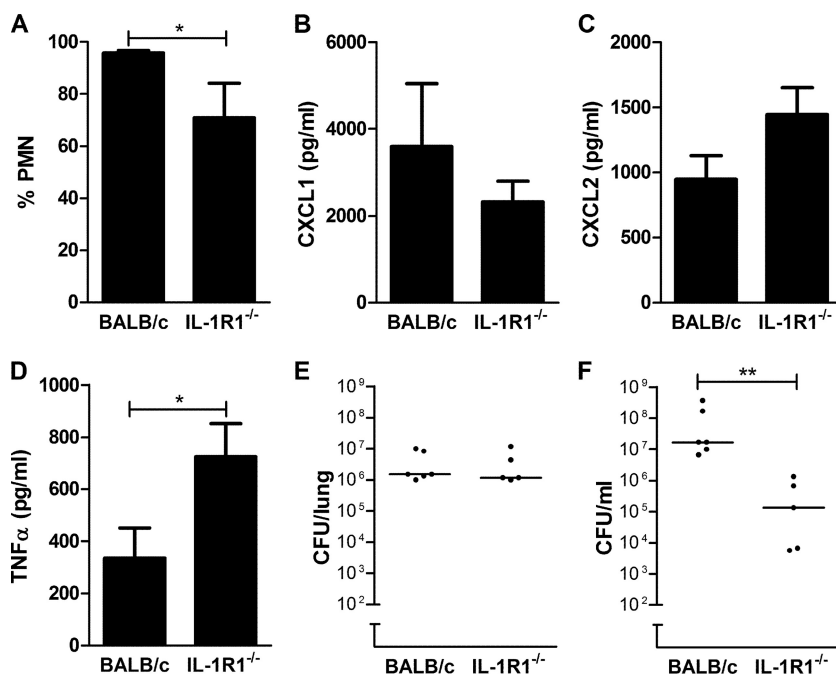
FIG 8 Modulation of IL-1 signaling can reduce the severity of invasive disease in fulminant pneumococcal infection. (A) Percentage of neutrophils (PMN) in bronchial alveolar lavage (BAL) fluid from BALB/c control mice and mice deficient in IL-1 type 1 receptor on a BALB/c background (IL-1R1$^{-/-}$) 24 h after intratracheal instillation of 1×10^7 CFU type 4 pneumococci. Levels of CXCL1 (B), CXCL2 (C), and TNF-α (D) in BAL fluid from the same experiment as that described for panel A. CFU of bacteria in lung homogenates (E) and blood (F) in the same experiments as that described for panel A. *, $P < 0.05$, unpaired t test; **, $P < 0.01$, Mann-Whitney test.

primary macrophage that was also in the early stage of differentiation. Our confirmation *in vivo*, using two different strains of mice, that modulation of IL-1 signaling influences CXCL1/2 expression and polymorphonuclear cell numbers in the lung during pneumococcal pneumonia confirmed *in vivo* the validity of this approach. Other studies have supported our findings that IL-1 contributes to host defense *in vivo* against pneumococci, although in the case of the lung, the effects were most important in the first 24 to 48 h after infection (45, 67).

IL-1 dependent neutrophilic inflammation has improved bacterial clearance in a previous model of pneumococcal pneumonia (46), and our studies with moderate bacterial inocula were consistent with this. However, in fulminant disease, neutrophilic inflammation and reactive oxygen species generation by polymorphonuclear leukocytes may adversely affect clinical outcomes (33, 34), and we observed that IL-1-dependent neutrophilic inflammation enhanced levels of tissue invasion and bacteremia. In some settings, neutrophil depletion can improve outcomes in murine models of fulminant pneumonia (33). We have previously highlighted the potential importance of leukocyte-derived IL-1 in the initiation of airway inflammation (37, 38, 42). The data shown here provide further support for the concept that targeting IL-1 in airway infection may in some settings be able to limit damaging effects of excessive inflammation by suppressing signaling between monocytes/macrophages and airway epithelial cells. This may be more tractable than manipulation of other pathways, such as the TNF-α pathway, which may have more significant overall effects in regulating host responses to pneumococci (46). Alternatively, since TNF-α was raised in the absence of IL-1 signaling in the *Il1r1*$^{-/-}$ mice, it might be that the combined inhibition of IL-1 and TNF-α signaling could be more effective at inhibiting CXC

chemokine generation. Any potential benefits would need to be weighed carefully against the possible harmful effects of inhibiting recruitment of the minimum number of polymorphonuclear leukocytes required to clear microorganisms. The timing of administration is likely to be key. Administration would need to be delayed until after the early stages of the host response and would ideally involve identifying a subgroup of patients with selective markers indicating that an excessive inflammatory response might develop and predispose the individual to complications, such as acute respiratory distress syndrome (ARDS), which may occur following pneumonia (61). Previous studies using IL-1Ra in the early stages of pneumonia evolution have not shown substantial alteration in inflammatory outcomes or survival in a murine model (47), but targeting such an intervention to select patients with markers of more severe inflammation might be tractable.

In conclusion, we present evidence for a synergistic relationship between alveolar epithelial cells and macrophages in the early inflammatory response to *S. pneumoniae*. We demonstrate a critical role for IL-1 signaling in CXC chemokine expression and neutrophil recruitment to the murine lung during pneumococcal pneumonia and suggest that in fulminant disease therapeutic modulation of this pathway, as could be achieved with IL-1Ra, could reduce the extent of invasive disease.

ACKNOWLEDGMENTS

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