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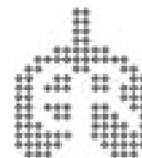

Legionella pneumophila-induced NF-κB- and MAPK-dependent cytokine release by lung epithelial cells

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ABSTRACT: *Legionella pneumophila* causes community-acquired pneumonia with high mortality, but little is known about its interaction with the alveolar epithelium. The aim of this study was to investigate whether *L. pneumophila* infection of lung epithelial cells (A549) resulted in pro-inflammatory activation.

***L. pneumophila* infection induced liberation of interleukin (IL)-2, -4, -6, -8 and -17, monocyte chemoattractant protein-1, tumour necrosis factor-α, IL-1β, interferon-γ and granulocyte colony-stimulating factor, but not of IL-5, -7, -10, -12 (p70) or -13 or granulocyte-macrophage colony-stimulating factor. The present study focused on IL-8 and found induction by *L. pneumophila* strains 130b, Philadelphia 1, Corby and, to a lesser extent, JR32. Knockout of *dotA*, a central gene involved in type IVB secretion, did not alter IL-8 induction, whereas lack of flagellin significantly reduced IL-8 release by Legionella. Moreover, p38 mitogen-activated protein kinase (MAPK) was activated and kinase inhibition reduced secretion of induced cytokines, with the exception of IL-2 and granulocyte colony-stimulating factor. In contrast, inhibition of the MAPK kinase 1/extracellular signal-regulated kinase pathway only reduced the expression of a few cytokines. *L. pneumophila* also induced binding of nuclear factor-κB subunit RelA/p65 and RNA polymerase II to the *il8* promoter, and a specific inhibitor of the inhibitor of nuclear factor-κB complex dose-dependently lowered IL-8 expression.**

Taken together, *Legionella pneumophila* activated p38 mitogen-activated protein kinase- and nuclear factor-κB/RelA pathway-dependent expression of a complex pattern of cytokines by human alveolar epithelial cells, presumably contributing to the immune response in legionellosis.

KEYWORDS: Alveolar epithelium, bacteria, cytokines, signal transduction pathways

An important causative agent of severe community-acquired pneumonia, *Legionella pneumophila* is the second most commonly detected pathogen in cases of pneumonia admitted to intensive care units in industrialised countries [1]. Approximately 15% of legionellosis appears in community outbreaks. Although >40 Legionella species are known, the majority of human infections are caused by *L. pneumophila* serogroup 1 [2]. *L. pneumophila* is a Gram-negative facultative intracellular pathogen of amoeba in natural and man-made aquatic environments. Infection of humans occurs after inhalation of contaminated water aerosol droplets. *L. pneumophila*-containing phagosomes initially do not fuse with lysosomes and the bacteria induce remodelling of their membrane-bound compartment into an endoplasmic reticulum-like organelle [3]. This remodelling depends on the defect in organelle trafficking (Dot)/intracellular multiplication (Icm) type IVB secretion apparatus of *L. pneumophila* [4]. Besides delivery of proteins by the type IVB secretion system, *L. pneumophila* contains a battery of additional virulence factors, including a type II secretion apparatus [3]. Sequencing of *L. pneumophila* serogroup 1 genomes revealed ~3,000 genes, among which are many genes with possible function in manipulation of host cell signalling [5].

According to the Legionella–host interaction, recent studies demonstrated that *L. pneumophila* lipopolysaccharide was recognised by Toll-like receptor (TLR)2 and flagellin by TLR5 [6, 7]. A stop codon in the human TLR5 gene leads to increased susceptibility to legionellosis [7]. Interestingly, although some studies have suggested a minor role of TLR4 in legionellosis [6], HAWN *et al.* [8] recently showed that TLR4

polymorphisms are associated with resistance to Legionnaires' disease.

Essential results according *L. pneumophila* pathogenesis were obtained by analysing infection of protozoans or immune cells such as macrophages. However, lung epithelial cells constitute a first mechanical and immunological barrier against airborne pathogens and are important sources of cytokines in the lung [9, 10]. Activation of pro-inflammatory signalling pathways in lung epithelial cells, including p38 mitogen-activated protein kinase (MAPK)- and nuclear factor-κB (NF-κB)-dependent gene transcription, by bacterial infection, contribute significantly to cytokine release [10, 11]. Although Legionella efficiently infects and stimulates lung epithelial cells [7, 12], mechanisms of *L. pneumophila*-induced activation of and cytokine release in lung epithelial cells are widely unknown. Therefore, the pro-inflammatory activation of lung epithelial cells by Legionella infection was analysed in detail.

In the present study, it was shown that *L. pneumophila* induced the release of several important cytokines in human alveolar epithelial A549 cells, *e.g.* interleukin (IL)-2, -4, -6, -8 and -17, monocyte chemoattractant protein (MCP)-1, tumour necrosis factor (TNF)-α, IL-1β, interferon (IFN)-γ and granulocyte colony-stimulating factor (G-CSF), as well as activating the p38 MAPK, extracellular signal-regulated kinase (ERK) and NF-κB pathways. Blocking p38 MAPK reduced secretion of all cytokines in Legionella-infected cells, with the exception of IL-2 and G-CSF, whereas blocking the ERK pathway diminished only release of IFN-γ, IL-1β, IL-6 and TNF-α. By addressing expression of IL-8 as a model cytokine in more detail, the important role of p38 MAPK- and NF-κB/RelA-dependent gene transcription in the activation of *L. pneumophila*-infected epithelial cells was verified. Moreover, activation of IL-8 expression was reduced by a flagellin deletion mutant, implying a role for TLR5 or possible intracellular receptors in Legionella sensing by A549 cells. However, IL-8 expression in A549 cells was not affected by a *dotA*-knockout mutant, suggesting that the type IVB Dot/Icm secretion system and intracellular replication are not needed for IL-8 expression in A549 cells.

MATERIALS AND METHODS

Cell lines

Alveolar epithelial cell line A549 was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Ham's F-12 medium with L-glutamine and 10% foetal calf serum (Life Technologies, Karlsruhe, Germany) without antibiotics. The NF-κB-dependent reporter cell line, A549 6Btkluc, was a kind gift of R. Newton (Dept of Biological Sciences, University of Warwick, Coventry, UK). These cells contain a stably integrated plasmid with three tandem repeats of the sequence 5′-AGCTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGA-3′, which contains two copies of the decameric NF-κB binding site upstream of a minimal thymidine kinase promoter (-105–51) driving a luciferase gene.

Infection with bacterial strains and isogenic mutants

L. pneumophila sg1 strains 130b (ATCC BAA-74, kindly provided by N.P. Cianciotto, Northwestern University Medical School, Chicago, IL, USA [13]), Philadelphia 1 (ATCC 33152, kindly provided by B. Neumeister, Tübingen University, Tübingen, Germany [14]), JR32 wildtype [15] and JR32 *dotA* mutant (LELA 3118, both kindly provided by H. Shuman, Columbia University, New York, NY, USA [16]), and Corby wildtype and a Corby *flaA*, defective in flagellin, mutant (both kindly provided by K. Heuner, Würzburg University, Würzburg, Germany) were routinely grown on buffered charcoal–yeast extract (BCYE) agar for 2–3 days at 37°C [17] and subsequently inoculated into plain RPMI medium at an optical density at 660 nm of 0.2–0.4. A549 cells (1×10^5 cells·mL^{-1}) were infected with 1×10^5–1×10^8 colony-forming units (cfu)·mL^{-1} *L. pneumophila*, *i.e.* at a multiplicity of infection of 1:1–1:1,000, in 1 mL epithelial cell growth medium for the indicated times. Extracellular bacteria were not routinely killed with antibiotics. *L. pneumophila* strains did not grow significantly in epithelial cell growth medium, as controlled by serial dilutions plated on BCYE agar.

In order to verify intracellular infection, A549 cells were incubated with the added bacteria for 2 h with or without kinase inhibitors, gentamicin (100 μg·mL^{-1}) was added for a further 2 h and then the cells were washed three times with plain medium, to remove unbound bacteria, and treated with 10% (weight/volume) saponin (Sigma Chemical Company, Munich, Germany) to lyse the host cells. Serial dilutions were plated on BCYE agar.

Interleukin-8 ELISA

Confluent A549 cells were infected as indicated in a humidified atmosphere for 15 h. After incubation, supernatants were collected and processed for IL-8-quantification by sandwich ELISA, as described previously [11, 18]. In some experiments, medium was changed after certain time periods (1, 2 or 4 h) with or without gentamicin (100 μg·mL^{-1}) and then the cells were incubated for the remaining time in the same medium before IL-8 was analysed in the supernatant.

Bioplex protein array system

Confluent A549 cells were infected as indicated in a humidified atmosphere for 15 h. After incubation, supernatants were collected and cytokine release was analysed using the Bioplex Protein Array system (BioRad, Hercules, CA, USA) and beads specific for IL-2, -4, -5, -6, -7, -8, -10, -12 (p70), -13 and -17, MCP-1, TNF-α, IL-1β, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF, according to the manufacturer's instructions [19, 20].

Western blotting

For determination of p38 MAPK and ERK phosphorylation, A549 cells were infected as indicated, washed twice and harvested. Cells were lysed in buffer containing Triton X-100 (Sigma Chemical Company), subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis and blotted on to Hybond ECL membrane (Amersham Biosciences, Freiburg, Germany). Immunodetection of phosphorylated MAPK was carried out using phospho-specific p38 MAPK or ERK antibodies (Cell Signaling, Frankfurt, Germany) [11]. Degradation of inhibitor of NF-κB (IκBα) was analysed using a rabbit polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) as described previously [11]. In all experiments, unphosphorylated ERK2 and p38

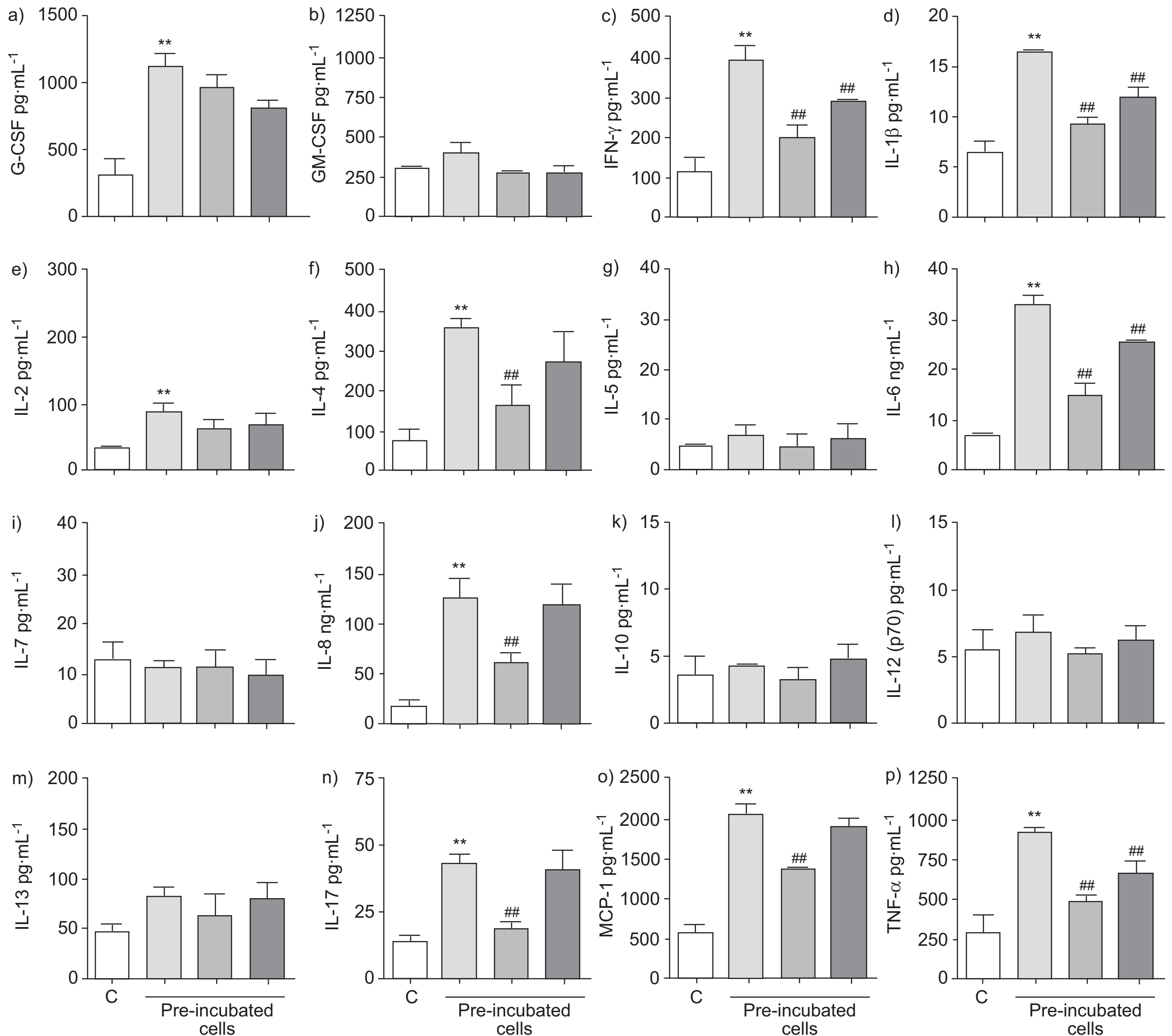

FIGURE 1. *Legionella pneumophila*-induced release of a cytokine pattern mainly dependent on p38 mitogen-activated protein kinase (MAPK) activity: a) granulocyte colony-stimulating factor (G-CSF); b) granulocyte-macrophage colony-stimulating factor (GM-CSF); c) interferon (IFN)-γ; d) interleukin (IL)-1β; e) IL-2; f) IL-4; g) IL-5; h) IL-6; i) IL-7; j) IL-8; k) IL-10; l) IL-12 (p70); m) IL-13; n) IL-17; o) monocyte chemoattractant protein (MCP)-1; and p) tumour necrosis factor (TNF)-α. A549 cells (1×10^5 cells·mL^{-1}) were pre-incubated (30 min) with medium alone (■), 5 μM SB202190 (p38 MAPK inhibitor; ■) or U0126 (MAPK kinase 1 inhibitor; ■) and infected with *L. pneumophila* 130b (1×10^7 colony-forming units·mL^{-1}; uninfected control (C; □)). Cytokine release in the supernatant was measured by Bioplex assay. Data are presented as mean±SEM. **: p<0.01 *versus* C; ##: p<0.01 *versus* infected cells without pre-incubation with inhibitors (in at least three independent experiments).

MAPK (Santa Cruz Biotechnologies) were detected simultaneously in order to confirm equal protein load. Proteins were visualised by incubation with secondary IRDye 800- or Cy5.5-labelled antibodies, respectively (Odyssey infrared imaging system; LI-COR Inc., Bad Homburg, Germany) [11, 21]. All primary antibodies were used at a dilution of 1:200 and all secondary antibodies at a dilution of 1:2,000.

RT-PCR

For analysis of IL-8 and reduced glyceraldehye-3-phosphate dehydrogenase (GAPDH) gene expression in A549 cells, total RNA was isolated by means of the RNEasy Mini kit (Qiagen, Hilden, Germany) and reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega, Heidelberg, Germany). The complementary DNA generated was amplified by PCR using specific intron-spanning IL-8 and GAPDH primers [11]. All primers were purchased from TIB MOLBIOL (Berlin, Germany). After 35 amplification cycles, PCR products were analysed on 1.5% agarose gels, stained with ethidium bromide and subsequently visualised. In order to confirm use of equal amounts of RNA in each experiment, all samples were checked for GAPDH mRNA expression.

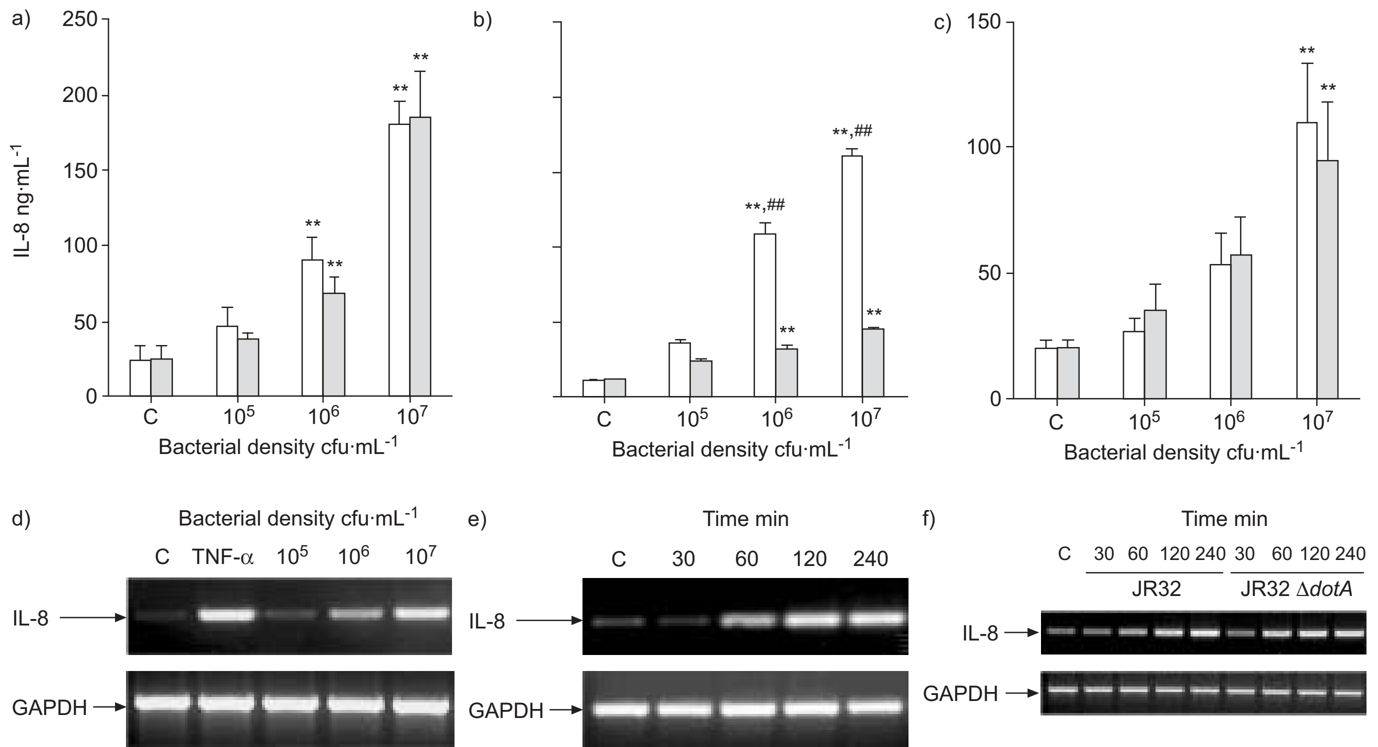

FIGURE 2. Induction of flagellin-dependent *dotA*-independent interleukin (IL)-8 expression in alveolar epithelial cells by various *Legionella pneumophila* strains. A549 cells (1×10^5 cells·mL^{-1}) were infected with *L. pneumophila*: a) 130b (□) and Philadelphia 1 (■); b) Corby wildtype (□) and Δ*flaA* knockout mutant (■); c) JR32 wildtype (□) and Δ*dotA* knockout mutant (■); d, e) 130b; and f) JR32 wildtype and Δ*dotA* knockout mutant. a–c) IL-8 release into the supernatant following treatment at the indicated bacterial densities was measured by ELISA after 15 h. Data are presented as mean ± SEM. d–f) IL-8 and reduced glyceraldehye-3-phosphate dehydrogenase (GAPDH) mRNA expression were determined by RT-PCR: d) 4 h after infection with the indicated concentrations of *L. pneumophila* or after incubation for 4 h with 100 ng·mL^{-1} tumour necrosis factor-α (TNF-α); and e, f) at the indicated time points after infection with 1×10^7 colony-forming units (cfu)·mL^{-1} *L. pneumophila* (representative gels of three are shown). **: p<0.01 *versus* uninfected control (C); ##: p<0.01 *versus* Philadelphia 1 or mutant.

Reporter gene assay

A549 cells stably transfected with a NF-κB-dependent luciferase reporter plasmid [22] were cultured in 12-well plates in Dulbecco's modified Eagle medium (Life Technologies). Cells were incubated with Legionella for 15 h and lysed, and then luciferase activity was measured using a luciferase reporter gene assay (Promega, Mannheim, Germany).

Chromatin immunoprecipitation

A549 cells in 75-cm^2 culture flasks were infected with *L. pneumophila* 130b as indicated and then subjected to a chromatin immunoprecipitation assay, as previously described, using anti-p65 or anti-RNA polymerase II antibodies (both Santa Cruz Biotechnologies) [11, 20]. The *il8* promoter was amplified by PCR using HotstarTaq polymerase (Qiagen) and specific primers as follows: 5'-AAGAAAACTTTCGTCATACTCCG–3' (sense); and 5'–TGGCTTTTTATATCATCACCCTAC-3' (antisense). PCR amplification of the total input DNA in each sample was performed as a control [18, 19].

Statistical methods

Data are presented as mean ± SEM of at least three independent experiments. One-way ANOVA was used for numerical data shown in the figures. Main effects were then compared using Newman–Keuls post-test. A p-value of <0.01 was considered significant.

RESULTS

Cytokine release

In order to characterise inflammatory activation of human alveolar epithelial cells by *L. pneumophila*, 1×10^5 A549 cells were infected with *L. pneumophila* strain 130b at an infection dose of 1×10^7 cfu·mL^{-1}, *i.e.* a multiplicity of infection of 1:100. Cytokine release was analysed using a Bioplex assay. After incubation for 15 h, significant induction of IL-2, -4, -6, -8 and -17, MCP-1, TNF-α, IL-1β, IFN-γ and G-CSF, but not of IL-5, -7, -10, -12 (p70) or -13 or GM-CSF was observed (fig. 1). Pre-incubation of A549 cells with the specific p38 MAPK inhibitor SB202190 (Calbiochem-Merck, Darmstadt, Germany) reduced levels of IL-4, -6, -8 and -17, MCP-1, TNF-α, IL-1β and IFN-γ, but not of IL-2 or G-CSF (TNF-α was obtained from R&D Systems, Wiesbaden, Germany). Inhibition of MAPK kinase 1 (MEK1) by U0126 reduced release of only IFN-γ, IL-1β, IL-6 and TNF-α (fig. 1). Incubation with inhibitors alone showed no cytotoxicity and did not alter cytokine expression or infection of epithelial cells (data not shown).

Flagellin- and dotA-dependency of interleukin-8 expression

Alveolar epithelial cell activation was analysed in more detail by addressing the expression of the important chemotactic

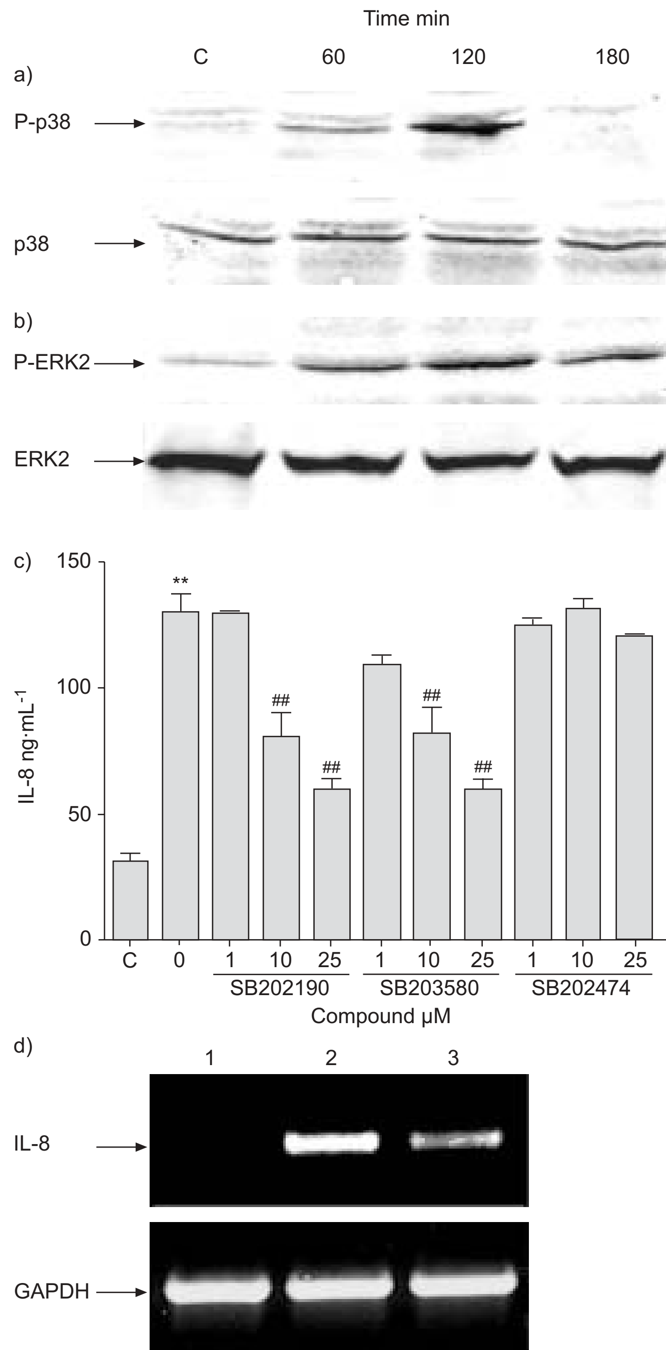

FIGURE 3. *Legionella pneumophila*-induced interleukin (IL)-8 release dependency on p38 mitogen-activated protein kinase (MAPK) activation in alveolar epithelial cells. A549 cells (1×10^5 cells·mL^{-1}) were infected with *L. pneumophila* 130b (1×10^7 colony-forming unit·mL^{-1}). Phosphorylation (P) of a) p38 MAPK and b) extracellular signal-regulated kinase (ERK)2 were determined at the indicated time points by Western blot analysis (representative blots of three are shown). c, d) A549 cells were pre-incubated (30 min) with 10 µM SB202190 (c and d), SB203580 (c) and SB202474 (c) and infected with *L. pneumophila* 130b. c) IL-8 release in the supernatant was measured by ELISA after 15 h. Data are presented as mean ± SEM. d) IL-8 mRNA was detected by RT-PCR after 4 h (lane 1: uninfected control (C); lane 2: cells pre-incubated with medium alone; lane 3: pre-incubated cells). GAPDH: reduced glyceraldehye-3-phosphate dehydrogenase. **: $p<0.01$ *versus* C; ##: $p<0.01$ *versus* infected cells not pre-incubated with inhibitors (in at least three independent experiments).

cytokine IL-8. A549 cells were infected with various concentrations of *L. pneumophila* strains 130b, Philadelphia 1, JR32 and Corby for 15 h (fig. 2). *L. pneumophila* 130b, Philadelphia 1 and Corby similarly induced dose-dependent IL-8 release (fig. 2a and b), whereas JR32-provoked cytokine secretion was lower (fig. 2c). *L. pneumophila* 130b also induced dose- (fig. 2d) and time-dependent (fig. 2e) expression of IL-8 mRNA. Gene expression started as early as 60 min after infection. A *flaA* knockout mutant, defective in flagellin production, resulted in strongly reduced IL-8 release by *L. pneumophila* Corby-infected A549 cells over 15 h (fig. 2b). In contrast, a *dotA* knockout mutant, defective in a gene essential for the establishment of a functional *L. pneumophila* type IVB secretion apparatus [3], did not alter IL-8 release by *L. pneumophila* JR32-infected A549 cells over 15 h (fig. 2c) or the time course of IL-8 mRNA induction up to 4 h (fig. 2f).

Interleukin-8 release dependency on p38 mitogen-activated protein kinase activation

Next, *L. pneumophila*-induced activation of mitogen-activated kinase pathways was analysed. A549 cells were infected with *L. pneumophila* 130b, and phosphorylation of p38 MAPK (fig. 3a) and ERK2 (fig. 3b) was assessed by Western blot analysis. *L. pneumophila* infection induced phosphorylation of both kinases within 60–120 min. Blocking p38 MAPK with SB202190 reduced IL-8 mRNA accumulation (fig. 3d). Moreover, p38 MAPK inhibitors SB202190 and SB203580 (Calbiochem-Merck) dose-dependently reduced *L. pneumophila*-induced IL-8 release (fig. 3c). Control compound SB202474 (Calbiochem-Merck) had no effect on cytokine release (fig. 3c). Neither inhibitors nor control compound reduced cell number or induced morphological signs of cytotoxicity.

Interleukin-8 release dependency on nuclear factor-κB activation

Activation of the IL-8 promoter is considered to require activation of the transcription factor NF-κB. IκBα kinase complex was blocked using the specific peptide inhibitor, IκB kinase NF-κB essential modulator-binding domain (IKK-NBD; Biomol, Plymouth Meeting, PA, USA), and a dose-dependent reduction in IL-8 secretion by *L. pneumophila* 130b-infected A549 cells observed (fig. 4a). In A549 cells, transfected with a NF-κB-dependent reporter gene construct, dose-dependent induction of reporter gene expression was found (fig. 4b). IKK-NBD displayed no cytotoxicity and did not alter infection of A549 by *Legionella* strain 130b or basal cytokine expression (data not shown). Western Blot analysis revealed degradation of cytosolic NF-κB inhibitor IκBα starting 60 min after stimulation of A549 cells with *L. pneumophila* 130b (fig. 4c), and chromatin immunoprecipitation showed recruitment of NF-κB/p65 and RNA polymerase II to the *il8* promoter (fig 4d). IKK-NBD did not reduce cell number or induce morphological signs of cytotoxicity. These data indicate that *L. pneumophila* induces IL-8 expression by activation of the canonical NF-κB pathway.

Effect of gentamicin on interleukin-8 release

In order to address the importance to cellular activation of *L. pneumophila* remaining extracellular, the medium was changed after infection periods of 1, 2 and 4 h in the presence and absence of gentamicin and IL-8 release analysed after a total infection time of 15 h (fig. 5). Removal of extracellular bacteria

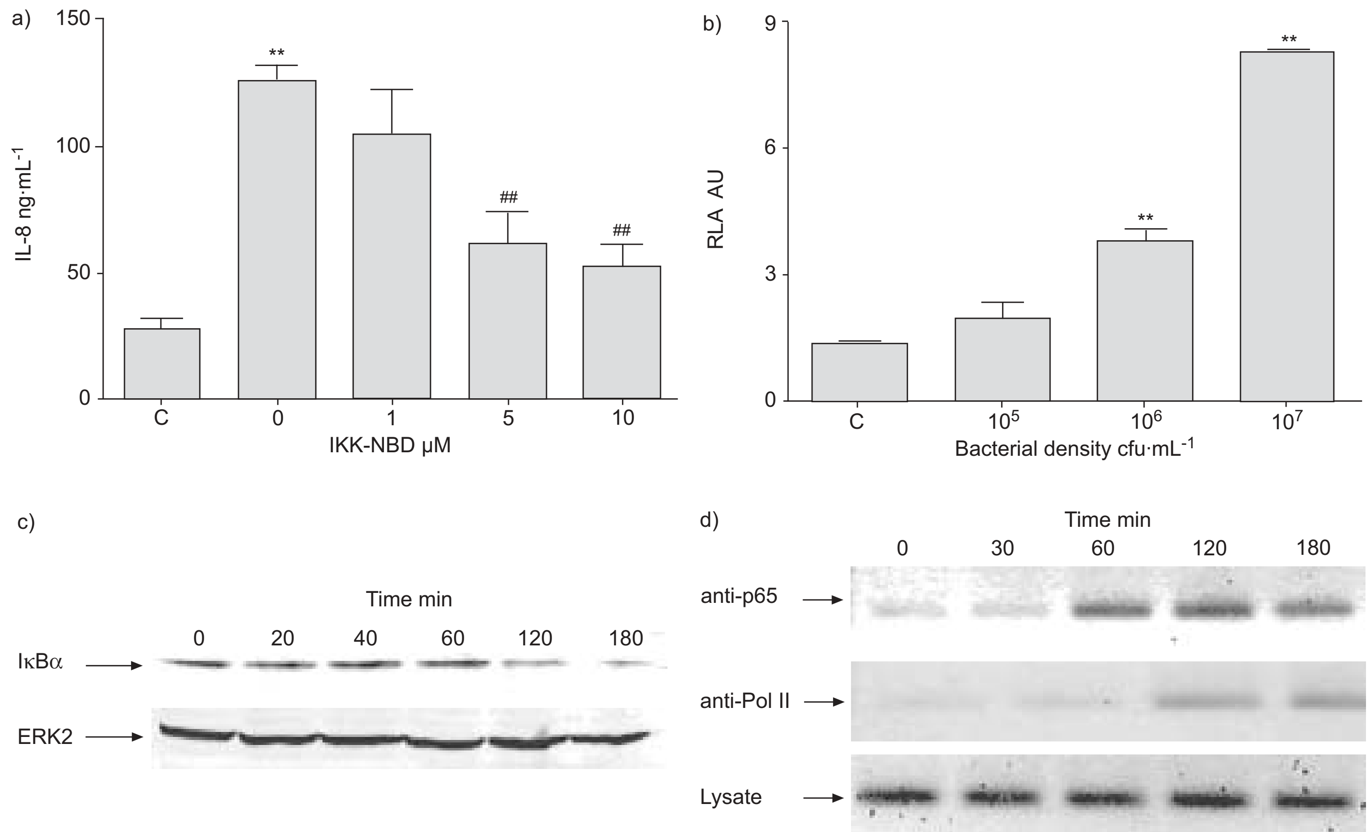

FIGURE 4. *Legionella pneumophila*-induced interleukin (IL)-8 release dependency on nuclear factor-κB (NF-κB) activation in alveolar epithelial cells. a) A549 cells (1×10^5 cells·mL^{-1}) were pre-incubated with the indicated concentrations of inhibitor of NF-κB (IκB) kinase NF-κB essential modulator-binding domain and infected with *L. pneumophila* 130b (1×10^7 colony-forming units (cfu)·mL^{-1}). IL-8 release in the supernatant was measured by ELISA. b) Relative luciferase activity (RLA) was determined in A549 cells stably transfected with a NF-κB-dependent reporter gene construct 6 h after infection with *L. pneumophila* 130b. Data are presented as mean±SEM. c) Degradation of IκBα was determined at the indicated time points after infection with 1×10^7 cfu·mL^{-1} *L. pneumophila* 130b by Western blot analysis (representative blots of three are shown). d) Recruitment of NF-κB/p65 and RNA polymerase II (Pol II) to the *il8* promoter was determined at the indicated time points after infection with 1×10^7 cfu·mL^{-1} *L. pneumophila* 130b by chromatin immunoprecipitation (precipitating antibodies are as indicated; the initial cell lysate without immunoprecipitation is also shown for comparison; representative blots/gels of three are shown). AU: arbitrary unit; ERK: extracellular signal-regulated kinase. **: $p<0.01$ *versus* uninfected control (C); ##: $p<0.01$ *versus* infected cells without pre-incubation with inhibitors (in at least three independent experiments).

early in infection resulted in significantly reduced IL-8 release in comparison with late removal after 4 h of infection. Moreover, killing of extracellular bacteria with gentamicin further reduced IL-8 release.

DISCUSSION

In the present study, it was found that *L. pneumophila* 130b induced release of a complex cytokine pattern by human alveolar epithelial cell line A549. Detailed analysis of IL-8 release showed similar IL-8 expression in cells infected with *L. pneumophila* strains 130b, Philadelphia 1 and Corby. IL-8 secretion depended on activation of the p38 MAPK and canonical NF-κB/RelA-pathway, and, to a lesser extent, the MEK1–ERK1/2 pathway (fig. 6).

Lung epithelial cells have important functions in innate immunity, *e.g.* they recognise pathogens, including bacteria, *via* TLRs and release antibacterial peptides, as well as chemotactic and pro-inflammatory cytokines [9, 10]. Although alveolar epithelial cells were infected efficiently by Legionella *in vitro* [12, 23], and *in vivo* in guinea pigs [12], knowledge about host immunoreaction against *L. pneumophila* mainly arises from studies with human monocytes/macrophages and animal studies in the Legionella-permissive *naip5* locus-defect A/J mouse strain [24].

Since chemokine synthesis is important for orchestration of the innate and adaptive immune response, the chemokine pattern released by *L. pneumophila*-infected alveolar epithelial cells was analysed first. For this purpose, the well-established human alveolar epithelial cell line A549, which Legionella infects efficiently, as shown in several studies [12, 23], was used.

L. pneumophila-infected A549 cells released the chemoattractants IL-8 (for polymorphonuclear neutrophils (PMNs)) and MCP-1 (for monocytes), the T-helper cell (Th) type 1 cytokines IL-2, TNF-α and IFN-γ, and the Th2 cytokines IL-4 and IL-6 within 15 h. In addition, the pro-inflammatory cytokines IL-1β and IL-17 and the myeloid growth factor G-CSF were secreted (fig. 6). In accordance with the present findings, CHANG *et al.* [23] described IL-6 and IL-8, as well as TNF-α, expression in Legionella-infected A549 cells.

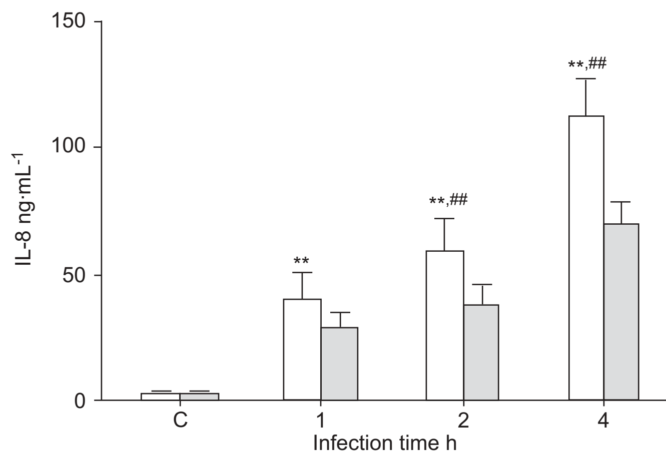

FIGURE 5. *Legionella pneumophila*-induced interleukin (IL)-8 release was reduced by gentamicin. A549 cells (1×10^5 cells·mL^{-1}) were infected with *L. pneumophila* 130b (1×10^7 colony-forming units·mL^{-1}). The medium was changed after the indicated time period, with (■) or without (□) addition of gentamicin. IL-8 release in the supernatant was measured by ELISA after a total incubation time of 15 h. **: p<0.01 *versus* uninfected control (C); ##: p<0.01 *versus* infected cells with addition of gentamicin (in at least three independent experiments).

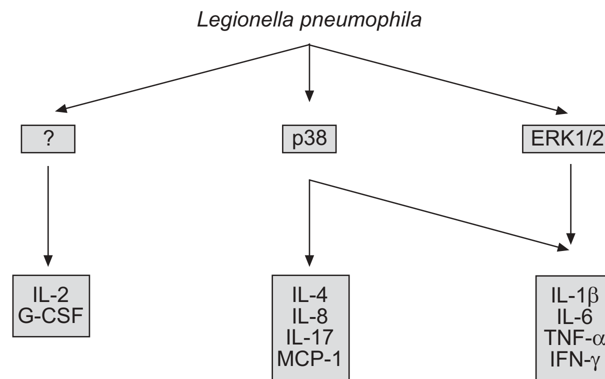

FIGURE 6. *Legionella pneumophila* induces release of a cytokine pattern differentially dependent on p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) 1/2 activity. *L. pneumophila* infection of the human alveolar A549 cell line leads to activation of p38 MAPK and ERK1/2 and to cytokine release. Secretion of interleukin (IL)-2 and granulocyte colony-stimulating factor (G-CSF) are not effected by kinase inhibition; IL-4, -8 and -17 and monocyte chemoattractant protein (MCP)-1 are blocked by p38 MAPK inhibition; and IL-1β, IL-6, tumour necrosis factor (TNF)-α and interferon (IFN)-γ also depend on ERK1/2 activity.

In humans, Legionella infection increases serum levels of, for example, IFN-γ and IL-6, -12 and -10 [25, 26]. In experimental studies using murine models or isolated macrophages, mainly cytokines attracting and activating PMNs and moncytes/macrophages have been analysed. In experimental Legionella pneumonia in A/J mice, TATEDA *et al.* [27] found induction of the chemotactic cytokines chemokine (CXC motif) ligand 1 (KC), macrophage inflammatory protein (MIP)-2 and chemokine (CXC motif) ligand 5 (LIX), recruiting PMNs into the lung. Accordingly, attachment of *L. pneumophila* to cultured mouse peritoneal macrophages increased steady-state levels of cellular mRNAs encoding the cytokines IL-1β, IL-6 and GM-CSF, and the chemokines MIP-1β, MIP-2 and KC [28]. Since PMN recruitment was seen in Legionella pneumonia in humans [29] and mice [30], and its blockage increased mortality in the A/J mouse model [27], alveolar epithelium may play an important role in orchestrating the immune response against Legionella.

Recruited monocytes were activated by the Th1 cytokines IFN-γ and TNF-α; IFN-γ promotes Legionella clearance in macrophages [31], and transgenic overexpression of IFN-γ in A/J mice reduces the bacterial burden [32]. Since IFN-γ was also found to be crucial to immune defence against *Listeria monocytogenes* in mice [33] and humans [34], an important role of IFN-γ in host defence against intracellular bacteria, including Legionella, has to be considered. Similarly, TNF-α promoted *L. pneumophila* clearance in human monocytes and proved to be protective in mice [35]. As release of both cytokines was reduced by inhibition of p38 MAPK, this pathway might be crucial to an effective immune response in *L. pneumophila* infection.

L. pneumophila-infected A549 cells did not, however, release Th1 cytokine IL-12 (p70), Th2 cytokines IL-5, -10 or -13, or the lymphoid and myeloid growth factors IL-7 and GM-CSF. Interestingly, high IL-12 (p70) levels accompanied decreased mortality in A/J mice with *L. pneumophila* infection [27]. Expression was also found in human *L. pneumophila* pneumonia [25], and it could be produced by Legionella-exposed dendritic cells [36], but alveolar epithelium does not seem to be a source of this cytokine. In contrast, IL-10 reversed the Legionella-protective effects of IFN-γ [37]. Taken together, *L. pneumophila*-infected human alveolar epithelial cells secreted chemotactic CC and CXC chemokines, as well as Th1 and Th2 chemokines (fig. 6). Of these, TNF-α, IL-1β, -6 and -8 and G-CSF were considered as uniform inflammatory reaction factors, *e.g.* induced by TLR2, whereas IFN-γ, IL-2, -4 and -17 and MCP-1 seem to be part of a pathogen-specific reaction [38]. Thus, Legionella-infected alveolar epithelial cells may potently and specifically contribute to the regulation of the host immune response in legionellosis.

In order to gain more insight into alveolar epithelial cell activation by *L. pneumophila*, expression of the important chemotatic cytokine IL-8 was analysed in more detail. *L. pneumophila* serogroup 1 strains 130b, Philadelphia 1, JR32 and Corby induced IL-8 secretion by infected A549 cells. Philadelphia 1-derived strain JR32 induced IL-8 expression to a lesser extent than the other strains in lung epithelial cells, underlining existing differences between these strains. In accordance with the recent findings of HAWN *et al.* [7], REN *et al.* [39] and MOLOFSKY *et al.* [40], experiments using a Corby *flaA* knockout mutant strain indicated that recognition of flagellin by TLR5 or other possibly intracellular receptors seems to be essential to the early induction of IL-8 release in alveolar epithelial cells *in vitro*. Moreover, removal or killing (gentamicin) of extracellular bacteria reduced IL-8 release by A549 cells significantly.

In addition, data obtained using a JR32 *dotA* knockout mutant indicated that type IVB-secreted effectors do not seem to be

essential to the early induction of IL-8 release in alveolar epithelial cells *in vitro*. Furthermore, this implies that bacterial replication is not necessary for induction of IL-8 release. However, CHANG *et al.* [23] have found that knockout of *dotG/icmE* in *L. pneumophila* strain 80-045 reduced cytokine expression at later time points [23]. *L. pneumophila* genomes showed marked plasticity and diversity, as recently demonstrated, for example, for the strains Paris and Lens [5], and showed different expression patterns of pathogenetic factors. The physiological importance of such differences is furthermore highlighted by, for example, the observation that Philadelphia 1-derived strain JR32 induced IL-8 expression to a lesser extent in lung epithelial cells. Thus, it cannot be ruled out that the importance of a particular virulence factor may vary between infections with different *L. pneumophila* strains. Moreover, different genes within the *icm/dot* loci were manipulated by mutagenesis. In accordance with CHANG *et al.* [23], the time course of IL-8 mRNA induction by the JR32 strain or *dotA* knockout mutant did not differ up to 4 h. Overall, it seems reasonable that recognition of extracellular Legionella by TLRs initially contributes to alveolar epithelial activation [6, 7].

Activation of p38 MAPK has been shown to contribute to bacteria-related expression of IL-8 in infected lung epithelial cells [11], and WELSH *et al.* [41] found that p38 MAPK and Janus kinase were activated early during the uptake of *L. pneumophila* by macrophages. In lung epithelial cells, p38 MAPK activation 60 min after infection was critical to the release of all induced cytokines, with the exception of IL-2 and G-CSF, but seems not to be necessary for invasion of the cells (data not shown). Thus, cell-specific effects should be considered concerning replication of *L. pneumophila*. Interestingly, although ERK2 kinase was activated in infected epithelium, ERK kinase activity contributed to a lesser extent to epithelial cell activation with respect to cytokine and chemokine release. Besides p38 MAPK activity, stimulation of IL-8 expression was dependent upon activation of the transcription factor NF-κB in *L. pneumophila*-infected epithelium, as was shown for *Streptococcus pneumoniae* in lung epithelial cells [11]. Further experiments addressing the role of the different pathogenic factors of Legionella, such as lipopolysaccharide, flagella or hydrolysing enzymes, for the activation of these important pro-inflammatory pathways are needed in order to gain more insight into the molecular mechanisms involved.

In summary, it is shown here that *Legionella pneumophila* infection induced strong chemokine and cytokine release in human alveolar epithelial A549 cells. Expression of these molecules depends predominantly upon activation of the p38 mitogen-activated protein kinase pathway and nuclear factor-κB-dependent gene transcription in A549 cells. Overall, activation of alveolar epithelium seems to contribute significantly to the orchestration of the immune response in legionellosis.

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